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EDITORIAL

Mucosal mast cells are pivotal elements in inflammatory bowel disease that connect the dots: Stress, intestinal hyperpermeability and inflammation

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Abstract

Mast cells (MC) are pivotal elements in several physiological and immunological functions of the gastrointestinal (GI) tract. MC translate the stress signals that has been transmitted through brain gut axis into release of proinflammatory mediators that can cause stimulation of nerve endings that could affect afferent nerve terminals and change their perception, affect intestinal motility, increase intestinal hyperpermeability and, in susceptible individuals, modulate the inflammation. Thus, it is not surprising that MC are an important element in the pathogenesis of inflammatory bowel disease and non inflammatory GI disorders such as IBS and mast cell enterocolitis.

Key words: Mast cells; Intestinal permeability; Stress, Inflammatory bowel disease; Irritable bowel syndrome; Intestinal barrier

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Mast cells (MC) of the intestinal mucosa are key elements in several biological processes. For example, they are an important component in allergic responses to exogenous antigens and they act in concert with IgE to increase the release of MC mediators in allergic reactions. Recently the role of MC in non-allergic phenomena has been getting more attention. In fact, MC are an important component of the mucosal innate immune response[1]. Thus, it is not surprising that these cells are involved in several inflammatory disease processes such as bronchiectasis[2], idiopathic pulmonary fibrosis[3], bronchiolitis obliterans with organizing pneumonia[4], sarcoidosis[5], glomerulonephritis[6] and rheumatoid arthritis[7]. In the gastrointestinal (GI) tract, similar to other mucosal surfaces, Mast cells are part of the allergic response to luminal antigens and of protective innate immune responses.

Mast cells in the GI tract also serve as end effectors of the brain-gut axis (BGA). The BGA is composed of main regulatory cores in the central nervous system that are connected to peripheral (enteric and autonomic) nervous systems through a series of networks of afferent and efferent nerves. One role of the BGA is to transmit information from the brain to the GI tract regarding the perception and/or experience of stressful events.

Upon activation of the BGA by stress, Mast cells release a wide range of neurotransmitters and other proinflammatory molecules. These mediators include histamine, heparin, chondroitin sulfate, chymase, carboxypeptidase, tryptase, platelet activating factor, prostagalanin (PGD2), leukotriene (LTC4) and a variety of interleukins such as IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-13, IL-16, IL-18, IL-25, TNF-alpha, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor, macrophage chemotactic peptide (MCP)-1, 3&4, regulated on activation of normal T cell-expressed and secreted protein (RANTES), and eotaxin[8].

The release of these mediators can profoundly affect GI physiology. For example, tryptase can activate PAR-2 receptors on epithelial cells, resulting in modulation of tight junction proteins and increases in permeability through paracellular pathways in the intestinal epithelium[9]. Such increases expose the submucosal immune system to lumen-derived food antigens and bacterial by-products, which will result in immune system activation[10,11]. This is clinically important because an increased mucosal permeability and activation of the mucosal immune system are the two major players in mucosal inflammation in inflammatory bowel disease (IBD). PAR-2 receptors are not limited to epithelial cells and the presence of this receptor on afferent nerve terminals and MC themselves has been shown. Thus, activation of PAR-2, can result in release of proinflammatory mediators from nerve

endings which may cause neurogenic inflammation[12] or even potentiate MC release by creating a positive feedback loop[13,14].

IBD is believed to result from an abnormal responses to normal pro-inflammatory factors in the gut lumen in a susceptible individual with immune dysregulation[15]. The origins of this disease are probably multi-factorial, with interplay between genetic and environmental factors[15,16]. This interplay results in initiation of inflammatory processes and creation of vicious cycles (involving positive feedback loops) that cause sustained, uncontrolled inflammation and tissue damage. However, for luminal factors such as bacterial antigens to initiate an inflammatory cascade, they must be able to bypass the intestinal barrier[17,18]. Indeed, as suggestive above, a decreased intestinal barrier integrity (leaky gut) has been implicated in the pathogenesis of IBD[17-20]. In fact, activation of the BGA by stressful situations and by the associated degranulation of MC in the gut mucosa can result in intestinal hyperpermeability and activation of the mucosal immune function.

Nevertheless, the mechanisms through which MC play a role in the pathogenesis of IBD are not well known. For example, there is a wide variation in the number of MC in IBD in different reports. A few studies have shown a mild to marked increase in the number of MC in subjects with active IBD[21-23]. King *et al*[26] and other researchers reported that MC number was not different between controls and subjects with inactive IBD[24-26]. Surprisingly, in the report by King *et al*[26] the number of MC increased in the area of demarcation between involved and non-involved colon and the number of MC dropped significantly in areas of active inflammation. In our own recent study, we did not find any significant differences in the number of MC in subjects with IBD compared to healthy controls. In addition we showed that there was no increase in the number of MC after stress in human subjects[27]. This contrasts with animal studies in which the number of MC increased after stress[28].

Although there is controversy regarding the number of intestinal MC in IBD, there is consensus that there is a close association among stress, BGA activation, and MC mediated mechanisms in IBD[21,29-35]. For example, studies in animal models of IBD showed that stress results in increased intestinal permeability and worsening of hapten-induced colitis in rats[36]. Stress did not affect gut permeability in MC-deficient rats and failed to cause epithelial mitochondrial damage in a rat model, indicating that stress-induced intestinal hyperpermeability is MC-dependent[28]. In human studies, stress [modeled using cold pressor test (CPT)] in healthy subjects caused activation of mucosal mast cells and release of proinflammatory mediators in the jejunum[37]. This study reaffirmed the finding that was previously showed in animal studies and reaffirmed the BGA activation in humans activates MC in GI mucosa in healthy subjects. Finally, we recently showed that stress (CPT) caused more pronounced MC activation and degranulation in patients with inactive IBD than in healthy controls. The activation of mucosal MC was associated with mucosal oxidative damage[27]. The mechanism for the exaggerated MC response to stress in IBD patients is not known but could be one of the important factors involved in IBD flare up. In fact, it remains to be seen, whether the exaggerated response of mucosal MC to stress in IBD subjects is a primary phenomenon due to an inherently abnormal MC or whether it is a secondary phenomenon due to the inflammatory environment of the MC. After further investigation we recently reported that MC in the intestinal mucosa of patients with IBD have reduced immunostaining of c-kit receptors compared to MC from healthy controls[27,38]. Mucosal MC are identified in intestinal tissues by antibodies against the CD117 (c-kit) antigen[29]. C-kit is a transmembrane, tyrosine kinase containing, growth factor receptor expressed by MC, and its presence on MC membranes represents maturity of the cells[30-32]. In our report, we compared the results of immunostaining with markers of mast cell degranulation (using electron microscopy) and observed that a lack of c-kit immunostaining is not associated with MC activity and degranulation. Whether this MC abnormality underlies MC overactivity in IBD requires further investigation.

Considering MC as the end effector of the BGA, it is not surprising that MC have an important role in the pathogenesis of other stress-related GI disorders such as irritable bowel syndrome (IBS). Barbara showed that the number of MC in ileum of subjects with IBS is increased[39,40]. He also showed that there is a close proximity of the nerve ending and mucosal MC[41]. He noted that MC activation and the close proximity of MC to nerve fibers are correlated with the severity of perceived abdominal painful sensations. The mediators released from MC interact with nerves supplying the gut leading to altered gut physiology and increased sensory perception. This proposes the notion of nerve↔MC activation in stressful situations. In fact, abnormal intestinal permeability has been reported in at least one subgroup of diarrhea -predominant IBS patients[42]. Although, there is a lack of clear histological inflammation in IBS, the apparent presence of a biochemical inflammatory process in IBS is an emerging topic. An abnormal proinflammatory cytokine profile has been reported in subjects with IBS[43,44]. Some researcher have also connected MC and functional bowel disorders such as IBS through allergic responses to food antigens and food intolerance[45]. MC enterocolitis is a new term that was coined by our group and includes a subgroup of IBS with intractable diarrhea who have normal routine histology but an increased number of MC [more than 20 per high power field (HPF)] in special staining for MC. These patients respond well to medicine that curbs the release of proinflammatory MC mediators such as histamine type I and II blockers[46]. Thus, it is not surprising that researchers are now proposing the possibility of using, in management of IBS, drugs that have the potential to control MC[40].

In conclusion, MC is an important component of gastrointestinal tract physiological and immunological functions. As the end effector of the BGA, MC translate the stress signals into release of proinflammatory mediators that can stimulate gastrointestinal nerve endings and affect its perception, change intestinal motility, cause intestinal hyperpermeability and, in susceptible individuals-

those with hyperreactive intestinal immune systems modify the inflammation. Despite the apparent importance of this element in the pathogenesis of several inflammatory and non-inflammatory GI disorders, our knowledge about the role of MC in these disorders is only rudimentary. Further research that more precisely characterizes the role of MC in these diseases could open new doors toward new therapies for IBD and other common GI ailments.

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EDITORIAL

Tissue toxicity induced by ionizing radiation to the normal intestine: Understanding the pathophysiological mechanisms to improve the medical management

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At the present time, more than one-half of all cancer patients are treated with radiation therapy. Despite a good therapeutic index, radiotherapy can disable normal tissue injury to normal tissues in long-term cancer survivors. Thus, an important challenge to modern radiation therapy is to increase the tolerance of normal tissues, in order to improve the quality of life of the patients, and to enhance local tumor control using dose escalation and/or new biological radiosensitizers[1]. The recent progress made by 3D-conformal and intensity-modulated radiation therapy has reduced radiation-induced complications especially in dose-limiting organs like the intestine[2]. Yet, acute intestinal complications do occur but are generally transient, whereas low and mild grade chronic gastrointestinal side effects continue to influence the patient's quality of life. Because the clinical evolution of delayed intestinal toxicity is progressive and inevitable, these complications are of much concern in clinical practice and further improvement in the management of such patients is required.

The current serie published in the *World Journal of Gastroenterology* contains several articles reviewing the most recent research in the field of radiation-induced intestinal toxicity the current therapeutic advances according to the patient's symptoms as well as constructive proposals for the improvement of the medical management of such patients. The proposed improvements depend upon the development of reliable diagnostic tests that are able to identify the underlying causes of the symptoms. The contribution by Lutgens *et al*[3] provides evidence that citrullinemia can be used in clinical and experimental settings as a biomaker of epithelial cell loss. The review by Kruse *et al*[4] addresses the recent advances in the use of micro array for the prediction of radiosensitivity and damage to normal tissues as well as their main limitations.

The physiopathological, cellular and molecular mechanisms of radiation-induced toxicity have been discussed according to the cellular compartments of the intestine and their implication to acute and chronic intestinal toxicity. Tissue exposure to ionizing radiation stimulates the local production of reactive oxygen species which induce replicative and apoptotic death of epithelial and microvascular endothelial cells of the intestinal mucosa. However, the intestinal response to radiation injury cannot be restricted to a simple cell-killing process but depends upon continuous and integrated pathogenic processes involving cell differentiation and crosstalk between the various cellular components within the extracellular matrix. Otterson[5] reviews the impact of irradiation on gastrointestinal motility with special emphasis on the enteric nervous system. Molla *et al*[6], Wang *et al*[7] discuss the role of radiation-induced vascular damage in the acute and delayed intestinal response to ionizing radiation. The radiation-induced alterations of intracellular signaling pathways lead to the transactivation of specific target genes such as genes coding for paracrine factors including vasoactive factors, thrombogenic agents, pro/anti inflammatory mediators and growth factors. The review by Verrechia *et al*[8] focuses on the role of TGF-β1 in fibrogenesis. TGF-β1 is a potent fibrogenic growth factor that triggers alterations in the resident cell phenotypes that subsequently modify cell to cell interactions and tissue composition. Finally, the extracellular matrix itself may contribute to the self-perpetuation of these wound healing signals by the release of growth factors and by constant activation of cell phenotype *via* membrane-associated receptors. The long-term persistence of these phenotypical alterations may have an impact on the activity of neighboring cells (mesenchymal, endothelial, epithelial or immune cells) and may lead to a possible amplification of the wound healing signals that perpetuate fibrosis. This aspect has been well

discussed by Haydont *et al*[9].

It is clear from these articles that the normal intestinal response to radiation injury cannot be restricted to a simple cell-killing process. Therefore, the previous concept of a primary cell target in which a single-cell type (whether it is epithelial or endothelial cell) dictated the whole tissue response to radiation injury should be replaced by the concept of coordinated multicellular response that may lead to either tissue recovery or to the development of complications[10]. The recent advances in this field should lead in the near future to the development of biologically-based therapeutic strategies which can be used clinical practice. These strategies would also be applicable to treat radiation injury in the event of radiation accidents or acts of terrorism and would help to improve the therapeutic management of other chronic intestinal diseases.

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Biomarkers for radiation-induced small bowel epithelial damage: An emerging role for plasma Citrulline

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Abstract

Reduction of cancer treatment-induced mucosal injury has been recognized as an important target for improving the therapeutic ratio as well as reducing the economic burden associated with these treatment related sequellae. Clinical studies addressing this issue are hampered by the fact that specific objective parameters, which enable monitoring of damage in routine clinical practice, are lacking. This review summarizes pros and cons of currently available endpoints for intestinal injury. The metabolic background and characteristics of plasma citrulline, a recently investigated biomarker specifically for small intestinal injury, are discussed in more detail.

Key words: Biomarker; Citrulline; Small bowel; Radiation injury

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INTRODUCTION

An increase in the use of multiple treatment modalities is characteristic for current developments in curative cancer treatment. Whereas this strategy has yielded superior treatment results in a variety of solid tumors, treatment related acute toxicity has increased as well[1-6]. Severe radiation induced intestinal injury occurring during a treatment course has a detrimental effect on treatment outcome in cancer patients due to necessary reductions in treatment intensity and/or treatment interruption. In addition, this acute type of epithelial gut damage has also been suggested as one of several mechanisms contributing to late treatment related sequellae[7]. Cancer treatments related epithelial gastrointestinal toxicity has also been recognized as a significant economic burden[8]. Hence, prevention and/or reduction of epithelial gut damage is expected to have a significant clinical and socio-economic impact. However, clinical studies addressing treatment induced gut damage are hampered by the fact that objective parameters, which enable monitoring of damage in routine clinical practice, are lacking. In case of radiation treatment for pelvic and/or abdominal cancers the small bowel is an important dose-limiting organ with regard to both early and late treatment related morbidity. The clonogenic crypt cell is a central target of intestinal epithelial radiation damage[9-12]. Radiation will result in an impairment or loss of cell production and eventually in the loss of functional cells, becoming manifest within days or weeks following single dose or fractionated radiation[9]. A wide diversity of functional disorders has been observed following ionizing radiation such as changes in trans-epithelial transport processes[13,14], gut barrier function[15], motility dysfunction[16,17], or the absorption of various nutrients such as carbohydrates, amino acids, proteins, vitamins and bile acid[18-26]. Some of these functional changes have been correlated with the epithelial cell mass available for absorption[23-26] suggesting a cellular basis in at least part of radiation induced functional disorders.

BIOMARKERS FOR EPITHELIAL INTESTINAL DAMAGE

Clinical symptoms

Clinical symptoms are most commonly used as a surrogate endpoint during and following treatment. Clinical symptoms of acute radiation enteritis include anorexia, nausea, vomiting, abdominal cramps and diarrhea. These symptoms may occur immediately following the start of treatment, although more usually, radiation sequellae become manifest during the 2nd or 3rd wk of fractionated treatment and lasting 2-6 wk following treatment. Whereas very early symptoms are attributable to altered intestinal motor activity, mucosal injury is the prominent feature underlying symptoms later on during the course

of treatment, although altered intestinal motor activity is another contributing factor throughout the treatment course and following treatment[27-29]. Beside the fact that toxicity-grading systems are not used uniformly by investigators[30], they are being adjusted on a regular basis. More importantly however, clinical symptoms correlate poorly with objective parameters of gut damage such as altered morphology[28,31], sugar permeability tests[32-34] or treatment related parameters[30], illustrating the complexity of the pathophysiology of clinical symptoms related to cytotoxic treatment induced small bowel damage[35-37].

Because of the limitations related to the assessment of morphological endpoints in patients, investigators have used several surrogate endpoints for measuring small bowel dysfunction.

Mucosal transport and barrier function

Mucosal transport and barrier function is another frequently used item for measuring small bowel dysfunction. Surrogate endpoints are the assessment of gut barrier function through measuring absorption of test markers[38,39] or tests for nutrient malabsorption[20,40], bile acid or vitamin B12 absorption[19,22,41-43]. These function tests are qualitative tests mainly suited for diagnostic purposes. The endpoints used do not address damage to target cells. Consequently, they lack a dose response relationship. Although not as troublesome as taking mucosal biopsies, these tests are impractical for monitoring purposes during and following radiation treatment. Enterocyte transport has been used as a surrogate endpoint for epithelial cell mass. Overgaard *et al*[23] demonstrated a dose response relationship for jejunal glucose absorption in mice following single dose upper abdominal irradiation. A linear correlation was observed between jejunal glucose absorption and the absorptive surface. Kirichenko *et al*[26] used a nuclear scintigraphic technique to quantify active enterocyte transport in mice. At 3.5 d after single dose whole body irradiation (WBI) absorption of the isotope correlated significantly with a surrogate endpoint for the jejunal absorptive surface, i.e. the number of cells per villus. A strong correlation was observed between absorption and radiation dose at this time point. For the dose points used in these experiments, i.e. 4, 6, 8 and 12.5 Gy, no correlation was seen between jejunal crypt regeneration, radiation dose and absorption. The results of both experiments[23,26] indicate a cellular basis for the absorptive function and a correlation with the absorptive area. Both function tests were investigated for their applicability as a clinical assay for radiation-induced epithelial cell loss in the gut and indirectly for quantification of radiation damage to the target cell for epithelial small bowel damage. To date none of these assays have been introduced in clinical practice for routine use during and following fractionated treatment, mainly for practical reasons.

Diamine oxidase

Diamine oxidase (DAO), a cytoplasmic enzyme found in almost all organs is present in a particularly high concentration in the epithelial cells of the small intestine[44-46]. Following injury to intestinal epithelial cells DAO is released into the intestinal lumen and intercellular space where it is taken up by lymphatics and blood vessels[45]. Circulating DAO is rapidly cleared by the liver[47]. The plasma DAO activity has been suggested as a candidate marker for measuring ischemic small bowel injury[48-50]. Ely *et al*[51] demonstrated a radiation dose-dependent decline of ileal tissue and plasma DAO activity in rats. Nadir values were observed at 3 d after radiation. At this time point a linear dose-response relationship was demonstrated for plasma and tissue DAO activity at a dose range of 0-6 Gy and 2-8 Gy, respectively. DeBell *et al*[52] investigated the time course of tissue and plasma DAO activity changes following irradiation. They found that the decline of plasma DAO activity preceded the decline of jejunal tissue DAO activity. In addition, the calculated RBE values for both parameters were not the same. These data do not support a direct correlation between the changes of plasma DAO activity and intestinal tissue DAO activity as was in fact also the case for the observation made by Bounous *et al*[49] These authors observed a 7.5 and 1.4 -fold increase in serum DAO activity 24 and 30 h following the onset of symptoms in a patient with a lethal acute intestinal ischemia.

Fatty acid-binding proteins

Fatty acid-binding proteins (FABP) are small (15 kDa) cytoplasmic proteins. Intestinal-type FABP (I-FABP) and liver-type FABP (L-FABP) are produced in small intestinal enterocytes, mainly in the villi, not in the crypt[53,54]. I-FABP has been demonstrated to be a sensitive biomarker for intestinal disease associated with tissue necrosis. Upon small bowel enterocyte necrosis I-FABP and L-FABP are readily shed into the circulation[53]. In ischemic bowel disease a rapid increase in plasma and urinary I-FABP concentration is observed[55,56]. In contrast, I-FABP and L-FABP were not elevated in patients with intestinal disease not associated with a significant degree of tissue necrosis[57]. In transplant recipients histologic graft rejection was not preceded by increased levels of serum I-FABP[58]. Taken together, I-FABP and L-FABP seem to be sensitive biomarkers for ischemic bowel disease. However, its use for intestinal damage initially targeting clonogenic crypt cells, as in radiation induced intestinal damage[9-12] and transplant rejection[59], has been disappointing so far.

Calprotectin

Calprotectin is a protein abundant in neutrophils. The fecal concentration of calprotectin has been identified as a sensitive biomarker of intestinal inflammation[60]. In patients with Crohn's disease the marker correlates with changes in intestinal permeability[61]. The test is highly sensitive. The marker was tested in a validated animal model for late intestinal radiation injury[62,63]. Fecal excretion of transferrin, the rodent analogue of calprotectin, the first 2 wk after treatment correlated with validated endpoints for acute and late intestinal radiation injury. Interestingly, the high sensitivity of the test allows treatment of a limited volume of small bowel. However, in

contrast to these experimental conditions the marker does not allow discrimination of anatomical sites of intestinal injury due to a low specificity[64].

CITRULLINE: A BIOMARKER FOR VIABLE SMALL BOWEL ENTEROCYTES

While radiation-induced tissue damage is unlikely to be expressed or quantified by a single functional or morphological parameter[36], an assay measuring damage to relevant target cells involved in the initiation of tissue damage is of great importance for both experimental and clinical research. Ideally, such an assay must be tissue-specific, display a dose-response relationship and in case of the small intestinal epithelium also a volume-response relationship. In addition, the assay must be easily accessible in clinical practice and independent of experimental conditions such as concurrent medical conditions, medication and nutritional status. Citrulline is a candidate biomarker fulfilling most of these criteria. The assay assesses radiation-induced epithelial cell loss, an important initiating factor in the pathogenesis of acute and chronic intestinal radiation injury and one of several pathophysiological mechanisms underlying clinical symptoms. Citrulline is a nitrogen end product of small bowel enterocyte metabolism. Plasma citrulline has been identified as a biomarker for functional small bowel enterocyte mass under various clinical and experimental conditions. In addition to surgery[65-68], celiac and non-celiac diseases[69], viral enteritis[70] and acute cellular rejection following small bowel transplantation[70-75], cytotoxic treatments was identified as another event associated with decreased plasma citrulline level due to epithelial cell loss[30,76-79]. As a whole, plasma citrulline seems to be a quantitative parameter independent of the underlying cause for epithelial cell loss.

Small intestinal intermediary metabolism

The small bowel epithelium plays an important role in the intermediary metabolism of amino acids, particularly glutamine, citrulline and arginine[80,81] thereby conditioning the availability of dietary amino acids to extra-intestinal organs[82]. Intestinal dysfunction resulting from intestinal diseases or injuries affect intermediary and inter-organ metabolism[83-85]. Hence, any factor affecting the intestinal mucosal cell mass will have an impact on protein and amino acid metabolism[83,86-90]. Since the pioneering work of Windmueller and Spaeth during the 1970's many research groups have demonstrated that amino acids are the major fuel for the small bowel epithelium, both under conditions of fasting and feeding[91-97]. Windmueller and Spaeth identified glutamine as the quantitatively most important arterial energy source[91,98-100] for the rat jejunum in fasted animals. Measurements in different species consistently demonstrated a concentration dependent high rate of intestinal glutamine extraction from the blood. Thus, 25%-33% of the total plasma glutamine is extracted by the small bowel in each single pass[98]. Glutamine is the most abundant amino acid in plasma and plays a key role in whole body protein and amino acid metabolism[98,101]. Organs may be classified as glutamine producers and as glutamine consumers[102]. Skeletal muscle is by far the most important producer and the small bowel the most important consumer. The gut epithelium has been identified as the predominant site of glutamine uptake and metabolism[98]. Of all epithelial cells, enterocytes are the cells mostly responsible for glutamine utilization[103,104]. The first step in enteral glutamine catabolism is the conversion to glutamate and ammonia by the mitochondrial enzyme glutaminase in a non-reversible reaction[105]. The intestinal uptake of glutamine from the blood varies with the availability of substrate in the lumen. However, the intestinal metabolism of plasma glutamine is sustained during competitive luminal substrate provision, even under conditions of luminal overloading with glutamate[91,98]. The gut epithelial cell has access to glutamine from the arterial blood supply and the gut lumen[98]. The metabolic fate of glutamine from both routes is nearly identical indicating a common metabolic pool[91]. Major glutamine carbon products are CO_2 (55%-65%), lactate (8%-16%), citrate (2%-7%), citrulline (4%-6%), proline (5%-6%), alanine (0.5%-4%) and ornithine (0.5%-2%). Major glutamine nitrogen products are ammonia (23%-36%), alanine (33%-36%), citrulline (10%-34%) and proline (7%-10%).

An endproduct of glutamine metabolism

Citrulline was identified as an endproduct of nitrogen glutamine metabolism in the rat intestine accounting for 27.6% of metabolised glutamine[96,98,99]. Citrulline is an intermediate in the urea cycle[106,107], which is comprised of 5 enzymes, 2 being mitochondrial [(CPSI) and (OCT)] and 3 being cytosolic enzymes (arginino succinate synthethase (ASS), arginino succinate lyase (ASL) and arginase). Windmueller and Spaeth[80] did not detect any urea-cycle intermediate following luminal administration of citrulline to the intestinal mucosal cells and concluded that intestinal mucosal cells contain an incomplete urea cycle. However, others suggested a complete urea cycle in rodent enterocytes[108,109]. Wu finally demonstrated urea synthesis in porcine enterocytes from ammonia, glutamine and arginine in a dose-dependent manner, thus providing the evidence that, in addition to periportal hepatocytes[106], small intestinal enterocytes[104,110] contain a complete urea cycle. Whereas an activity was observed of all urea cycle enzymes, the activity of OCT was by far the highest of all (i.e. a factor 10-20)[110]. In contrast to hepatocytes in which CPSI and ASS are considered the regulatory enzymes due to an exceedingly high arginase activity, in enterocytes the arginase activity seems to be the limiting factor for urea synthesis[110]. Given the high rate of glutamine/glutamate metabolism[91,93,96,111] and the relative abundant OCT activity[110] in small intestinal enterocytes, the majority of citrulline produced from glutamine/glutamate[80,104] will not be further metabolised in the urea cycle but instead released in the portal circulation. Thus only 5% of the glutamine-derived ammonia was converted to urea indicating the low capacity of urea synthesis from glutamine (or ammonia) in enterocytes[110]. Hence, although the small intestinal mucosa contains a metabolically significant urea cycle, the liver is without doubt the major

organ for urea synthesis in mammals[106,112]. Furthermore, citrulline can be effectively regarded as an endproduct of glutamine/glutamate metabolism of intestinal enterocytes as suggested by Windmueller and Spaeth[80,98] and confirmed in many studies since then[66,69,83,86-88,90,104,111,113-115].

Pathways for citrulline synthesis

The synthesis of citrulline from glutamine involves 5 mitochondrial enzymes; phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate synthase (P5CS), ornithine aminotransferase (OAT), OCT and CPSI with P5CS being the key regulatory enzyme[104,116-118]. P5CS is unique to small intestinal enterocytes[116,119-121]. PDG converts glutamine to glutamate and ammonia. Glutamate is then converted to pyrroline-5 carboxylate by P5CS. Pyrroline-5-carboxylate is then converted to ornithine by OAT. Glutamine derived ammonia plus HCO_3^- are converted to carbamoyl phosphate by CPSI. Carbamoyl phosphate and ornithine are finally converted to citrulline by OCT. Pyrroline-5-carboxylate is a common precursor of both ornithine and proline. For a long time, glutamine and glutamate have been considered the only precursor for pyrroline-5-carboxylate. Wu *et al*[122] have demonstrated proline oxidase (PROox) activity in porcine enterocytes with the synthesis of citrulline and arginine from proline being another important pathway for citrulline synthesis. This pathway involves 4 mitochondrial enzymes, being PROox[123], OAT, OCT and PCSI. Proline is converted to pyrroline-5-carboxylate by PROox[124]. The subsequent metabolic steps are the same as for citrulline synthesis from glutamine, involving OAT, CPSI and OCT. As a consequence, glutamine-derived nitrogen intermediates such as glutamate and ammonia are necessary for the synthesis of citrulline from proline[122]. Based on the relative enzyme activities[104,125] PROox and CPSI are suggested key regulatory enzymes in citrulline synthesis from proline[122]. Small intestinal PROox activity is relatively high, i.e. 10- and 6-fold greater than the activity in the liver and the kidney of piglets, respectively[123]. Furthermore, the total cell mass of small intestine is relatively large compared to the liver and kidneys, respectively, i.e. 162% (liver) and 970% (kidneys) in 6 wk old pigs[126]. Hence, the small intestine may be a major site of proline degradation and subsequent synthesis of citrulline from proline[122]. In contrast to glutamine, the luminal proline derived from the diet is the most important source of proline for citrulline synthesis[98,122].

Metabolic fate of citrulline released into the portal vein

Under physiologic conditions there is no appreciable uptake of citrulline by the liver[80]. Labelled citrulline was supplied to the liver by a continuous portal infusion at a concentration 1.5 times the usual portal blood concentration. Less than 10% of the labelled citrulline had disappeared from the perfusate after about 40 passes clearly indicating that very little citrulline in the portal blood released by the intestine is metabolised by the liver[80]. Thus citrulline produced and released by the small intestine simply passes through the liver and reaches the systemic circulation. Subsequently the kidney is the major consumer of circulating citrulline extracting about 35% of arterial citrulline in each pass[80,86]. The relevance of this pathway is demonstrated quantitatively by the increase of the plasma citrulline level observed in patients with renal failure[127].

Source of circulating citrulline

It is now generally accepted that the small intestinal absorptive epithelial cell is the major source of circulating citrulline[104,122,128]. Windmueller and Spaeth investigated the existence of alternative sources of circulating citrulline[80]. Within 5 min after exclusion of either the intestine alone or all portal drained viscera from the circulation, plasma citrulline concentration fell by only 27% and 20%, respectively. Hence, more than 70% of the plasma citrulline concentration is sustained. Based on the high rate of citrulline uptake by the kidney accounting for 83% of the citrulline released by the small bowel, it can be estimated that clearance of citrulline from the plasma should be complete after 4.3 min in the rat in the absence of any input[80]. These findings indicate the existence of extra-splanchnic production sites and/or storage sites of citrulline. The cerebrospinal fluid[129] and skeletal muscle[130] are known sites with citrulline concentrations exceeding that of plasma but could not be identified as citrulline releasing sites[80]. Measurement of arteriovenous concentration differences across the hindquarter after complete removal of the portal drained viscera revealed a small net release of citrulline accounting for only 24% of citrulline uptake by the kidney under physiological conditions. Hence, whereas skeletal muscle may be considered a storage site for citrulline, it is not a substantial source for circulating citrulline under normal physiological circumstances[80]. The liver does not release citrulline unless provided with un-physiologically high doses of ammonium in conjunction with high concentrations of ornithine or proline in the perfusate, indicating that all the citrulline formed from ornithine or proline is converted to arginine[80]. This is probably due to the efficient metabolic channelling of citrulline to ASS and the high activity of type I arginase in hepatocytes leading to a subsequent rapid hydrolysis of arginine into urea and ornithine[131]. Hence, despite the results observed with the organ exclusion experiments performed by Windmueller and Spaeth, no other site but the intestine has been identified so far that releases significant amounts of citrulline under physiological conditions. The role of the small intestine as the major source for circulating citrulline is demonstrated by experiments in which the plasma citrulline concentration is reduced by means of small bowel targeted interventions such as specific inhibitors of OAT[116] or OCT[132], yielding a similar decrease of plasma citrulline concentration as observed after small bowel resection[83,86,113,114]. Furthermore, strong lines of evidence have been obtained since then through clinical observations which are in agreement with this concept[66,69,71,73,87,90,133,134]. Experimental and clinical data suggest a non-homogenous distribution of citrulline production. The distribution of P5CS activity in rats was 26%, 31%, 33% and 10% in the duodenum, upper jejunum, lower jejunum and ileum,

respectively[120]. The release of citrulline measured as venous minus arterial concentration in patients admitted for elective gastrointestinal surgery was 30.4 ± 4.0 μmol/L and 8.4 ± 1.7 μmol/L for the jejunum and ileum, respectively[111].

Determinants of intestinal citrulline synthesis and plasma citrulline level

The activity of intestinal citrulline-synthesizing enzymes changes as a function of the feeding regimen, i.e. during the suckling and (post) weaning period. Weaning-induced changes in plasma cortisol levels are suggested to play a role in the difference observed between suckling and weaning animals, rather than developmental changes related to age[135-138]. Except for the interaction of metabolites with specific enzymes[137-141], substrate availability is another determinant of the citrulline production by enterocytes[104,122,140,142]. Several inborn errors[143-148] may give rise to specific changes in citrulline concentration. Enhanced NO synthase activity in patients with SLE has been associated with hypercitrullinemia[149]. Taken the central role of glutamine metabolism in the small intestinal citrulline synthesis[104,122], any metabolic condition substantially influencing intestinal glutamine metabolism is likely to have a major impact on citrulline synthesis as well. In this respect cumulative data indicate an important role for glucose metabolism[140,141,150]. A major determinant, however, under steady state conditions for the rate of citrulline released into the portal and subsequently the systemic circulation is the actual number of functional enterocytes[65-69,83,86,87,90,113,114,134,151]. This was further demonstrated by clinical data on small bowel transplantation[70-75,152]. In addition to a variation in citrulline synthesis, alterations in citrulline utilization will have an influence on the plasma citrulline concentration[80,119,127].

Plasma citrulline a surrogate endpoint for enterocyte mass

Effectively, citrulline can be regarded as an endproduct of glutamine and/or proline metabolism of intestinal enterocytes[80,98,104,110,122]. Several enzymes are involved in the synthesis of citrulline from glutamine and/or proline[104,110,122]. Whereas OAT[116,121] can be categorized as a ubiquitous enzyme, OCT, CPSI, PROox are highly polarized and P5CS is extremely polarized[119]. Thus high activities of OCT[153], CPSI[153] and PROox[121,123] are found in the small intestine and liver. P5CS activity is almost exclusively found in small intestinal enterocytes[116,120,121]. This unique enzymatic profile, the unique role of the small intestine in whole body glutamine metabolism[96,154] and the relatively high small bowel enterocyte cell mass[126] make the small bowel epithelium the most important source of circulating citrulline[128,131]. Taken together these data indicate a high specificity for circulating citrulline, i.e. small intestinal enterocytes. Thus under steady state conditions, citrulline can be considered a marker for the functional epithelial cell mass of the small bowel, a concept amply demonstrated in experimental and clinical studies[65-75,80,83,86-88,90,104,111,113-116,132-134,152]. Of notice, the lower plasma citrulline level observed in short bowel patients was sustained up to one year after treatment[66] emphasizing its strict dependence on the epithelial cell mass. As such, plasma citrulline concentration has been proposed as a biological marker for viable small bowel epithelium[65-67,69,71,73-75,152]. Crenn *et al*[69] have recently correlated plasma citrulline concentration with histologically graded villous atrophy in 42 patients with celiac and 10 patients with non-celiac villous atrophy disease. These authors identified a threshold value of 10 μmol/L (25% of the mean normal baseline value) to be predictive for severe and extensive villous atrophy and 20 μmol/L to be predictive for severe villous atrophy, whatever the extent. The plasma level of citrulline was thus indicative for the degree of villous atrophy. This finding is indicative for a possible volume effect. Crenn *et al*[69] demonstrated the use of plasma citrulline for monitoring treatment response in patients with celiac disease, indicating the simplicity of the marker in clinical practice. The accuracy of the assay has been assessed for various clinical settings. Crenn *et al*[66] measured plasma citrulline level in 57 patients with nonmalignant short bowel syndrome defined by a postduodenal remnant small bowel length of less than 200 cm. Minimal follow up was 2 years after definite digestive circuit modification. The threshold of plasma citrulline that best discriminated short bowel patients from controls was 30 μmol/L yielding a sensitivity, specificity, PPV and NPV of 77%, 75%, 76% and 77%, respectively. The best threshold of plasma citrulline for discrimination of transient from permanent intestinal failure was 20 μmol/L yielding a sensitivity, specificity, PPV and NPV of 92%, 90%, 95% and 85%, respectively. In a series of 52 patients with celiac and nonceliac villous atrophy Crenn *et al*[69] correlated plasma citrulline and mucosal atrophy assessed by endoscopic mucosal biopsies. The threshold of plasma citrulline for discrimination between nondestructive and destructive mucosal lesions (modified Marsh classification) was 20 μmol/L yielding a sensitivity, specificity, PPV and NPV of 95%, 90%, 88% and 96%, respectively. Gondolesi *et al*[75] measured plasma citrulline in 49 intestinal transplant recipients within 12 h before or after endoscopic biopsies taken according to a protocol (i.e. twice weekly for 6 wk, once weekly until 6 mo and monthly until 1 year postintestinal transplant). The sensitivity and specificity of the citrulline assay for diagnosing transplant rejection in adults was 80% and 58%, respectively.

PLASMA CITRULLINE: A SURROGATE ENDPOINT FOR RADIATION INDUCED EPITHELIAL CELL LOSS

Taken together, plasma citrulline is a candidate marker for measuring radiation-induced epithelial small bowel damage. The data indicate that this biomarker is tissue-specific, i.e. small intestinal epithelium. The biomarker corresponds with an important morpholocal endpoint, i.e. mucosal atrophy, and is easily accessible in clinical practice. Although experimental[120] and clinical data[111] suggest a non-homogenous distribution of citrulline production, a volume effect is suggested by the data provided by Crenn *et al*[66,69].

A decrease of intestinal absorptive function following irradiation has been correlated to the loss of

functionally active enterocytes constituting the absorptive mucosal surface[23-26]. The correlation between radiation-induced epithelial cell loss and plasma citrulline level was demonstrated in mice by Lutgens *et al*[76] Following treatment with a single whole body irradiation (WBI) (dose range 0-14.9 Gy) blood and jejunal tissue were sampled for analysis. At 84 h and 4 d after WBI a dose response relationship was observed for plasma citrulline level. At this time point plasma citrulline correlated with mucosal surface, a surrogate endpoint for functional enterocyte mass. Plasma citrulline level decreased as a function of dose and time after WBI. Whereas the time effect was significant for all dose levels used, a significant dose-response relationship was observed only at d 4 after WBI. Remarkably, a rapid decline of plasma citrulline was observed at the first 2 d after WBI independent of the WBI doses used whereas recovery was more rapid for the lowest dose (i.e. 8 Gy) and incomplete during the observation period for the highest dose levels used (i.e. 11 and 12 Gy). This time and dose pattern is in agreement with the radiation effect on the hierarchically structured intestinal epithelium[155]. Interestingly, using the epithelial surface lining as a parameter did not yield significant changes except for the 4 d time point for the highest dose levels (i.e. 11 and 12 Gy) whereas for citrullinemia significant changes were observed for all dose levels used at the 4 d time point. Furthermore, plasma citrulline levels remained significantly decreased at the 11 d time point. For the dose range used in our experiments, mean values for mucosal surface lining ranged between 56% and 130% of control values, whereas for citrullinemia mean values ranged between 6% and 121% of control values. Thus citrullinemia seems to be more sensitive for detecting and monitoring small bowel radiation-induced epithelial cell loss than the representative morphologic endpoint used in these experiments. After WBI doses of 1-3 Gy no effect on citrullinemia could be demonstrated whereas this parameter was inversely proportional to WBI doses of 3-12 Gy. The threshold dose for the citrulline assay (about 3 Gy) is significantly lower as compared to the microcolony assay (about 8 Gy). Furthermore, in contrast to the microcolony assay the citrulline assay permits repeated measurements within the same animal. Therefore the citrulline assay and the microcolony assay are supplementary, both with regard to the dose range as with regard to their applicability.

The use of plasma citrulline as an assay for acute small bowel epithelial radiation injury was demonstrated by Lutgens *et al*[76] Amifostine was administered to mice as a radioprotective agent with a consistently found dose modification factor (DMF) of 1.6 using the microcolony assay as endpoint[156]. The DMF observed for citrulline (1.5) was in complete agreement with literature data. Vanclee *et al*[79] have used the citrulline assay to demonstrate a protective effect of keratinocyte growth factor on cytotoxic treatment induced intestinal injury.

The feasibility of plasma citrulline as a surrogate marker for radiation-induced small bowel injury was demonstrated by Lutgens *et al*[30] in a prospective clinical study in patients treated with fractionated radiotherapy for abdominal and/or pelvic cancer sites. A dose and volume effect was observed using dose volume histogram parameters and plasma citrulline levels as endpoints. Median nadir citrulline levels were observed during the 3rd wk of fractionated radiotherapy. This time course of plasma citrulline was further established in two clinical studies using archive material of patients treated with intensive myeloablative therapy[77,78]. Following conditioning treatment with high dose chemotherapy and fractionated WBI nadir plasma citrulline levels were observed around 7 d after hematopoietic stem cell transplant. Sensitivity and specificity of the citrulline assay were better compared to standard endpoints used for assessment of gut damage[77].

CONCLUSION

Radiation-induced small bowel damage is unlikely to be expressed or quantified by a single functional or morphological parameter. Several biomarkers are currently available differing with respect to kinetics, related target cells and pathophysiological processes involved and the convenience for clinical use. It is thus challenging to choose a (set of) biomarker(s) that is best suited to a specific experimental or clinical setting. Citrulline is a promising candidate biomarker. A dose-response relationship[76] and a correlation with epithelial cell mass[76,79] have been recently demonstrated in experimental studies. The time course of plasma citrulline following radiation[30,76,78] is in agreement with well known radiation effects on the hierarchically structured intestinal epithelium[155] and clinical observations of acute intestinal injury. The feasibility of the marker was demonstrated in a series of patients treated with fractionated radiotherapy for pelvic and/or abdominal cancers[30]. Unlike most other used endpoints, the citrulline assay can be applied to both experimental and clinical settings facilitating translational research. Also citrulline can be repeatedly measured enabling monitoring of treatment effects. Finally, the assay is simple to apply and relatively cheap. Like surgery[65-68], celiac and non-celiac disease[69] and acute cellular rejection following small bowel transplantation[70-73,75,152], ionizing irradiation has been demonstrated to be an additional event associated with reduced small bowel epithelial cell mass that can be monitored by plasma citrulline[30,76-79].

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Radiation-induced intestinal inflammation

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Abstract

Radiation induces an important inflammatory response in the irradiated organs, characterized by leukocyte infiltration and vascular changes that are the main limiting factor in the application of this therapeutic modality for the treatment of cancer. Recently, a considerable investigative effort has been directed at determining the molecular mechanisms by which radiation induces leukocyte recruitment, in order to create strategies to prevent intestinal inflammatory damage. In these review, we consider current available evidence on the factors governing the process of leukocyte recruitment in irradiated organs, mainly derived from experimental studies, with special attention to adhesion molecules, and their value as therapeutic targets.

Key words: Inflammation; Radiation; Leukocyte; Endothelium; Adhesion molecules

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INTRODUCTION

Treatment of malignant tumours by radiotherapy is limited by the need to avoid acute and late damage to healthy tissue. Acute and late effects are increasingly being observed because of the introduction of new aggressive treatment protocols with combined modalities, specially radiotherapy plus chemotherapy protocols, or the application of new technologies allowing to escalate the dose, such as intensity modulated radiotherapy in prostate cancer[1].

Acute radiation damage is most prominent in tissues with rapid proliferating cells, such as skin or alimentary tract. Symptoms develop when functional cell are lost as a part of normal tissue turnover, and are not replaced because of the damage produced to the stem-cell compartment. Normally, there is a compensatory proliferation within the stem cell compartment followed by replacement of functional cells and a final recovery. Nevertheless, sometimes radiation can cause irreversible damage to the vital cellular components.

Vascular injury is also a key determinant of both acute and chronic organ dysfunction associated with irradiation of the gastrointestinal tract. Generally the acute vascular changes resolve, at least in part, but many eventually progress leading to obliterative endarteritis, producing intestinal ischemia and contributing to more extensive mucosal injury, ulceration and necrosis[2]. Progress in molecular and cellular biology has shown that normal tissue response to irradiation not only depends on the response of a single target cell type. Radiation dose delivery can affect the interactions between different cellular systems. This process relies on a dynamic equilibrium and radiation can result in the overproduction of a number of proinflammatory mediators and profibrotic cytokines which produce vascular injury and activation of the coagulation cascade[3].

The objective of this review is to define molecular implication in radiation-induced intestinal inflammation in order to improve therapeutic strategies to prevent radiation side effects.

REGULATION OF LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS

Multistep model of leukocyte recruitment

Intravital microscopy studies have documented an early inflammatory response, appearing only a few hours after irradiation, characterized by leukocyte infiltration into the irradiated organs[4,5]. This early inflammatory response has been implicated in the vascular alterations that result from radiation damage[6].

The development of an inflammatory response is a finely regulated process that involves sequential leukocyte-endothelial cell interactions composed of rolling, activation, adhesion and emigration phases. Adhesion molecules expressed on the surface of endothelial cells (postcapillary venules) and leukocytes, serve to ensure an ordered sequence of cell to cell interactions that

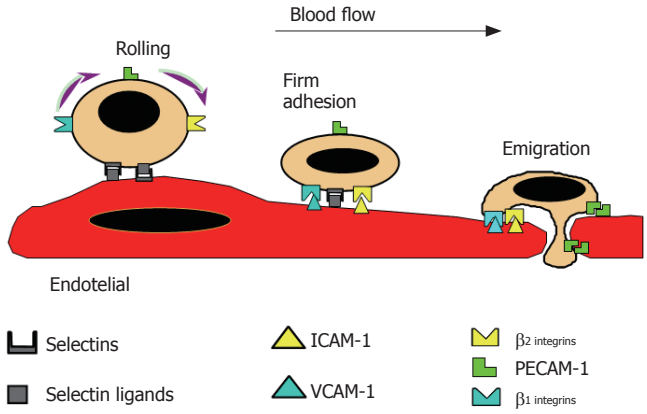

Figure 1 Scheme showing the multistep model of leukocyte-endothelial cell interactions. The leukocyte and endothelial cell receptors that contribute to the different steps (rolling, firm adhesion and emigration) are also illustrated. Reprinted with permission. (review Gastroenterology 1998).

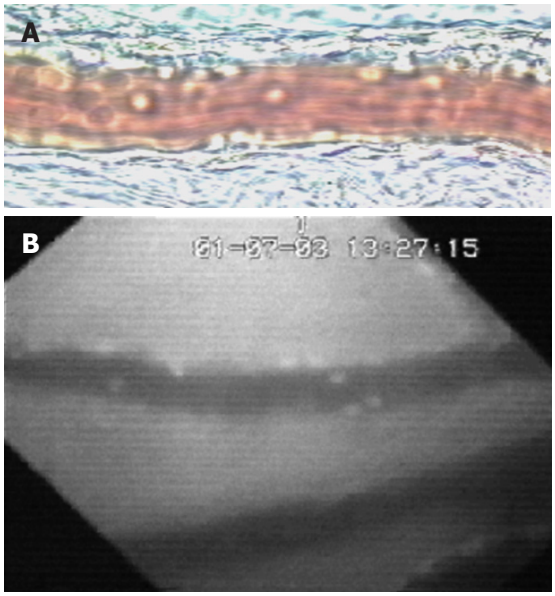

Figure 2 Image of mesenteric (**A**) and small bowel (**B**) venules was obtained by Intravital microscopic. Rolling and firm adherent leukocytes can be easily identified by transillumination (**A**) or fluorescent staining (**B**).

sustain leukocyte adherence to vascular endothelium and subsequent transendothelial migration into the inflamed tissue. The initial event is a weak adhesive interaction that results in leukocyte rolling along the endothelial cell lining of postcapillary venules. Subsequently, there is a strengthening of these adhesive forces that lead leukocytes to become firmly attached to the endothelium and remain stationary (adherence). Finally, leukocytes migrate into the interstitium through spaces between adjacent endothelial cells (Figure 1). These adhesive interactions are regulated by sequential activation of different families of adhesion molecules expressed on the surface of leukocytes and endothelial cells.

Intravital microscopic studies of radiation-induced leukocyte-endothelial cell adhesion have revealed an increased number of rolling leukocyte in mesenteric and intestinal venules (Figure 2) 2 h after irradiation, with a marked increase in the number of firmly adherent leukocytes noted after 6 h and 24 h and decrease 14 d after irradiation[4,8,9]. An increase in oxygen radical production in the vascular wall has been documented as early as 2 h after irradiation with a more intense oxidant stress observed at 6 h, this second burst being produced mainly by infiltrating inflammatory cells.

An increase in the microvascular permeability of mesenteric venules has clearly been shown 6 h after abdominal irradiation. Furthermore, there is evidence indicating that the phenomena of leukocyte adherence plays an important role in mediating these increases in permeability because the immunoneutralization of β2-integrin (CD18) or ICAM-1 results in attenuation of radiation-induced leukocyte adherence and albumin leakage[8].

A possible explanation for the delayed recruitment of adherent and migrant leukocytes in postcapillary venules after radiation exposure is an increased expression of endothelial adhesion molecules. In addition to cytokines are proteins released by irradiated tissues and are implicated in the acute response phase to irradiation[10].

Molecular determinants of leukocyte-endothelial cell adhesion selectins

Selectins and their ligands mediate the phenomenon of leukocyte rolling. Selectines, designed as L-, P-, and E-selectins, represent a family of adhesive receptors expressed on leukocytes (L), platelets and endothelial cells (P), or endothelial cells alone (E). Formed P-selectin is stored in endothelium-specific storage granules called Weibel-Palade bodies and in the alpha granules of platelets. It is then redistributed to the cell surface of platelets and endothelial cells within minutes upon stimulation[11].

Several studies have involved E- and P-selectin in mediating leukocyte-endothelial cell interactions in late and acute inflammation[12,13].

Using P-selectin deficient mice, Mayadas *et al*[14] showed that the initial leukocyte recruitment to the peritoneal cavity in experimentally induced inflammation was entirely P-selectin dependent.

Different studies have reported that *in vivo* P-selectin expression increases after irradiation in lung tissue[15] and in intestinal tissue. Hallahan *et al*[16] showed *in vitro* that the lowest dose that induced translocation of P-selectin to the cell membrane was 2 Gy. Confirmatory evidence has been provided showing that P-selectin expression is significantly up-regulated in response to abdominal irradiation, and this regulation is dose- and time-dependent[6]. However, lack of P-selectin does not afford protection against radiation-induced inflammatory intestinal alterations probably because leukocyte adhesion is not solely a consequence of an increase in P-selectin mediated rolling; there is a release of proinflammatory mediators like PAF[5] or LTB4[17] that may directly affect firm leukocyte adhesion.

Nevertheless, the heterogeneity in constitutive and induced P-selectin expression in different vascular beds suggests that its role in acute inflammation may depend on the tissue in which damage occurs[18].

The role of E-selectin in radiation-induced intestinal inflammation has not been clearly defined. Hallahan *et al*[19] showed that thoracic irradiation increases E-selectin expression in the pulmonary vascular endothelium of mice. In contrast, another study did not detect expression

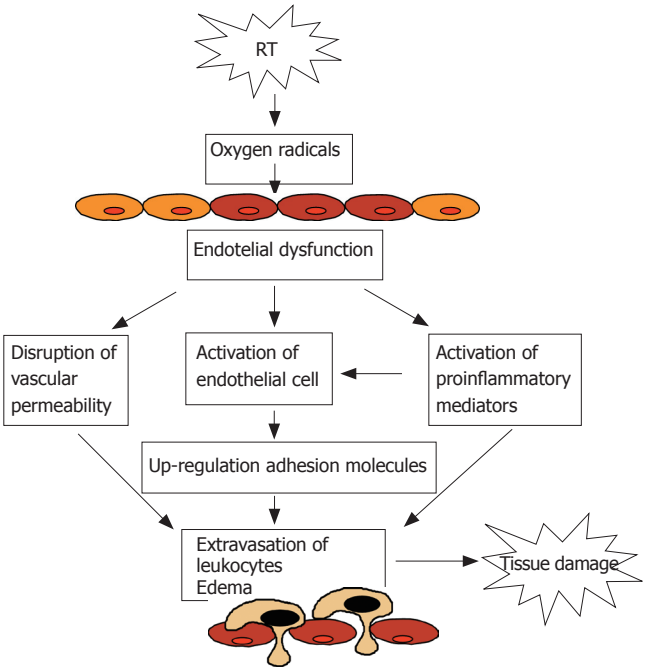

Figure 3 Mechanism proposed to explain leukocyte-cell adhesion and tissue damage caused by irradiation.

of this molecule in wild-type or in P-selectin deficient mice after abdominal irradiation at different doses (4, 10 Gy) and time (2 h or 24 h) points[6]. Recent studies have suggested that E-selectin could be involved in the anti-inflammatory response after radiotherapy, mediating the drop in leukocyte adhesion after low dose irradiation (0.3-0.6 Gy) *in vitro*[20].

Immunoglobulin superfamily

Extravasation of leukocytes from the vasculature requires the participation of molecules of the immunoglobulin superfamily (e.g., ICAM-1 and VCAM-1), which are expressed on the surface of endothelial cells and interact with their respective counter-receptors on leukocytes (integrins) to facilitate inflammatory cell extravasation[10].

A consistent body of evidence indicates[21,22] that ICAM-1 expression is up-regulated following irradiation. Studies performed in experimental models of radiation enteritis[23] support the view that ICAM-1 plays a pivotal role in determining leukocyte adhesion to intestinal venular endothelium in early time points after irradiation; these results were confirmed by two approaches used to test ICAM-1 function by monoclonal antibodies and ICAM-1 deficient mice[9]. Even though the role of a particular determinant of leukocyte recruitment in response to an inflammatory stimulus varies among different organs, the important role of ICAM-1 has been also documented in irradiated lung[22,24]. The molecular determinants of late radiation-induced inflammation seems to differ from the acute condition. It has been shown that at late time points (14 d) ICAM-1 expression returns to baseline levels and blockade of ICAM-1 by monoclonal antibodies has no longer an effect on leukocyte adhesion in intestinal normal tissue. By contrast, VCAM-1 seems to be a key determinant of leukocyte infiltration in the irradiated intestine at late time points, because expression of this adhesion molecule is upregulated late after irradiation, and the increase in leukocyte adhesion is abrogated by VCAM-1 immunoblockade[9]. In other chronic inflammatory models like experimental colitis, such as inflammatory bowel disease, VCAM-1 has also been shown to play a key role as a determinant in leukocyte recruitment in intestinal tissue[25]. These findings support the hypothesis that ICAM-1 and VCAM-1 induction by ionizing radiation mediated in leukocyte recruitment. Pharmaceuticals that block these molecules may prevent radiation-mediated inflammation in normal tissue.

Regulation of endothelial adhesion molecular expression

Regulation of adhesion molecules is determined in part by activation of transcription factors. Of the many transcription factors that have been described, NF-κB and activation protein-1 (AP-1) seem to be particularly relevant to the regulation of endothelial cell adhesion molecules[26-28]. NF-κB is believed to play a pivotal role in the inducible expression of many genes, including cytokines in gut immune and inflammatory response. *In vitro*[29,30] and *in vivo*[31], irradiation induced a cascade of inflammatory responses that involved the transcription factor NF-κB.

Activation of NF-κB in nuclear extracts of intestinal samples in irradiated rats was detectable 30 min after irradiation and was maximal at 60 min, with a progressive decline in the amount of this transcription factor in nuclear extracts over the following 60 min[17]. The radiation-induced inflammatory response is preceded by the NF-κB transcription factor that up-regulated ICAM-1 expression on endothelial cells[32,33]. These findings provide the rationale for manipulation of NF-κB system as a mean of controlling transcription-dependent cellular events that are involved in radiation-induced inflammatory response.

Other important mechanisms that seem to regulate endothelial cells expression was the transcription factor AP-1. The studies suggest that oxidative stress affect AP-1 and NF-κB differently and can be explained by the differential binding of AP-1 and NF-κB to the IL-8 promoter[34]. These cell specific activation signals in endothelial cells may influence the leukocyte recruitment and inflammatory reactions.

CONCLUSIONS

Many factors come into play in the development of radiation induced intestinal damage.

The data derived from *in vivo* and *in vitro* models of radiation-induced intestinal inflammation are generally consistent with the notion that activated endothelial cells produce inflammatory mediators and induce the up-regulation of CD11/CD18 on leukocytes, which along with the oxidant stress cause an NF-κB-dependent increase in expression of endothelial cell adhesion molecules. This results in firm leukocyte adhesion to the vascular endothelium and subsequent extravasation of inflammatory cells into the inflamed tissue (Figure 3). Leukocyte infiltration of irradiated tissues is one the initial histological changes of radiation-induced organ damage.

The recognition that leukocyte recruitment is so critical in the pathogenesis of radiation-induced acute and chronic organ damage has resulted in an intensive effort to define the molecular mechanisms that underlie these cell- cell adhesive interactions. Therapeutic potential of adhesion molecules inhibition provides the opportunity to develop therapeutic strategies that can prevent inflammatory response.

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TOPIC HIGHLIGHT

Marie-Catherine Vozenin-Brotons, PhD, *Series Editor*

Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy

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Abstract

This review summarizes the current state of knowledge regarding the role of endothelial dysfunction in the pathogenesis of early and delayed intestinal radiation toxicity and discusses various endothelial-oriented interventions aimed at reducing the risk of radiation enteropathy. Studies published in the biomedical literature during the past four decades and cited in PubMed, as well as clinical and laboratory data from our own research program are reviewed. The risk of injury to normal tissues limits the cancer cure rates that can be achieved with radiation therapy. During treatment of abdominal and pelvic tumors, the intestine is frequently a major dose-limiting factor. Microvascular injury is a prominent feature of both early (inflammatory), as well as delayed (fibroproliferative) radiation injuries in the intestine and in many other normal tissues. Evidence from our and other laboratories suggests that endothelial dysfunction, notably a deficiency of endothelial thrombomodulin, plays a key role in the pathogenesis of these radiation responses. Deficient levels of thrombomodulin cause loss of vascular thromboresistance, excessive activation of cellular thrombin receptors by thrombin, and insufficient activation of protein C, a plasma protein with anticoagulant, anti-inflammatory, and cytoprotective properties. These changes are presumed to be critically involved in many aspects of early intestinal radiation toxicity and may sustain the fibroproliferative processes that lead to delayed intestinal dysfunction, fibrosis, and clinical complications. In conclusion, injury of vascular endothelium is important in the pathogenesis of the intestinal radiation response. Endothelial-oriented interventions are appealing strategies to prevent or treat normal tissue toxicity associated with radiation treatment of cancer.

Key words: Endothelial cells; Thrombomodulin; Proteinase-activated receptors; Radiation injuries; Radiation enteropathy

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INTRODUCTION

There are currently more than 10 million cancer survivors in the United States[1]. The exponential increase in the cancer survivor population has led to a stronger focus on reducing treatment-related side effects, thus prompting a more proactive approach aimed at acquiring a better understanding of the molecular and cellular basis of treatment-related side effects, and at developing interventions to ameliorate or prevent long term toxicities of cancer therapy.

Approximately 70% of all cancer patients receive radiation therapy at some point during the course of their disease and radiation therapy plays a critical role in 25% of all cancer cures[2]. Recent advances in treatment delivery, such as the development of dose-sculpting techniques, have led to an overall reduction in normal tissue exposure during radiation therapy. Nevertheless, normal tissue radiation toxicity remains the single-most important dose-limiting factor in radiation therapy and a major obstacle to uncomplicated cancer cures.

More than 200 000 patients in the United States undergo localized radiation therapy for abdominal, pelvic, and retroperitoneal malignancies each year. During

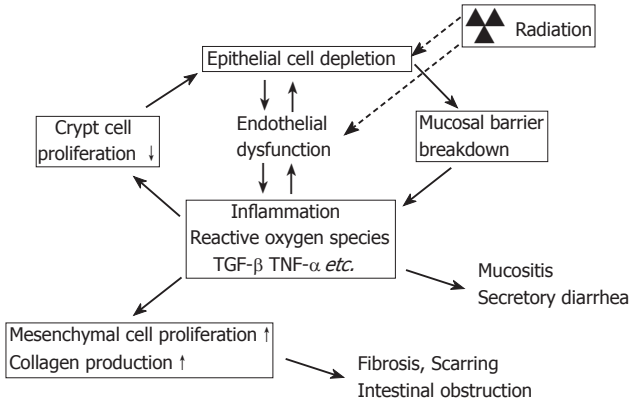


Figure 1 Model of interaction between epithelial and endothelial radiation injury in the intestine demonstrating how endothelial dysfunction may exacerbate the early intestinal radiation response and "drive" the cycle of chronicity of intestinal radiation fibrosis. Radiation causes epithelial crypt cell death, leading to insufficient replacement of the villus epithelium, and breakdown of the epithelial barrier that normally separates intestinal tissue from the intraluminal contents of the intestine. Simultaneously, radiation causes endothelial dysfunction, notably loss of thromboresistance and increased expression of chemokines and adhesion molecules. The combination of loss of epithelial barrier function and endothelial dysfunction enhances the post-radiation inflammatory response, inhibits restitution of the epithelium, and promotes extracellular matrix deposition.

treatment of such tumors, the bowel is almost invariably exposed and the risk of intestinal radiation injury (radiation enteropathy) is often the most important dose-limiting factor.

Radiation enteropathy is classified as early (acute) or delayed (chronic). Early radiation enteropathy occurs during or shortly after radiation therapy. It is a consequence of death of rapidly proliferating crypt cells, resulting in epithelial barrier breakdown and mucosal inflammation (radiation mucositis). Delayed radiation enteropathy, by convention, occurs three months or later after radiation therapy. Chronic radiation enteropathy is characterized by vascular sclerosis and progressive intestinal wall fibrosis, leading to intestinal dysfunction (e.g., dysmotility or malabsorption) and structural injury (e.g., stricture formation, fistulas, or perforation). In addition to radiation-induced cell death, radiation enteropathy is the result of a complex interplay among a plethora of pathophysiological processes, including activation of the coagulation system, inflammation, epithelial regeneration, tissue remodeling and collagen deposition. These processes are orchestrated by a large number of cell types and interacting molecular signals, including cytokines and growth factors, as well as various molecules on the endothelial cell surface[3]. Functional perturbation of these endothelial cell molecules is collectively referred to as endothelial dysfunction.

ENDOTHELIAL DYSFUNCTION IN EARLY AND DELAYED RADIATION ENTEROPATHY

Effects of ionizing radiation on the vascular endothelium

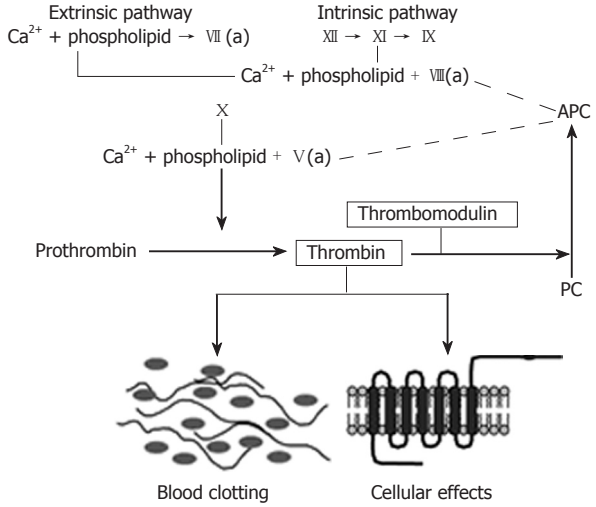
Endothelial cells form the inner lining of blood vessels and cover a total surface area of 4000-7000 m^2[4]. Endothelial cells are highly dynamic and participate in a multitude of physiological functions, including maintenance of blood fluidity, control of vasomotor tone, trafficking of cells and nutrients, and growth of new blood vessels[5]. Under normal conditions, endothelial cells maintain an antithrombotic and anticoagulant balance by exerting molecular control of platelet aggregation, coagulation and fibrinolysis[6].

An increasing body of evidence shows that injury of the microvasculature plays a central role in early and delayed radiation responses in many normal tissues, including the intestine. Notably, microvascular injury may be responsible for the unique self-perpetuating nature of chronic radiation fibrosis[7-13]. A model depicting how endothelial cell dysfunction may contribute to and sustain post-radiation inflammatory and fibroproliferative responses in the intestine is shown in Figure 1.

The high radiation sensitivity of the microvasculature is to a large extent attributable to the endothelial cells[14]. Radiation induces a plethora of morphological and functional alterations in endothelial cells, including apoptosis, detachment from the basement membrane, and increased endothelial permeability, resulting in fibrin deposition in the interstitial space[15,16].

The role of endothelial apoptosis in early intestinal radiation toxicity, particularly in the so-called acute gastrointestinal radiation syndrome, has been a much debated issue for a number of years. The debate originated from reports that mice deficient in the enzyme acid sphingomyelinase are protected from radiation-induced endothelial cell apoptosis, and that these mice also exhibit decreased levels of crypt cell apoptosis and decreased lethality after total body irradiation[17]. Because endothelial cell apoptosis, but not apoptosis of the crypt epithelium, is sphingomyelin-dependent, the interpretation of this finding, together with a substantial body of additional supportive evidence, was that endothelial cell apoptosis appears to be a major contributor to early intestinal radiation toxicity and that there may be a causal relationship between endothelial cell apoptosis and crypt cell apoptosis. There has, however, been considerable controversy related to the extent and significance of endothelial apoptosis in the intestinal microvasculature after radiation exposure, and to whether or not there is a direct relationship between endothelial apoptosis and apoptosis in the crypt epithelium[18]. Despite this controversy, it may be possible to reconcile these seemingly contradictory findings. It is well known from other areas of gastrointestinal pathophysiology that genetic manipulations or pharmacologic interventions that preserve the intestinal microcirculation after an insult have a protective effect on the gut epithelium and the intestinal mucosa. Therefore, it is conceivable that radiation-induced endothelial cell apoptosis may be the bellwether, or "tip of the iceberg" that indicates a state of dysfunction of the intestinal microvasculature, and that it is the state of endothelial dysfunction that adversely affects the radiation tolerance and/or repair capacity of the crypt epithelium.

Loss of thromboresistance is a major feature of endothelial dysfunction after exposure to ionizing radiation. Radiation induces adhesion and aggregation of platelets and development of platelet-fibrin thrombi[19-22],



Figuge 2 The coagulation cascade. Simplified diagram of the coagulation "cascade" with the intrinsic, extrinsic, and common pathways. Note how thrombomodulin, located on the luminal surface of endothelial cells, forms a complex with thrombin, which is converted from a pro-coagulant to an anticoagulant and how activated protein C (APC) limits thrombin generation by feed-back into the intrinsic and common coagulation pathways. See text for further details.

as well as adhesion of inflammatory cells to the endothelium[23-25] with subsequent perivascular leukocyte infiltration. The molecular basis underlying the loss of endothelial thromboresistance is complex and includes increased expression of tissue factor[26,27], von Willebrand factor (vWF)[28-30], and platelet activating factor (PAF)[31]; reduction in fibrinolytic activity[32-34]; and radiation-induced reduction in the expression of prostacyclin (PGI2), the PGI2 receptor[35-37], and thrombomodulin (TM)[12,38]. Studies performed in our laboratory suggest that radiation-induced loss of TM may play a particularly important role in the pathogenesis of radiation enteropathy.

The thrombomodulin-protein C system

Endothelial TM is a transmembrane glycoprotein located on the luminal surface of endothelial cells in most normal blood vessels. TM forms a complex with thrombin, and essentially converts thrombin from a pro-coagulant to an anticoagulant by changing its substrate specificity. Thrombin, when in complex with TM, no longer cleaves fibrinogen to form fibrin and no longer activates cellular thrombin receptors, but instead activates protein C, thereby limiting further thrombin generation and counteracting thrombin's many coagulant, inflammatory, and fibroproliferative effects (Figure 2). In addition, both TM and activated protein C (APC) have important intrinsic anti-inflammatory properties.

Recent studies have demonstrated the importance of TM in attenuation of inflammatory responses in a variety of settings, such as, endotoxin-induced tissue damage, glomerulonephritis, and atherosclerosis[39-42]. One mechanism by which TM exerts its anti-inflammatory properties involves APC. APC inhibits leukocyte chemotaxis and leukocyte adhesion, suppresses inflammatory cytokine production, reduces endothelial cell apoptosis, and maintains endothelial cell barrier function[43-48]. In addition, recent studies have shown that TM has potent intrinsic anti-inflammatory properties by virtue of its N-terminal domain binding and inhibiting high mobility group box 1 protein (HMGB1)[49].

Clinical and preclinical studies performed in our laboratory have shown that radiation causes a striking (80%-90%) and sustained reduction in endothelial TM expression in intestinal microvasculature[12,50,51]. The reduction in TM appears to be due to a combination of direct oxidative damage [52,53], and down regulation of TM at the gene expression level by radiation-induced inflammatory cytokines such as interleukin 1 (IL1), tumor necrosis factor α (TNFα) and transforming growth factor β (TGFβ)[54-57], and increased release of TM from the endothelial cell membrane into the circulation (ectodomain shedding) by granulocyte proteinases and other inflammatory mediators[58].

Thrombin and cellular thrombin receptors

In the normal situation, thrombin is rapidly removed from the microcirculation by complex formation with TM. Local deficiency of TM, such as occurs after irradiation, leads to decreased thrombin clearance and insufficient protein C activation, resulting in accumulation of thrombin. Moreover, the expression of tissue factor, a critical initiator of thrombin generation, can also be triggered by radiation, both *in vitro* and *in vivo*[26,27]. Hence, radiation enhances thrombin generation both through the intrinsic and the extrinsic pathway.

Thrombin induces gap formation between endothelial cells, resulting in increased vascular permeability[59-62]. Consequently, thrombin may pass through the endothelial cell layer into the vessel wall and extravascular tissues. Studies performed in our laboratory show that radiation causes deposition of enzymatically active thrombin on the vascular endothelium, in the vascular wall of small arteries, as well as in the extravascular connective tissue[27]. Thrombin bound to extracellular matrix remains functionally active and able to generate fibrin and interact with surrounding cells[63,64]. We have demonstrated increased deposition of fibrin in irradiated intestine that co-localizes with enzymatically active thrombin[27].

Thrombin, in addition to its central role in coagulation, activates a variety of cell types including endothelial cells, smooth muscle cells, leukocytes, and platelets, thereby enhancing many inflammatory and fibroproliferative processes. For example, thrombin has chemotactic activity for monocytes and leukocytes and stimulates the migration of these cells to sites of injury[65]. Thrombin stimulates fibroblast chemotaxis[66], fibroblast proliferation[67,68], and fibroblast procollagen production[69]. Thrombin also enhances proliferation and migration of smooth muscle cells (SMC) and promotes SMC procollagen synthesis[70-72].

The cellular effects of thrombin are mediated by activation of cell surface thrombin receptors, proteinase activated receptors (PARs), a 4-member G-protein coupled receptor subfamily. Proteinase activated receptor 1 (PAR_1) is the biologically most relevant among the PARs[71-75]. Studies performed in our laboratory show that radiation upregulates PAR_1 expression in endothelium, SMC,

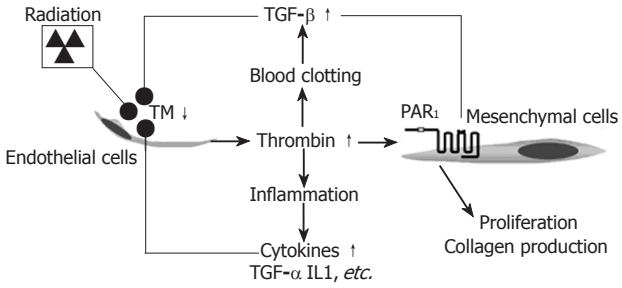


Figure 3 Proposed model linking radiation-induced endothelial dysfunction to chronic inflammation and progressive intestinal fibrosis via chronic PAR_1 activation. Radiation causes TM deficiency in endothelial cells, leading to insufficient "scavenging" of locally formed thrombin. Thrombin exerts pro-coagulant, pro-inflammatory, mitogenic, and pro-fibrogenic effects on mesenchymal cells (smooth muscle cells, fibroblasts, and myofibroblasts), as well as other cell types in the irradiated tissue. Feed-back by cytokines and other inflammatory mediators sustains the endothelial TM deficiency and thus contributes to the chronicity of radiation injury.

and myofibroblasts, particularly in areas of fibrosis[27]. Increased expression of PAR_1 in SMC may be particularly important in the context of intestinal wall fibrosis. This is because, in the intestine, SMC rather than fibroblasts are the predominant producers of collagen. Analogous to our observations in radiation enteropathy, upregulation of PAR_1 occurs in a number of other vascular disorders, including neointima formation after mechanical injury[73], as well as in response to injury-related cytokines and growth factors. Figure 3 depicts a model for how deficient levels of TM after radiation exposure, with subsequent increased thrombin formation and upregulation of PAR_1, may contribute to and sustain inflammatory and fibroproliferative responses in irradiated tissues. Consistent with this model, *in vivo* studies performed in our laboratory have confirmed that scavenging active TGFβ1[76], inhibiting platelet aggregation[77], inhibiting thrombin function[27], mucosal immunomodulation[78], or inhibiting PAR_1 (unpublished data, 2005) all ameliorate various aspects of early and/or delayed radiation enteropathy. These studies are consistent with the notion that thrombin is a key link between downregulated TM and radiation-induced vascular and intestinal fibrosis.

Platelets

Thrombin, in addition to the properties described above, also has major effects on blood platelets. Platelets are the first cellular elements at the site of endothelial injury, where they initiate the hemostatic and inflammatory responses and contribute to the local cytokine milieu[79]. *In vivo* and *in vitro* studies have demonstrated that radiation enhances platelet adhesion[80] and platelet aggregation in the microvascular network[19,20]. The anti-platelet agent, acetylsalicylic acid (ASA, aspirin) may ameliorate certain aspects of intestinal and renal radiation toxicity[81-83].

Platelet adhesion, aggregation, and secretion are regulated by several mediators that are recognized by platelet surface receptors. Thrombin is a powerful platelet agonist and PARs mediate most of the actions of thrombin on platelet function[84]. Hence, PAR_1 activating peptide (PAR_1-AP) triggers complete platelet aggregation similar to the aggregation induced by thrombin. Adenosine diphosphate (ADP) is stored in platelet granules and is released in response to primary agonists, including thrombin. Thus, part of the response of platelets to thrombin is via autocrine and paracrine effects by secreted ADP[85]. In fact, some studies suggest that PAR_1-AP-induced aggregation may be entirely dependent on release of ADP[86]. ADP potentiates multiple platelet responses including the initiation of platelet aggregation (by receptor P2Y1) and the subsequent full aggregation and stabilization of platelet aggregates (by receptor P2Y12)[87,88]. Recent studies from our laboratory and others show that inhibition of ADP-induced platelet aggregation by clopidogrel or ticlopidine ameliorates early and delayed intestinal radiation toxicity[77,89].

Activated platelets directly elicit an inflammatory response by the production of free radicals and by the release of potent inflammatory mediators, such as, TGFβ, PAF, thromboxane, platelet derived growth factor, and IL1, which all contribute to chemoattraction and activation of inflammatory cells[79,90]. The ubiquitous proinflammatory, immunosuppressive, and fibrogenic growth factor, TGFβ, has been implicated in radiation injury such as skin, liver, heart, kidney, lung and intestine[76,91-93]. Platelets contain TGFβ in about 100-fold higher amounts than other types of cells or tissues. We have observed that TGFβ is expressed at significantly higher than normal levels after irradiation[94-96]. Moreover, our studies in radiation enteropathy were the first to demonstrate a mechanistic role for TGFβ in radiation-induced tissue toxicity[76].

Table 1 Potential pharmacological strategies for modulating post-radiation endothelial dysfunction to ameliorate development of radiation enteropathy and some of their respective limitations

Intervention	Major limitation
Platelet aggregation inhibitors	Narrow therapeutic window (bleeding)
Direct thrombin inhibitors	Narrow therapeutic window (bleeding)
Thrombin receptor blockers	Blocks only cellular thrombin effects
Recombinant thrombomodulin	Does not restore endothelial thrombomodulin
Activated protein C	Only partly blocks the effects of preformed thrombin
Statins	Non-specificity
Pentoxifylline	Non-specificity
Vitamin E	Non-specificity and variable efficacy

ENDOTHELIAL-ORIENTED APPROACHES TO MODULATE RADIATION ENTEROPATHY

As described in the previous sections of this review, radiation induces a plethora of changes in the microvascular endothelium. Some of these changes are transient, but may contribute to aspects of early radiation enteropathy. Other changes are sustained and may play direct roles in the pathogenesis of intestinal radiation fibrosis and in the mechanisms of chronicity and progression of injury. The postradiation shift in the thrombohemorrhagic balance toward procoagulation and the accompanying cellular effects that are the consequences of this shift represent particularly promising targets for intervention (Table 1).

Many of the conventional inhibitors of blood clotting have been tested in the attempt to ameliorate normal tissue radiation toxicity. The inconsistent results of these interventions are likely a result of the use of non-specific drugs with multiple actions, use of compounds with dose-limiting side-effects (primarily bleeding), and/or a too narrow focus on coagulation without appropriate consideration of the cellular effects of thrombin and the anti-inflammatory properties of the TM-protein C pathway. For example, while heparin is a highly effective anticoagulant, at therapeutic concentrations heparin reduces the affinity of thrombin for TM and the rate of protein C activation[97], and heparin administered at the time of irradiation actually exacerbates radiation-induced intestinal tissue injury[98]. The direct thrombin inhibitor, hirudin, ameliorates radiation enteropathy, but is less effective than an inhibitor of ADP-induced platelet aggregation, clopidogrel[27]. A possible explanation of these findings may be that thrombin inhibition also reduces thrombin-induced protein C activation and thereby the anti-inflammatory actions of APC. In contrast, inhibition of ADP-induced platelet aggregation targets processes downstream of thrombin and does not influence APC in the same manner. These observations are consistent with results from other studies showing that direct thrombin inhibition enhances leukocyte-endothelial cell interaction in endotoxin-induced sepsis[99] and, despite a favorable effect on collagen accumulation, does not affect inflammatory cell recruitment in bleomycin-induced lung injury[100].

Particularly attractive and presumably safe approaches to modulate radiation-induced endothelial dysfunction are to administer exogenous recombinant TM and APC, to restore endothelial cell TM, and/or to block the downstream effector of thrombin, PAR_1.

Recombinant human soluble TM (rhsTM) is composed of the active, extracellular domain of TM. rhsTM activates protein C[101], reduces thrombin generation[102], and prevents thrombosis *in vivo*[103-105]. The efficacy of rhsTM has been demonstrated in other situations associated with deficiency of endothelial TM, such as disseminated intravascular coagulation, experimental sepsis, and multiple system organ failure[42,105,106]. rhsTM also inhibits smooth muscle proliferation and vascular neointimal hyperplasia[107,108]. Although rhsTM has not yet been tested in the context of radiation toxicity, it is conceivable that rhsTM may be beneficial in normal tissue radiation toxicity. The objective would be to provide TM by the exogenous route for a limited period of time and thus allow TM to regenerate on the endothelial surface.

Synthetic TM mimics are compounds that change thrombin's substrate specificity in a fashion similar to TM and thus cause thrombin to activate protein C[109]. This is a new class of antithrombotic agents that exploits the powerful natural protein C anticoagulant pathway. This approach may be particularly appealing in the context of radiation enteropathy, because localized radiation does not cause protein C deficiency, but rather induces a decrease in local protein C activation due to lack of functional TM. However, while the TM mimics may have a superior therapeutic profile compared to direct thrombin inhibitors, TM mimics suitable for use *in vivo* are not yet available.

Replacement therapy with recombinant APC (rAPC) is another strategy that might allow endothelial function to recover and thus interrupt the vicious cycle that leads to radiation-induced organ dysfunction. APC possesses a number of properties that are different from those of conventional anticoagulants, including potent anti-inflammatory and cytoprotective activities[110-113]. Studies by others have shown that rAPC prevents the lethal effects of E. coli-associated sepsis in animal models and improves the outcome of patients with severe sepsis[114], and that short-term rAPC administration ameliorates lung fibrosis in bleomycin-induced lung injury[115]. Administration of rAPC during the early postradiation phase warrants investigation as an approach to mitigate radiation enteropathy development.

A particularly interesting approach to upregulate and/or restore endothelial TM is treatment with inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, for example, the lipid-lowering statins. In 2003, we and a Japanese group demonstrated independently that statins, in addition to inhibiting the biosynthesis of cholesterol, strongly upregulate TM gene expression, protein levels, and function[116,117] and counteract the effects of TNFα on endothelial TM[116]. It was subsequently shown that statins attenuate radiation pneumonitis[118] and early radiation-induced intestinal toxicity[119]. Whether the radioprotective properties of statins are indeed attributable to their effect on TM expression or to other non-lipid-related statin effects, and whether the beneficial effect of statins in experimental models of normal tissue radiation toxicity can be translated to the clinical situation remains to be shown.

Pentoxifylline as monotherapy or in combination with tocopherol (vitamin E) is another approach that may ameliorate normal tissue radiation toxicity in some tissues by decreasing endothelial dysfunction and restoring endothelial TM. Pentoxifylline is a methylxanthine derivative with potent hemorrheologic properties. It improves blood fluidity by multiple effects such as increasing the deformability of red blood cells and leukocytes, preventing the aggregation of platelets, and decreasing plasma viscosity. It was originally developed for treatment of regional microcirculation disorders such as intermittent claudication and cerebrovascular disease. However, recent studies have shown that pentoxifylline possesses anti-inflammatory and immunomodulatory properties[120-122] and can be used as an adjuvant in the treatment of a diverse group of diseases, including sepsis and severe acute respiratory distress syndrome. Pentoxifylline increases endothelial TM expression and prevents hypoxic- and TNFα-induced reduction in TM expression[123,124]. Pentoxifylline also inhibits TF expression and counteracts activation of the coagulation cascade by endotoxin[125]. Clinical studies suggest that pentoxifylline may reverse radiation-induced chronic skin and subcutaneous tissue fibrosis[126]. Beneficial effects have also been observed in radiation-induced ulcer healing, as well as in radiation-induced toxicity in lung, intestine, uterine, breast, and jaw muscles[127-131]. Nevertheless, a number

of negative animal studies[132,133] and several inconclusive clinical reports highlight the need for further studies to define the benefits, indications, and mechanisms of action of pentoxifylline in radiation fibrosis.

Inhibition of PAR_1 may prove to be a particularly effective strategy to reduce radiation-induced normal tissue toxicity. Because PAR_1 antagonists are specific for the cellular actions of thrombin, it does not interfere with formation of the thrombin-TM complex and therefore does not reduce activation of protein C. Furthermore, since PAR_1 inhibitors do not interfere with fibrin generation, they will likely be associated with fewer bleeding complications than other anticoagulants. Several peptide and non-peptide (small molecule) PAR_1 antagonists are under development[134,135]. Some act on the extracellular portion of the receptors[134], whereas others act as intracellular inhibitors of signal transduction from receptors to G proteins[135]. Studies of PAR_1 inhibition as an approach to reduce normal tissue radiation toxicity are currently underway in our laboratory.

CONCLUSIONS

Normal tissue toxicity, including intestinal radiation toxicity, is the main dose-limiting factor during radiation therapy of cancer. Radiation enteropathy adversely impacts the therapeutic efficacy of radiation therapy, as well as the quality of life of long term cancer survivors. Clinical and preclinical evidence strongly suggests that endothelial dysfunction plays a critical role in the pathogenesis of early and delayed radiation enteropathy. Various endothelial-oriented pharmacological interventions are currently under development for the purpose of preventing or treating radiation enteropathy. Strategies aimed at restoring or preserving endothelial TM or blocking the thrombin receptor, PAR_1, hold particular promise, especially if interventions can be targeted to specific tissues or cellular compartments.

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TOPIC HIGHLIGHT

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Transforming growth factor-β and fibrosis

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Abstract

Transforming growth factor-β (TGF-β), a prototype of multifunctional cytokine, is a key regulator of extracellular matrix (ECM) assembly and remodeling. Specifically, TGF-β isoforms have the ability to induce the expression of ECM proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM. Elevated TGF-β expression in affected organs, and subsequent deregulation of TGF-β functions, correlates with the abnormal connective tissue deposition observed during the onset of fibrotic diseases. During the last few years, tremendous progress has been made in the understanding of the molecular aspects of intracellular signaling downstream of the TGF-β receptors. In particular, Smad proteins, TGF-β receptor kinase substrates that translocate into the cell nucleus to act as transcription factors, have been studied extensively. The role of Smad3 in the transcriptional regulation of type I collagen gene expression and in the development of fibrosis, demonstrated both *in vitro* and in animal models with a targeted deletion of *Smad3*, is of critical importance because it may lead to novel therapeutic strategies against these diseases. This review focuses on the mechanisms underlying Smad modulation of fibrillar collagen expression and how it relates to fibrotic processes.

Key words: Collagen; Connective tissue growth factor; Fibrosis; Smad; Transforming growth factor-β

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INTRODUCTION

Fibrosis is a complex tissue disease whose predominant characteristics are the excessive and abnormal deposition of extracellular matrix (ECM) components[1,2], that may affect various organs, including lung, liver, kidney and skin. From a clinical point of view, fibrosis may be considered as a somewhat irreversible state of scar tissue, during which resolution of the healing process does not occur. Long-term activation of fibroblasts in the affected organs results in massive fibrous ECM deposition and excessive fibroblast/myofibroblast proliferation, thus contrasting with normal wound healing during which feedback mechanisms counterbalance the initial fibroblast activation into myofibroblasts[3].

Much attention is focused on the role of many cytokines and growth factors, a group of diverse molecules derived from blood cells such as platelets, or elaborated locally by mesenchymal and epithelial cells, that contribute to the fibrogenic process[1,4]. Among them, the profibrotic proteins transforming factor-β (TGF-β) and connective tissue growth factor (CTGF) are considered master switches for the induction of the fibrotic program. TGF-β induces fibroblasts to synthesize and contract ECM[5,6], and CTGF, induced by TGF-β, is considered as a critical downstream mediator of TGF-β effects on fibroblasts[7,8]. In this overview, we will discuss the progress made in understanding the central role of TGF-β in fibrotic diseases.

TGF-β AND RECEPTORS ACTIVATION

TGF-β activation

More than 60 TGF-β family members have been identified in multicellular organisms. Among these, there are three TGF-βs, five activins and at least eight Bone Morphogenetic Proteins (BMPs), all encoded by distinct genes (Figure 1)[9]. The three mammalian TGF-β isoforms, TGF-β1, 2, and 3 are secreted as latent precursor molecules (LTGF-β) that contain an amino-terminal hydrophobic signal peptide region, the latency associated peptide (LAP) region and the C-terminal potentially bioactive region[10]. The LTGF-β is usually complexed with latent TGF-β-binding proteins (LTBP), requiring activation into a mature form for receptor binding and subsequent activation of signal transduction pathways. The LTBP is removed extracellularly by either proteolic cleavage by various proteases such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases, or by

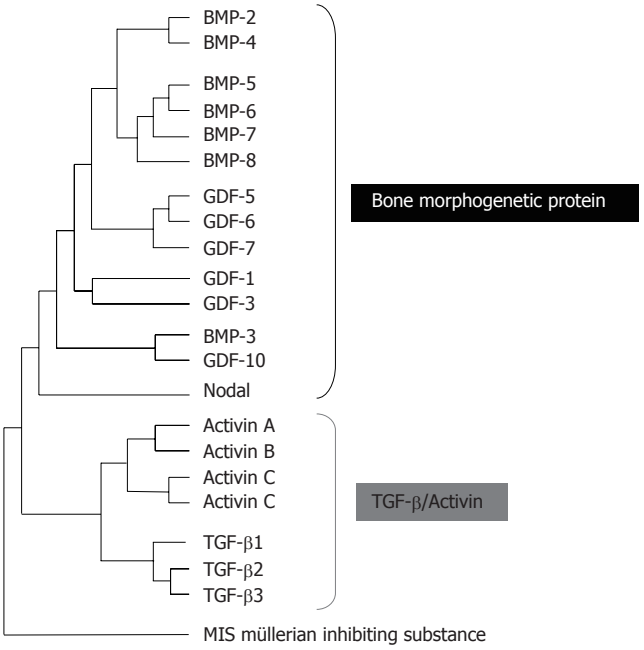

Figure 1 TGF-β family members.

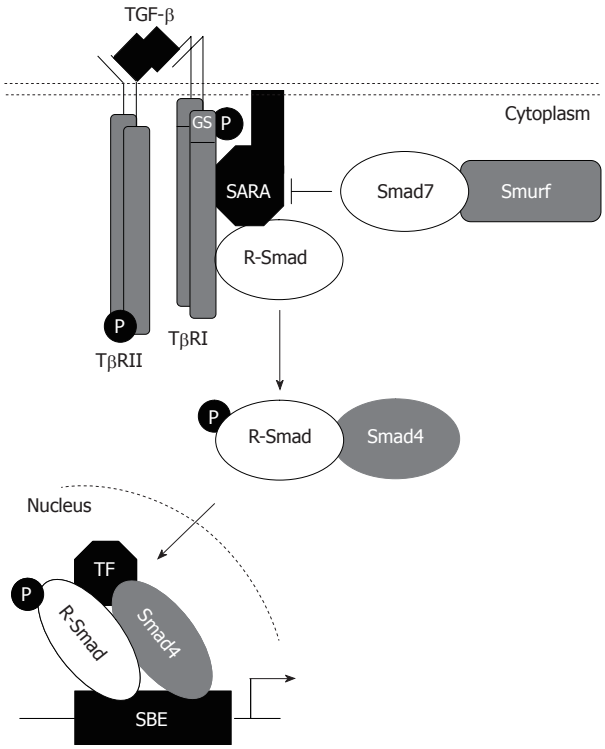

Figure 2 TGF-β/Smad signalling pathway.

physical interactions of the LAP with other proteins, such as thrombospondin-1[11].

TGF-β receptors

Signaling by TGF-β family members occurs through type Ⅰ (TβRI) and type Ⅱ (TβRⅡ) receptors (Figure 2). Five type Ⅱ and seven type I receptors, termed Activin-receptor-like kinases (ALKs) have been identified in vertebrates[12]. TβRI and TβRⅡ are similar transmembrane serine/threonine kinases, but type I receptors have a conserved Gly/Ser-rich (GS box) upstream from the kinase domain. In the absence of ligand, TβRI and TβRⅡ are present as homodimers in the plasma membrane[13]. Ligand binding induces the assembly of type I and type Ⅱ receptors into complexes, within which TβRⅡ phosphorylates and activates TβRI. This phosphorylation event is associated with activation of TβRI kinase and subsequent downstream signalling[12].

TGF-β SIGNALLING BY SMAD PROTEINS

Smad proteins

Signaling from activated TβRI to the nucleus occurs predominantly by phosphorylation of cytoplasmic protein mediators belonging to the Smad family[9]. The receptor-associated Smads (R-Smads; Smad1, 2, 3, 5 and 8) are recruited to activated TβRI by auxiliary proteins such as Smad Anchor for Receptor Activation (SARA)[14]. They all consist of two conserved Mad-homology (MH) domains that form globular structures separated by a linker region[15]. The N-terminal MH1 domain has DNA-binding activity, whereas the C-terminal MH2 domain has protein-binding and transactivation properties. Upon phosphorylation by activated TβRI on two serine residues within a conserved-SS(M/V)S-motif at the extreme C terminus, activated R-Smads form heteromeric complexes with a Co-Smad, Smad4, and are translocated into the nucleus where they may function as transcription factors directly or in association with other DNA-binding factors[9,12,16]. Finally, the inhibitory Smads, Smad6 and Smad7, act in an opposing manner to R-Smads to antagonize signaling. They compete with R-Smads for binding to activated TβRI and thus inhibit the phosphorylation of R-Smads and/or recruit E3-ubiquitin ligases to activated TβRI, resulting in receptor degradation[16]. Additionally, they may recruit protein phosphatase-1 (PP1) to the receptor complex, resulting in the dephosphorylation, thus inactivation, of the receptors via the catalytic subunit of PP1, GADD45[9]. Once in the nucleus, Smad proteins activate transcription through physical interactions and functional cooperation of DNA-binding Smads with sequence-specific transcription factors and with the coactivators CBP and p300. The R-Smads MH1 domain can bind directly to DNA except in the case of Smad2 where a 30 amino acid insertion in this domain prevents DNA binding. The minimal Smad3/4-binding element (SBE) contains only four base pairs, 5'-AGAC-3', but there are reports of binding to other G/C-rich sequences[9,16,17].

TGF-β REGULATION OF EXTRACELLULAR MATRIX GENE EXPRESSION

The net accumulation of collagen in tissue fibrosis is a result of an imbalance between enhanced production and deposition and impaired degradation of ECM components, mostly collagens (Figure 3). To date, about 25 types of collagens have been identified. All collagen molecules consist of three polypeptides, so-called α-chains.

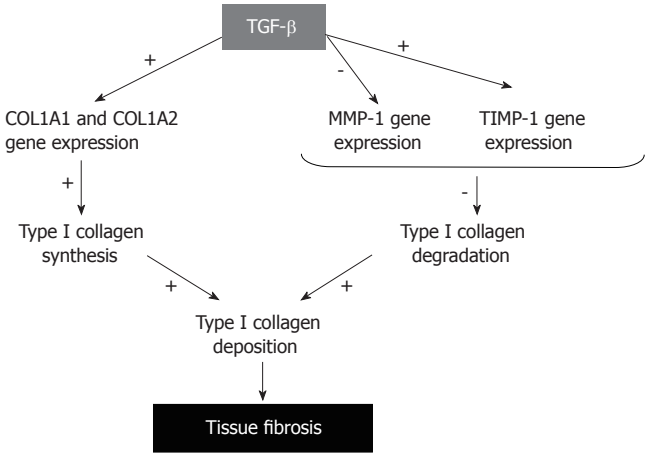

Figure 3 TGF-β, ECM gene expression and tissue fibrosis.

Some collagens are homopolymers with each of the three polypeptides being identical, while other collagens are heterotrimers with two or three distinct α-chains. Type I collagen, the major component of ECM is composed of two α1 (I) chains and one α2 (I) chain which are the products of two genes, *COL1A1* and *COL1A2*. After translation, the pro-α1 (I) and pro-α2 (I) polypeptides chains enter into the endoplasmic reticulum where specific proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine. This event allows the pro-α chains to combine with other chains by hydrogen bonds and form the triple helix procollagen structure. Procollagens are then secreted through the Golgi apparatus in the extracellular space, where the N-terminal and C-terminal propeptides are cleaved by specific proteases. The mature processed collagen molecules aggregates to form larger collagens[18]. Abnormalities in any step of type I collagen production may result in abnormally elevated synthesis of type I collagen which, in turn, causes tissue fibrosis.

Several studies have shown that exaggerated tissue deposition of type I collagen during the fibrotic process is largely due to an increase in the rate of transcription of the corresponding genes[2,19,20]. To date, numerous efforts have been made to identify the signal transduction pathways involved in the transcription of type I collagen genes by TGF-β. Original works demonstrated that TGF-β-responsive sequences regarding the human promoter of *COL1A1* are located between 174 and 84 bp from the transcription start site, which region contains a binding site for Sp1 and an element with the canonical NF-1 binding motif[21]. Regarding the human *COL1A2* promoter, original works demonstrated that a 135-bp region of the promoter within 330-bp of the transcription start site could confer responsiveness to TGF-β[22,23]. The minimal TGF-β-response element was further refined to the region between nucleotides -271 and -235. The latter contains potential overlapping cis-element for Smad and AP-1, which are both implicated in *COL1A2* transactivation by TGF-β[24]. Several Sp1 binding elements contribute to basal gene expression, and may represent targets for anti-fibrotic intervention[25]. Cooperation between Smad3 and Sp1 to transactivate the *COL1A2* promoter have also been described, and it has been shown that Smad-p300/CBP interactions are critical for TGF-β driven *COL1A2* gene transactivation[26,27]. Other transcriptional coactivators such as SRC-1 may also participate in TGF-β effects[28].

By the end of the year 2000, only approximately 12 genes were known to contain Smad-responsive regions, binding Smad complexes directly or indirectly. All Smad gene targets identified downstream TGF-β were Smad3-dependent including *COL7A1*[29], *PAI-1*[30], and *COL1A2*[31]. Using a combined cDNA microarray promoter transactivation approach, we have identified new Smad3/4 gene targets in cultured dermal fibroblasts: *COL1A1, COL3A1, COL5A2, COL6A1, COL6A3*, and *TIMP-1*. In addition, we identified 49 immediate-early TGF-β target genes. Their activation by TGF-β is rapid and does not require protein neo-synthesis or JNK activity. Furthermore, their activation was blocked by overexpression of the inhibitory Smad, Smad7, and did not occur in Smad3-deficient mouse fibroblasts. Thus, we demonstrated that the Smad signaling pathway is crucial for simultaneous activation of skin fibrillar collagen genes (*COL1A1, COL1A2, COL3A1* and *COL5A2*) by TGF-β[32]. Besides playing a large part in the regulation of the expression of ECM components, Smads have been identified as capable of mediating the inhibitory activity of TGF-β on interstitial collagenase (matrix metalloproteinase-1, MMP-1) gene activation by pro-inflammatory cytokines, such as IL-1β[33], another mean by which the Smad pathway is likely to contribute to exacerbated ECM deposition.

TGF-β IN HUMAN SKIN FIBROSIS DISEASES

Keloids represent a dysregulated response to cutaneous wounding that results in an excessive deposition of collagen with a severely debilitating outcome for the affected patients. Several studies have demonstrated that TGF-β1 is expressed at greater levels in keloid fibroblasts when compared with normal dermal fibroblasts[1]. In addition, increased expression of TβRI and TBRⅡ, and increased phosphorylation of Smad3 in keloid fibroblasts, have also been reported[34], supporting the hypothesis that TGF-β/Smad signaling plays a central role in keloid pathogenesis. Furthermore, the activation of Smad signaling, importantly that of Smad3, appears to be one facet of the complex epithelial-mesenchymal interactions in keloid pathogenesis, resulting in active keratinocyte proliferation and collagen production by fibroblasts[35].

Skin tissue fibrosis may also be a sequel of both radiotherapy or accidental exposure to gamma irradiation[36]. Superficial fibrosis is a sequel in humans after radiotherapy[37], and is characterized by induration of the dermis and the subcutaneous tissue. In cases of radiation accidents, high doses of radiation can be delivered to the skin and severe skin burns can be observed, resulting in the development of extensive fibronecrotic tissues[36,38]. The concept concerning the initiation of radiation damage proposes that a cascade of cytokines is initiated

immediately after irradiation, during the clinically silent period, persists for long periods of times, and leads to the development of late damage[39]. The involvement of TGF-β in this early cascade has been reported in various irradiated tissues including skin, intestine, mammary gland and lung[36]. For example, in skin fibrotic samples from soldiers that suffered accidental irradiation in Lilo, Georgia, 1997, gene expression studies for collagen type Ⅰ and Ⅲ, and TGF-β1 showed that these three genes are specifically overexpressed. In addition, TGF-β1 protein was overexpressed in fibronecrotic skin both in the scar epidermis and in the fibrotic dermis[36].

Systemic sclerosis (SSc) is a heterogeneous and generalized connective tissue disorder characterized by micro-vascular and larger vessel lesions, with consequent induration and thickening of the skin, fibrotic degenerative changes in muscles, joints and viscera, mainly the intestinal tract, the heart, the lungs and the kidneys. Although the mechanisms involved in the pathological increase of collagen expression in SSc have not been entirely elucidated, extensive recent efforts have been devoted to study the role of TGF-β signaling pathway by Smad proteins[40]. Immunohistochemical analysis of skin biopsies performed in non lesional areas from SSc patients and analysis of fibroblast cultures showed that Smad2 and Smad3 expression and their nuclear translocation were increased in these SSc patients[41]. More recently Dong *et al*[42]. reported reduction of Smad7 expression in SSc derived fibroblast cultures as compared to fibroblast cultures from unaffected areas of the same patients, suggesting that a defective Smad7 feedback inhibition could play a role in TGF-β hyper-responsiveness in SSc.

TGF-β has also been implicated as being a key mediator in a number of fibrotic diseases in organs other than skin. For example, an increased expression for TGF-β has been documented during the phase of tissue remodeling in several forms of acute or chronic lung disease[43], such as rapid progressive pulmonary fibrosis[44], idiopathic pulmonary fibrosis[45], scleroderma[46], or cystic fibrosis[47]. In the cardiovascular system, mounting evidence supports the notion that TGF-β1 stimulates the progression of cardiac fibrosis during cardiac hypertrophy and heart failure[48]. In the kidney, TGF-β is closely associated with renal interstitial fibrosis, in which normal glomerular tissue is replaced by ECM, leading to organ failure[49]. Epithelial-to-Mesenchymal transdifferentiation induced by TGF-β may contribute to tubular atrophy and generation of interstitial myofibroblasts, leading to concomitant tubulo-interstitial fibrosis[50]. In advanced liver fibrosis resulting in cirrhosis, liver failure, and portal hypertension, TGF-β fibrotic action is broadly associated with its ability to lead transdifferentiation of hepatic stellate cells into myofibroblasts[51].

Smad3, A KEY MEDIATOR OF FIBROTIC PROCESSES

The most direct evidence supporting the involvement of Smad3 in fibrosis came from the use of mice with a targeted deletion of *Smad3*[52]. For example, skin from *Smad3*$^{-/-}$ mice exposed to a single dose of 30 to 50 Gy of gamma-irradiation showed significantly less epidermal acanthosis and dermal influx of mast cells, macrophages, neutrophils and decreased expression of TGF-β than skin from wild type littermates suggesting that inhibition of Smad3 could decrease tissue damage and reduce fibrosis after exposure to ionizing radiations[53]. In another experimental model of fibrosis, mice deficient in Smad3 exhibited suppressed type I procollagen mRNA expression and reduced hydroxyproline content in the lungs compared with wild-type mice treated with bleomycin. Furthermore, loss of Smad3 greatly attenuated morphological fibrotic responses to bleomycin in the mouse lungs[54]. Likewise, transient overexpression of active TGF-β1 in lungs, using adenoviral vector-mediated gene transfer, resulted in progressive pulmonary fibrosis in wild-type mice, whereas no fibrosis was seen in the lungs of *Smad3*$^{-/-}$ animals[55]. Conversely, C57BL/6 mice with bleomycin-induced lungs receiving an intratracheal injection of a recombinant adenovirus expressing Smad7 demonstrated suppression of type I procollagen mRNA, reduced hydroxyproline content, and no morphological fibrotic responses in the lungs, indicated that gene transfer of Smad7 prevents bleomycin-induced lung fibrosis[56]. More recently, using mice with targeted deletion of Smad3, Roberts *et al*[57]. demonstrated that lack of Smad3 prevents the epithelial-to-mesenchymal transition of lens epithelial cells following injury, and attenuates the development of fibrotic sequelae. Together, these various experimental approaches demonstrate the direct implication of Smad3 activation downstream of TGF-β in the pathogenesis of pulmonary fibrosis.

CONNECTIVE TISSUE GROWTH FACTOR

Although TGF-β has long been regarded as a pivotal growth factor in the formation and maintenance of connective tissues and as a major driving influence in many progressive fibrotic diseases, attention has recently focused on the role of connective tissue growth factor (CTGF) in fibrosis. For example, Systemic sclerosis (SSc) fibroblasts demonstrate constitutive over-expression of CTGF that promotes migration, proliferation and matrix production. Specifically, in fibroblasts cultured from SSc lesions, CTGF mRNA and protein are constitutively expressed, even in the absence of exogenously added TGF-β[58]. In normal adult fibroblasts, TGF-β induces the expression of CTGF *via* a functional Smad3 binding site in the CTGF promoter. However, mutation of the Smad binding site does not reduce the high level of CTGF promoter activity observed in dermal fibroblasts cultured from lesional areas of scleroderma patients. Thus, the maintenance of the fibrotic phenotype in scleroderma fibroblasts, as visualized by excess CTGF expression, appears to be independent of Smad-dependent TGF-β signaling[59]. The increased level of CTGF protein and mRNA is also associated with the accumulation of fibroblasts/myofibroblasts and collagen deposition in the persistence of late intestinal radiation fibrosis[60]. Interestingly, Balb/c mice that lack CTGF induction upon stimulation with bleomycin, can be transformed into fibrosis-sensitive individuals by generation of

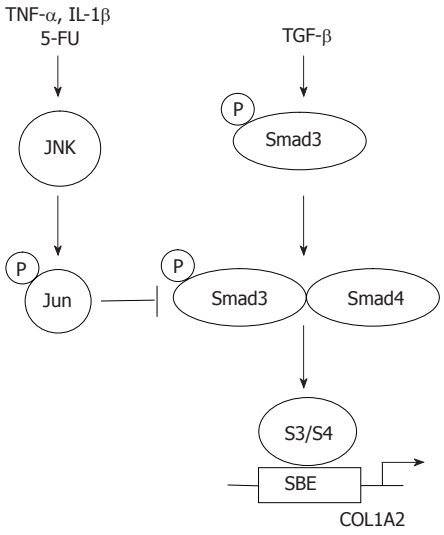

Figure 4 Inhibition of TGF-β-driven COL1A2 transcriptional activity by JNK.

a CTGF-rich environment using transient overexpression of CTGF by adenoviral gene transfer. In this context, silencing CTGF expression with siRNA demonstrated therapeutic potential to prevent liver fibrosis by inhibiting hepatic stellate cells activation[61]. Together these observations suggest that CTGF is an important mediator in the pathogenesis of fibrosis and can be act as an enhancer of TGF-β/Smad3 fibrotic response[62].

PERSPECTIVES FOR THERAPEUTIC INTERVENTION

Tremendous progress has been accomplished over the past several years in the understanding of the initial steps of TGF-β intracellular signalling. The identification of Smad proteins as direct links between the cell surface and the nucleus has allowed for the elucidation of critical events leading to gene activation by TGF-β. Specifically, an increasing body of evidence demonstrates that Smad3 plays a crucial role during the fibrotic process both *in vitro* and *in vivo*. These observations suggest that blocking the TGF-β/Smad3 pathways may promise opportunities for treatment of fibrotic diseases. In particular, several endogenous inhibitors of TGF-β/Smad3-mediated gene expression have been discovered. Firstly, Smad7 induction by IFN-γ, a well known anti-fibrotic cytokine, blocks TGF-β/Smad signalling pathway. In this context, halofuginone a low molecular weight plant alkaloid used as a coccidiostat for poultry, was effective in inhibiting dermal fibrosis in the tight skin mouse of scleroderma, and radiation-induced fibrosis[63-65]. Thus, halofuginone, which has demonstrated efficacy and tolerance in humans, could become an effective and novel therapy for example for liver fibrosis[66]. Secondly, activation of the MAP kinase JNK, whether by cytokines such as TNF-α or by pharmacologic molecules such as 5-fluoro-uracyl, blocks the transcriptional outcome of the TGF-β/Smad3 signaling pathway by induction of c-Jun phosphorylation which, directly interferes with Smad3-dependent transcription (Figure 4)[67-72]. Thirdly, cAMP was shown to inhibit TGF-β Smad3/4 dependent transcription via a protein kinase A-dependent mechanism[73]. However, several hurdles remain before the TGF-β/Smad3 pathway can be considered a perfect therapeutic target in situations such as fibrosis. The identification of alternate signalling pathways for TGF-β remains critically important. For example, the role of Smad2 downstream of TGF-β is rather poorly understood. Identification of Smad2 target genes will likely shed some light on alternate mechanisms by which TGF-β may affect connective tissue remodeling. Likewise, recent evidence for a role of the Rho pathway in the pathogenesis of radiation-induced enteritis suggest that inhibition of Rho pathway by pravastatin, an inhibitor of Rho isoprenylation, may also promise opportunities for new therapeutic perspectives[74].

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BASIC RESEARCH

In situ tumor vaccination with adenovirus vectors encoding measles virus fusogenic membrane proteins and cytokines

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Abstract

AIM: To evaluate whether intratumoral expression of measles virus fusogenic membrane glycoproteins H and F (MV-FMG), encoded by an adenovirus vector Ad.MV-H/F, alone or in combination with local coexpression of cytokines (IL-2, IL-12, IL-18, IL-21 or GM-CSF), can serve as a platform for inducing tumor-specific immune responses in colon cancer.

METHODS: We used confocal laser scanning microscopy and flow cytometry to analyze cell-cell fusion after expression of MV-FMG by dye colocalization. In a syngeneic bilateral subcutaneous MC38 and Colon26 colon cancer model in C57BL/6 and BALB/c mice, we assessed the effect on both the directly vector-treated tumor as well as the contralateral, not directly vector-treated tumor. We assessed the induction of a tumor-specific cytotoxic T lymphocyte (CTL) response with a lactate dehydrogenase (LDH) release assay.

RESULTS: We demonstrated *in vitro* that transduction of MC38 and Colon26 cells with Ad.MV-H/F resulted in dye colocalization, indicative of cell-cell fusion. In addition, in the syngeneic bilateral tumor model we demonstrated a significant regression of the directly vector-inoculated tumor upon intratumoral expression of MV-FMG alone or in combination with the tested cytokines. We observed the highest anti-neoplastic efficacy with MV-FMG and IL-21 coexpression. The degree of tumor regression of the not directly vector-treated tumor correlated with the anti-neoplastic response of the directly vector-treated tumor. This regression was mediated by a tumor-specific CTL response.

CONCLUSION: Our data indicate that intratumoral expression of measles virus fusogenic membrane glycoproteins is a promising tool both for direct tumor treatment as well as for tumor vaccination approaches that can be further enhanced by cytokine coexpression.

Key words: Adenovirus vectors; Measles virus fusogenic membrane glycoproteins; Colorectal cancer; Interleukins

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INTRODUCTION

Induction of tumor-specific immunity is an attractive approach to cancer therapy because of the possibility to harness the body's own defense mechanisms to destroy metastatic tumors and to provide long-term protection against tumor recurrence. The conceptual framework for immunotherapy depends on the presence of tumor-specific antigens and the ability to induce a cytotoxic immune response that recognizes tumor cells presenting antigens. Cytotoxic T lymphocytes (CTLs) recognize major histocompatibility complex (MHC) class I molecules complexed to peptides derived from cellular proteins presented on the cell surface in combination with costimulatory molecules[1]. However, immunotherapeutic application using tumor-associated antigens as a vaccine component is limited to patients with a defined cancer because only few antigens have been identified to date[2]. Some studies circumvent this limitation by utilizing tumor-cell lysates, which probably include both known and unknown antigens[3,4]. The tumor-cell lysate is a very attractive antigen source for the development of versatile cancer immunotherapy. In fact, several studies demonstrated that dendritic cells pulsed with tumor-cell lysates could offer the potential advantage of augmenting a broader T cell-immune response against uncharacterized tumors. However, this method is rather laborious and time-consuming, as the dendritic cells (DC) have to be prepared from the patient's blood for *ex vivo* pulsing with tumor cell lysates and are then reinfused[3-5].

The possibility of eliciting antitumor immunity by *in situ* vaccination by unmasking tumor antigens for

appropriate presentation in a cytokine environment stimulating cell-mediated immunity would abrogate the need to obtain and culture a patient's autologous tumor cells for manipulation *in vitro*, including transduction with cytokine genes, irradiation, and subsequent vaccination.

Using the fusogenic membrane protein G from vesicular stomatitis virus (VSV-G), which triggers cell fusion at pH 5.5, Linardakis *et al* recently demonstrated in a syngeneic murine B16 melanoma model that FMG expression can enhance the efficacy of a weak allogeneic vaccine[5]. Fusogenic membrane glycoproteins (FMG) were introduced as a new class of therapeutic genes for cancer gene therapy by Bateman *et al*[6], who demonstrated that FMG expression alone resulted in a significantly greater tumor growth control than suicide prodrug systems. For cancer gene therapy, glycoproteins from human immunodeficiency virus (HIV-1)[7], gibbon ape leukemia virus (GALV)[8,9], and measles virus (MV)[10] have been evaluated.

Intratumoral expression of viral fusogenic glycoproteins leads to syncytia formation of infected cells with adjacent cells, thereby increasing the dispersion of viruses throughout the tumor, lateral spread of the transgene, virus release and enhanced immunogenicity of tumor cells. In measles virus, the hemagglutinin (H) protein mediates attachment to its receptor on the target cell[11,12] and thus triggers conformational changes in the virus fusion (F) glycoprotein[13]. This leads to a biologically active fusogenic form of the F protein that interacts with the host cell membrane, causing virus-cell or cell-cell fusion[14]. Both H and F proteins are necessary for fusion to occur.

In this study, we assessed whether the intratumoral expression of measles virus fusogenic membrane proteins alone or in combination with local cytokine expression can serve as an *in situ* tumor vaccination strategy for colorectal cancer in two syngeneic bilateral subcutaneous colorectal cancer models in C57BL/6 and BALB/c mice. We evaluated the following five cytokines encoded by replication-defective adenovirus vectors: IL-2, which acts as a growth factor for T, B and natural killer (NK) cells and regulates T cell survival by promoting activation-induced cell death[15]; IL-12, which stimulates proliferation of T as well as NK cells[16]; IL-18, which regulates Th1 and Th2 immune responses[17] and stimulates IFN-γ production from immune cells[18]; IL-21, which has immunostimulatory effects on T and NK as well as dendritic cells[19] and promotes the proliferation of some B cells[19,20]; and GM-CSF, which acts mainly on $CD4^+$ and $CD8^+$ T cells and dendritic cells[21] but can also promote humoral immune responses[22,23].

In several clinical studies, systemic administration of cytokines has been evaluated for the treatment of cancer. High-dose cytokine therapy has proven to be effective in some cases, but there has been a considerable range of adverse side effects limiting the applicability[24,25]. In our study, the cytokines were expressed intratumorally, resulting in local high cytokine concentration and therefore reduced systemic side effects[26].

We monitored the anti-neoplastic effects of the directly vector-inoculated tumor and effects on the growth of the contralateral untreated tumor in a bilateral subcutaneous syngeneic colon cancer model in mice. In addition we analyzed the induction of a tumor-specific cytotoxic T lymphocyte (CTL) response. Our data indicate that intratumoral expression of MV-FMG particularly in combination with cytokine expression can serve as an in situ tumor vaccination strategy for colorectal cancer.

MATERIALS AND METHODS

Cells and cell culture

The human colon adenocarcinoma cell line HT-29 (ATCC HTB-38)[27] was purchased from the American Type Culture Collection (Manassas, VA), and the murine colon adenocarcinoma Colon26 cell line from CLS (Heidelberg, Germany). The murine colon adenocarcinoma cell line MC38 was a gift from Steven A. Rosenberg, NCI, NIH, Bethesda, MD. The human embryonic kidney cell line 293 was purchased from Microbix Biosystems (Toronto, ON).

Viruses

The adenovirus vector Ad.MV-H/F (previously named as Ad CMV F&H[28]), which carries a bicistronic expression cassette H/IRES/F encoding measles virus H and F, was kindly provided by Matthias Dobbelstein, Department of Molecular Oncology, University of Göttingen, Germany. The adenovirus vector Ad.IL-2 encoding human IL-2, which is cross-active in mice[29], has been described previously[30].

The adenovirus vectors Ad.IL-12, Ad.IL-18, Ad.IL-21 and Ad.GM-CSF encoding the murine cytokines IL-12, IL-18, IL-21, and GM-CSF, respectively, were generated using the AdEasy-1 system[31]. The cDNA for mIL12 (pNGVL3-mIL12[32]; kindly obtained from Alexander Rakhmilevich, Department of Human Oncology, University of Wisconsin-Madison, Madison, WI), mIL-18 (pCR3.1:IL-18[33]; kindly provided by Camille Locht, Laboratoire de Microbiologie Génétique et Moléculaire, Institut Pasteur de Lille, Lille, France), mIL-21 (pORF9-mIL21, InvivoGen, San Diego, CA), and mGM-CSF (pGT60mGM-CSF, InvivoGen) were cloned into the adenovirus transfer vector pAd.Track[31].

All adenovirus vectors used in this study were E1 and E3-deleted and produced in 293 cells. All viruses were purified with the Vivapure AdenoPACK 100 kit (Vivascience, Hannover, Germany). The adenovirus particle concentration in purified preparations was determined by spectrophotometry as described previously[34] and expressed as viral particles (VP)/mL. With the used ion-exchange column purification kit we obtained constant particle-to-PFU ratios of about 30:1. The vector Ad.MV-H/F was produced in the presence of the synthetic fusion inhibitory peptide Z-D-Phe-Phe-Gly-OH (10 μmol/L; Bachem AG, Bubendorf, Switzerland)[35]. The functionality of the cytokine encoding adenovirus vectors were determined using cytokine specific ELISA kits (Biosource International, Camarillo, CA and R&D Systems, Minneapolis, MN). For this, 500 000 293 cells were transduced at an MOI of 30 VP/cell in 1 mL with the vectors. Twenty-four hours after transduction with

Ad.IL-2, Ad.IL-12, Ad.IL-18, Ad.IL-21, or Ad.GM-CSF we detected in the supernatants 150, 500, 180, 130, and 500 pg of the respective cytokines.

Quantification of syncytia formation by confocal laser scanning microscopy and flow cytometric analysis

To detect cell fusion, the opposing fusion partners were cytosolically stained with CellTracker Green CMFDA or CellTracker Orange CMTMR (Invitrogen, Molecular Probes, Eugene, OR) according to the manufacturer's instructions and seeded in an equal ratio onto culture slides (BD Biosciences Pharmingen, San Diego, CA). Next morning, 95%-100% confluent cell monolayers were transduced with Ad.MV-H/F at a multiplicity of infection (MOI) of 1000 VP/cell (MC38 and Colon26) and 200 VP/cell (HT-29), respectively. The chosen MOI for all cell lines resulted with Ad5.GFP in ~100% transduction efficiency.

For flow cytometric analysis, 24 h after viral infection cells were detached by trypsin treatment, washed once with PBS and analyzed (FACSCalibur flow cytometer, Becton Dickinson Immunocytometry Systems, Mansfield, MA). For confocal laser scanning microscopy, 36 h after transduction, cells were washed and fixed with 2% paraformaldehyde. Slides were mounted and covered with thin cover slips before analyzing with the confocal laser scanning microscope TCS SP2 + DMIRE2 (Leica, Bensheim, Germany). Dual fluorescence, indicating membrane fusion, was quantified using the ImageJ (Version 1.36b, NIH, Bethesda, MA) software with the colocalization plug-in.

Animal studies

Six to eight week-old female C57BL/6 and BALB/c mice were obtained from Janvier (Le Genest-St-Isle, France). For the syngeneic bilateral subcutaneous syngeneic tumor model, C57BL/6 or BALB/c mice received subcutaneously 1×10^5 MC38 or Colon26 cells, respectively, in 100 μL into the right hind flank and 1×10^4 cells in 100 μL into the left hind flank. Animals were randomly assigned to treatment groups ($n = 5$ for each tumor model) when the tumor on the right hind flank reached a volume of some 200 mm^3 and the tumor on the left side was palpable.

Animals treated just with the Ad.MV-H/F or the cytokine encoding adenovirus vectors received 6×10^9 VP in 100 μL PBS on d 0 and 2 into the right tumor. When Ad.MV-H/F was administered in combination with the cytokine encoding adenoviral vectors, 3×10^9 VP of each vector in 100 μL PBS was injected on d 0 and 2 into the right tumor. At least once a week, minimum and maximum perpendicular tumor axes were measured using vernier calipers, and tumor volume was calculated using the simplified formula of a rotational ellipse ($l \times w^2 \times 0.5$). The skin thickness of 0.4 mm was subtracted from the measurements. Animals were maintained under specific pathogen-free conditions. To generate effector cells, mice were sacrificed and spleens were harvested and weighed 28 d after virus inoculation.

Immunohistochemistry

For sectioning, tumors were embedded in Jung tissue-freezing medium (Leica Instruments, Nussloch, Germany) as described previously[36]. A Leica CM1900 (Leica Instruments, Wetzlar, Germany) cryostat was used to prepare ten micron cryosections. Sections were transferred to microscope slides, followed by acetone fixation at room temperature for 2 min. After three times washing with phosphate buffered saline (PBS), sections were immunostained with rat anti-mouse CD11b (M1/70.15.11.5) fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Miltenyi Biotec Inc., Auburn, CA). Digital images were taken with a high-resolution still camera (Olympus DP50, Tokyo, Japan) attached to a fluorescence microscope (Olympus BX51, Tokyo, Japan).

CTL assay

We analyzed the cytotoxic T lymphocyte (CTL) response to tumor cells, using the lactate dehydrogenase (LDH) based CytoTox 96 (Promega) assay according to the manufacturer's instructions. In brief, target cells (MC38 or Colon26 cells) were plated at a density of 5000 cells per well in round-bottom 96-well plates. Target cells were then mixed with effector cells for 4 h at the indicated ratios. LDH release was determined measuring absorbance at 490 nm with a plate reader, and the specific lysis was calculated from triplicate samples as follows:

$$\text{specific lysis [\%]} = \frac{\text{Experimental } A_{490} - \text{Effector spontaneous } A_{490} - \text{Target spontaneous } A_{490}}{\text{Target maximum } A_{490} - \text{Target spontaneous } A_{490}} \times 100$$

Statistical analysis

The statistical software package SPSS 13 (SPSS Inc., Chicago, IL) was used for data analysis with indicated tests. $P < 0.05$ was considered significant.

RESULTS

MV-FMG expression induces cell-cell fusion and the formation of multinucleated syncytia of colorectal cells

First, using flow cytometry and confocal laser scanning microscopy, we analyzed whether transduction of confluent MC38 and Colon26 cell monolayers with the measles virus H and F encoding adenovirus Ad.MV-H/F results in dye colocalization, indicative of cell-cell fusion. The human colon cancer cell line HT-29 served as a positive control.

As shown in Figure 1A, flow cytometric analyses, 24 h after transduction with Ad.MV-H/F, revealed in the murine colon carcinoma cell lines a slight cell-cell fusion, whereas in the HT29 cells we observed extensive cell-cell fusion. We confirmed these data qualitatively by confocal laser scanning microscopy 36 h after transduction with Ad.MV-H/F (Figure 1B).

Regression of the vector-treated tumor by intratumoral expression of MV-FMG was enhanced by local cytokine expression

We evaluated whether the combination of MV-FMG expression and cytokine (IL-2, IL-12, IL-18, IL-21, or GM-CSF) expression results in an enhanced *in vivo* treatment efficacy of the directly vector-treated tumor and the contralateral tumor, when compared to single agent treatment of the treatment components, in a syngeneic bilateral subcutaneous MC38 colorectal tumor model

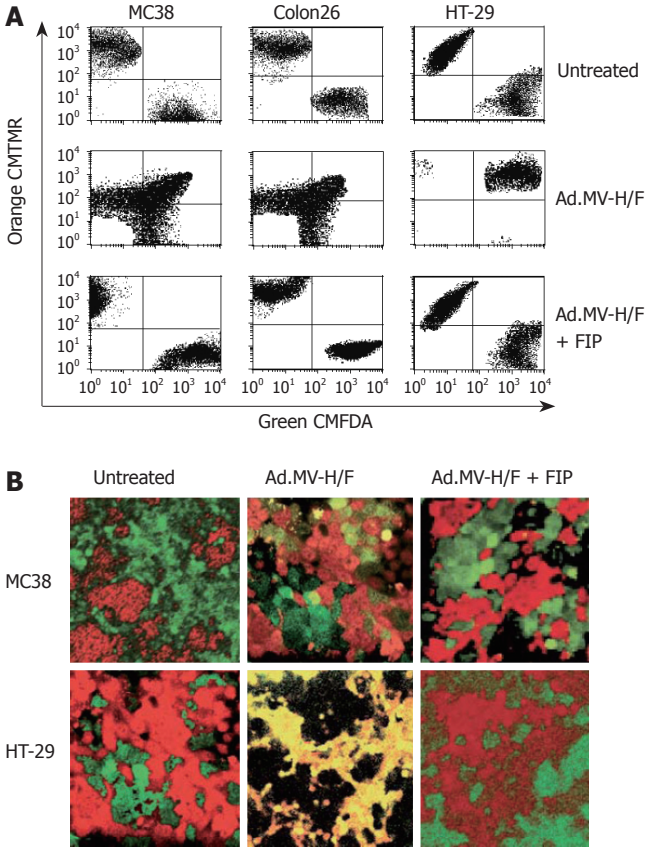

Figure 1 Quantification of cell-cell fusion by flow cytometry and laser scanning confocal microscopy. **A**: Twenty-four hours later cells were analyzed for dye colocalization by flow cytometry. As a control we used the synthetic fusion inhibitory peptide (FIP); **B**: In addition we analyzed the cells 36 h after transduction with Ad.MV-H/F by confocal laser scanning microscopy. Transduction of Colon26 cells with Ad.MV-H/F resulted in similar cell-cell fusion as in MC38 cells (data not shown). One representative experiment out of three is shown.

in C57BL/6 mice. As shown in Figure 2, intratumoral inoculation of Ad.MV-H/F alone resulted in a 51% reduction of the treated tumor at d 28 ($P < 0.005$, ANOVA with Tukey's HSD). Administration of IL-12, IL-18 or IL-21 encoding vector resulted in an about 10% to 47% reduction of directly treated tumor ($P < 0.01$, ANOVA with Tukey's HSD). Treatment with Ad.IL-2 and Ad.GM-CSF produced a 10% reduction of the directly treated tumor (P = NS, ANOVA with Tukey's HSD). Intratumoral administration of Ad.MV-H/F in combination with the IL-2, IL-12, IL-18, IL-21, or GM-CSF encoding vectors resulted in a 87% to 98% reduction of directly treated tumor, respectively ($P < 0.01$, ANOVA with Tukey's HSD).

To assess whether our results are unique to MC38 cells and C57BL/6 mice (H-2b), we repeated the syngeneic bilateral tumor model with Colon26 cells in BALB/c mice (H-2d), which have contrasting susceptibilities to certain intracellular pathogens[37,38]. The experimental design was identical to that described above for MC38 cells in C57BL/6 mice. As shown in Figure 2, the results are qualitatively similar to that obtained with MC38 cells.

The efficacy of MV-FMG expression as a tumor vaccine is enhanced by intratumoral cytokine expression

To determine whether intratumoral expression of measles virus H and F can serve as an in situ tumor vaccination, we monitored the tumor growth of the not directly vector-treated tumor on the left flank (Figure 2). Intratumoral expression of measles virus H/F resulted in a 54% reduction of the contralateral left tumor when compared to mock treated animals, respectively ($P < 0.001$, ANOVA with Tukey's HSD). Intratumoral treatment of animals with IL-2, IL-12, IL-18, IL-21, or GM-CSF encoding vectors resulted in a 8%, 20%, 46%, 32%, or 21% reduction of the not directly vector-treated tumor, respectively ($P \leqslant 0.05$, ANOVA with Tukey's HSD; P = NS for Ad.IL-2). The combination of Ad.MV-H/F with cytokine encoding adenoviral vectors resulted in an about 85% reduction of the not directly vector-treated tumor ($P < 0.001$, ANOVA with Tukey's HSD).

Intratumoral cytokine expression increased measles virus H and F expression induced splenomegaly

To analyze whether the observed growth regression of the not directly vector-treated tumor was immune mediated, we determined on d 29 the spleen weight of the animals (Figure 3A). We observed in both tumor models in animals treated with Ad.MV-H/F about 230% increased median spleen weight when compared to mock infected animals. When compared to mock infected animals, treatment with cytokine encoding adenoviral vectors resulted in about 30% increased spleen weight (P = NS, ANOVA with Tukey's HSD; $P < 0.05$ for Ad.IL-18). The combination of intratumoral Ad.MV-H/F inoculation with the cytokine encoding vectors resulted in a -300% increased spleen weight. The treatment combination of intratumoral MV-FMG and IL-18 expression resulted in the most pronounced splenomegaly. The spleens of representative mice of different treatment groups are shown in Figure 3B.

Local and distant anti-neoplastic effects are associated with the tumor infiltration of macrophages

Shown by immunohistochemistry (Figure 3C), the combination therapy consisting of Ad.MV-H/F and Ad.IL-18 resulted in a strongly enhanced infiltration of macrophages into the not directly vector-treated tumors, when compared to mock or single vector-treated animals.

Intratumoral cytokine expression enhanced measles virus H and F expression-induced tumor cell-specific cytotoxic T cell response

To analyze whether the observed effects on tumor growth regression of the not directly vector-treated tumors were mediated by tumor-specific lymphocyte response, we performed an LDH based cytotoxicity assay. As shown in Figure 4, the effector splenocytes derived from untreated mice without tumor did not lyse target tumor cells (MC38 or Colon26 cells). Splenocytes derived from mock treated tumor bearing animals did not lyse target tumor cells. A slight lysis of target cells was observed for splenocytes of animals treated with Ad.IL-2, Ad.IL-12, Ad.IL-21, or Ad.GM-CSF, while Ad.IL-18 treated animals had the highest CTL activity resulting in 25% cell lysis at an effector to target ratio of 100:1. Splenocytes of animals treated with Ad.MV-H/F showed a cytotoxicity of about 51% at a ratio of 100:1. The combination with the interleukin encoding adenoviruses resulted in a median

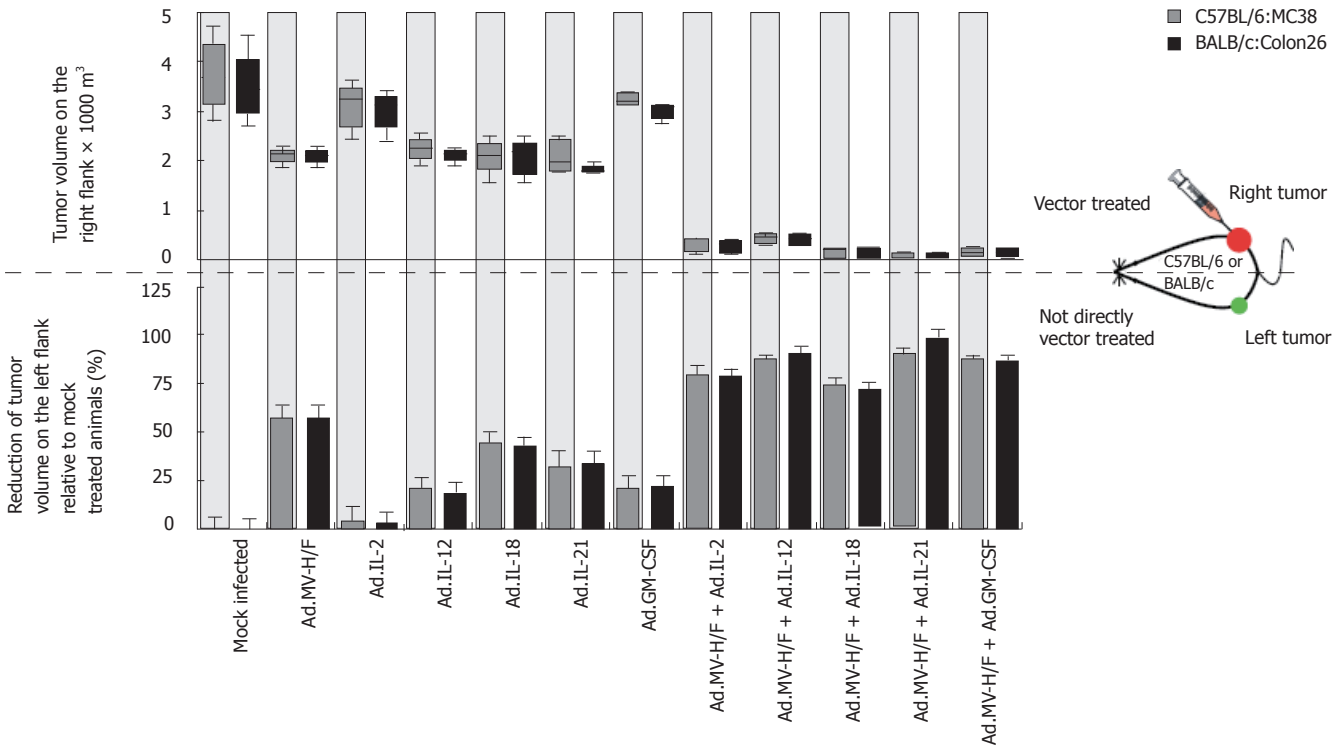

Figure 2 Local and immune-mediated tumor control in a syngeneic bilateral subcutaneous colon cancer model. **A**: The volume of the tumor on the right flank was measured at d 28 and presented as box-and-whisker plots, showing minimum, 25th percentile, median, 75th percentile, and maximum tumor volume; **B**: The volume of the tumor on the left flank, which did not receive direct viral vector injections, was measured at d 28 and the volume reduction relative to mock treated animals is presented as bar graphs (mean ± SD). Data of C57BL/6 are presented in white and the data of BALB/c are presented in orange.

cytotoxicity of 76% at an effector to target ratio of 100:1, whereas the highest cytotoxicity was observed with the splenocytes from Ad.MV-H/F and Ad.IL-21 treated animals.

DISCUSSION

The intratumoral expression of viral fusogenic membrane proteins is a promising approach for cancer gene therapy, since their expression in tumor cells is directly cytotoxic and associated with a local bystander effect[6] but can also induce an anti-tumor immunity[39,40]. Whether the expression of measles virus H and F in murine cells results in cell-cell fusion remains controversial. There have been reports that no cell-cell fusion occurs in murine cells upon expression of MV-FMG[41], while others demonstrated cell-cell fusion upon measles virus FMG expression in highly confluent murine cell monolayers[42,43].

In this study, we demonstrated dye colocalization by flow cytometry and confocal laser scanning microscopy in murine cells upon MV-FMG expression, indicating cell-cell fusion[44]. Fused murine cells were smaller and cell-cell fusion occurred to a lesser extent than in human cells, but fusion was clearly due to measles virus H and F expression since cell-cell fusion could be blocked by adding a measles virus-specific fusion inhibitory peptide[35].

The key findings of the colorectal cancer models in C57BL/6 and BALB/c mice can be summarized as the following. First, intratumoral expression of measles virus H and F by the adenovirus vector Ad.MV-H/F resulted, despite the limited intratumoral spread and transduction efficiency of the replication-defective adenovirus vectors, in tumor regression of the directly vector-treated tumors, confirming previous studies[6]. Due to the host specificity of adenovirus, generally human adenovirus will not infect murine cells productively[45]. Thus a trans-complementation of the replication-defective vectors for replication[46] to improve tumor transduction efficiency[47] is not possible in this model. Second, we confirmed that FMG expression can serve as a tumor vaccination platform[5,39], since we observed regression of the not directly vector-treated tumor. Third, intratumoral expression of IL-12, IL-18 and IL-21 resulted in reduction of both the directly vector-treated and the contralateral untreated tumor. However, in both models the intratumoral expression of IL-2 did not result in a regression of the contralateral tumor. There have been several studies examining the tumor therapy potential of interleukins, mostly IL-2 and IL-12, administered as recombinant proteins or expressed from DNA plasmid vectors in tumor vaccination trials mostly in combination with chemotherapy[48,49]. Fourth, intratumoral expression of MV-FMG in combination with the cytokines IL-2, IL-12, IL-18, IL-21 or GM-CSF, encoded by adenovirus vectors, resulted in a significantly improved treatment efficacy of the directly vector-inoculated tumors, but also of the contralateral, not vector-treated tumors, when compared to single agent therapy. Fifth, treatment of animals with the combination of Ad.MV-H/F and or cytokine expression induced tumor-specific cytotoxic T lymphocyte responses and a massive increased spleen weight. This suggests that the cytoreductive effects of MV-FMG expression alone and in combination with

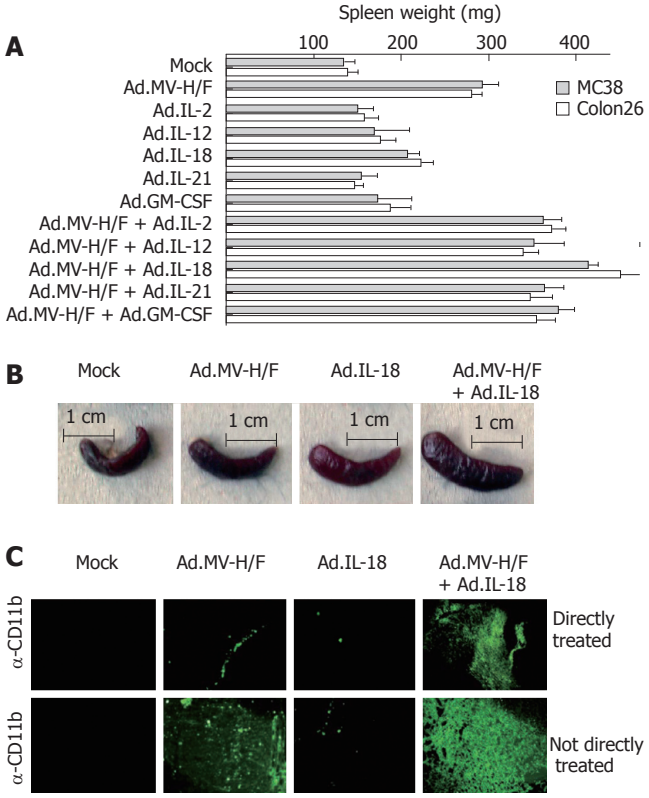

Figure 3 Effect of indicated treatments on the spleen weight and infiltration of tumors with macrophages. Treatment was carried out as described in Figure 2. **A**: At d 29 animals were euthanized and spleen weight was determined (mean ± SD); **B**: The spleens of representative mice of different treatment groups are shown; **C**: In addition, fourteen days after initiation of therapy continous serial sections of the directly and not directly vector treated tumors of spare animals were prepared and individually immunostained for indicated cells. Representative slides are shown; original magnification × 400. Similar data were obtained in the Colon26 tumor model (data not shown).

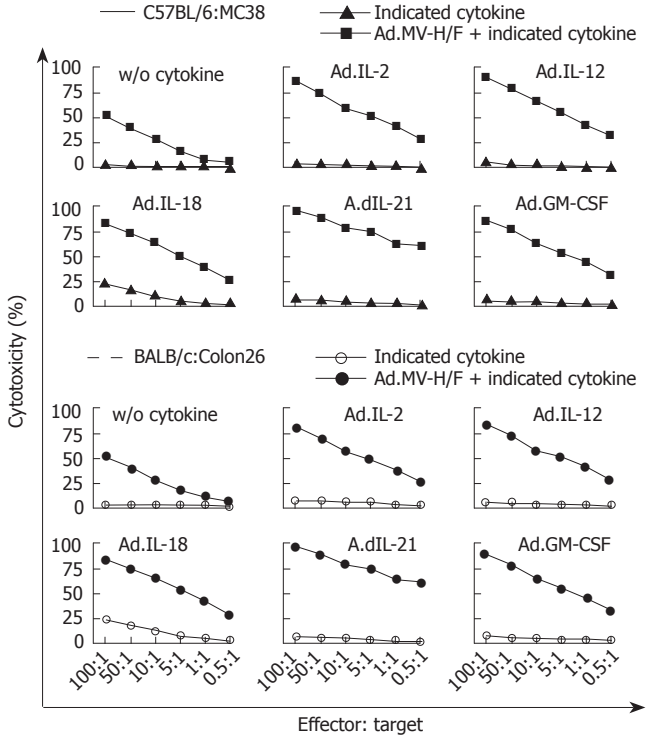

Figure 4 T cell mediated tumor regression by expression of MV-FMG alone or in combination with cytokines. Data of all animals were expressed as the percentage of specific release of three independent experiments (mean ± SD).

intratumoral cytokine expression on the not directly vector-treated tumors were immune mediated.

A conceivable mechanism for the induction of tumor-specific immunity by expression of measles virus fusogenic membrane proteins and cytokines are the xenogenization of tumor cells by presentation of viral antigens on the cell surface in conjunction with major histocompatibility complex class I molecules leading to cytotoxic T lymphocyte (CTL)-mediated tumor cell destruction[50,51]. Furthermore, the expression of FMG has been postulated to result in an efficient presentation of tumor antigens on antigen-presenting cells having taken up debris of apoptotic cells or exosomes of fused cells[52,53]. The impact of MV-FMG expression seen in our study confirms the findings published to date, demonstrating that dendritic cell (DC) maturation and naïve T cell activation are effectively primed upon contact with FMG-transduced, syncytia forming tumor cells[40]. Furthermore, we observed by immunohistochemistry a pronounced tumor infiltration with macrophages of animals that received intratumoral injections of adenovirus vector encoding measles virus H and F in combination with intratumoral IL-18 expression. Previously, Shimura *et al*[54] demonstrated that tumor-associated macrophages are inversely correlated with tumor progression in human prostate cancer, since macrophages provide important antigen-presenting functions[55].

In summary, our data demonstrate that the intratumoral expression of measles virus fusogenic membrane glycoproteins in combination with intratumoral cytokine expression gives the best results with regard to the anti-neoplastic effects on the directly vector-treated tumor, and also with regard to the induction of an anti-tumor immunity affecting an untreated tumor. To further improve the treatment efficacy, it should be advantageous to use an oncolytic vector expressing the FMG and cytokines, most likely resulting in a more efficient liberation of potential tumor-associated antigens[56].

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BASIC RESEARCH

Overexpression of the cholesterol-binding protein MLN64 induces liver damage in the mouse

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Abstract

AIM: To examine the *in vivo* phenotype associated with hepatic metastatic lymph node 64 (MLN64) overexpression.

METHODS: Recombinant-adenovirus-mediated MLN64 gene transfer was used to overexpress MLN64 in the livers of C57BL/6 mice. We measured the effects of MLN64 overexpression on hepatic cholesterol content, bile flow, biliary lipid secretion and apoptosis markers. For *in vitro* studies cultured CHO cells with transient MLN64 overexpression were utilized and apoptosis by TUNEL assay was measured.

RESULTS: Livers from Ad.MLN64-infected mice exhibited early onset of liver damage and apoptosis. This response correlated with increases in liver cholesterol content and biliary bile acid concentration, and impaired bile flow. We investigated whether liver MLN64 expression could be modulated in a murine model of hepatic injury. We found increased hepatic MLN64 mRNA and protein levels in mice with chenodeoxycholic acid-induced liver damage. In addition, cultured CHO cells with transient MLN64 overexpression showed increased apoptosis.

CONCLUSION: In summary, hepatic MLN64 overexpression induced damage and apoptosis in murine livers and altered cholesterol metabolism. Further studies are required to elucidate the relevance of these findings under physiologic and disease conditions.

Key words: Metastatic lymph node 64; Apoptosis; Cholesterol; Liver; StarD3

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INTRODUCTION

MLN64 (metastatic lymph node 64) cDNA was originally discovered as a highly expressed and amplified gene in certain breast, gastric, and esophageal cancers[1-3]. Although MLN64 could play a causative role in tumorigenesis, its amplification probably reflects the close genomic proximity (within 36 kb) to the oncogene c-*erb*-B2 (*her*-2/*neu*), which is invariantly coamplified[3,4]. The N-terminal of MLN64, the so-called MENTAL domain, includes four transmembrane helices, whereas the C-terminal domain contains the StAR-related lipid transfer domain (START)[5,6]. The latter domain is present in proteins involved in diverse cell functions[6,7] and exhibits 37% identity with StAR[5,6]. There is a family of proteins with homology to StAR, each containing the 200-210-aa START domain, in which MLN64 is known as StarD3[5,6].

Like StAR, the isolated MLN64 START domain binds[5] and transfers[8] cholesterol *in vitro* to the mitochondria and stimulates steroidogenesis when cotransfected with the cytochrome P450scc[8,9]. However, full-length MLN64 is less active in steroidogenic assays since its transmembrane domain localizes it to late endosomes with the START domain facing the cytosol[8,10]. For this reason, the role of MLN64 in steroidogenesis is still unclear, but it has been suggested that proteolysis could release the START domain to allow delivery of cholesterol to mitochondria[8]. This putative role for MLN64 in steroidogenesis has led to

speculation that high levels of MLN64 observed in some breast carcinomas could contribute to the progression of these tumors through increased intratumoral steroidogenesis[4].

The localization and topology of MLN64 in late endosomes suggest that this protein participates in the efflux of cholesterol from late endosomes and lysosomes, possibly together with other proteins such as Niemann-Pick type C (NPC)1 and NPC2[10,11]. NPC disease is a lysosomal cholesterol-storage neurodegenerative disorder that is caused by deficiency of either NPC1 or NPC2[12,13]. Interestingly, expression of a truncated MLN64 protein lacking the START domain caused accumulation of free cholesterol in lysosomes of COS-1 and CHO cells, causing an NPC-like cellular phenotype[8]. These results suggest that MLN64 plays a role in the maintenance of endosomal cholesterol flow and intracellular cholesterol homeostasis. However, the overexpression of full-length MLN64 also induced an increase in sterol accumulation in COS cells, arguing against a role for MLN64 in cholesterol efflux from this compartment[14]. Moreover, mice with targeted mutation of the MLN64 START domain were neurologically intact and fertile, and exhibited only modest alterations in cellular sterol metabolism[15], leaving unanswered the question of MLN64 function *in vivo*.

MLN64 expression is detected in all tissues[16]. In the liver, MLN64 could mobilize cholesterol into mitochondria where it should be oxidized in the first step of the acidic pathway for bile acid synthesis[17]. Indeed, Pandak *et al*[18] and Ren *et al*[19,20] demonstrated that overexpression of the closest homologue of MLN64 in liver cells, StAR, leads to an important increase in bile acid synthesis both *in vitro* and *in vivo*. However, MLN64 overexpression in primary rat hepatocytes produced only a 20% increase in the rate of bile acid synthesis, suggesting that the hepatic expression of this protein is not a major determinant of the transport of cholesterol to the mitochondria during bile acid synthesis[19].

Even though MLN64 has been suggested to participate in intracellular cholesterol mobilization and steroidogenesis, its physiological function remains unclear. Interestingly, MLN64 cDNA has also been associated with other cellular processes such as the actin-mediated dynamics of late endocytic organelles[14] and apoptosis, since a differential-display analysis identified the gene as being downregulated in retinoic acid-induced apoptosis in T-cell lymphoma[21]. In the present study, we studied the *in vivo* phenotype associated with MLN64 overexpression in liver using an adenovirus-mediated overexpression strategy. We found that mice with hepatic MLN64 overexpression exhibited significant liver damage and apoptosis, and some minor alterations to cholesterol metabolism.

MATERIALS AND METHODS

Animals and diets

C57BL/6J mice originally purchased from Jackson Laboratory (Bar Harbor, ME) were bred to generate our own colony. All mice had free access to water and a chow diet (< 0.02% cholesterol; Prolab RMH 3000, PMI Feeds, St. Louis, MO). In some experiments, 2-mo-old C57BL/6J mice were fed chow supplemented with 2% chenodeoxycholic acid (CDCA) for 1 or 2 d.

Protocols were performed according to accepted criteria for the humane care of experimental animals, and were approved by the review board for animal studies of our institution.

Table 1 Primers for gene expression analysis in MLN64-overexpressing mouse livers

Gene	Primers	Fragment size
bax	TATTGGTGAGTCGGATTGC (S) TGGACGGTCAGTGTCTGG (AS)	230 bp
mdm2	CCAACATGTCTGTGTCTACCG (S) ACAATGTGCTGCTGCTTCTC (AS)	216 bp
mln64	TCGACATCTTTGTTCTGGCT (S) GAGCAACTCAGAAAGGATGAC (AS)	148 bp
18 S	GTAACCCGTTGAACCCCATT (S) CCATCCAATCGGTAGTAGCG (AS)	151 bp

S: sense; AS: antisense.

Preparation and administration of recombinant adenoviruses

The recombinant adenovirus encoding the full-length murine MLN64 cDNA (Ad.MLN64), under control of the cytomegalovirus (CMV) promoter, was generated by homologous recombination in bacterial cells using the AdEAsy system (generously provided by Dr. Bert Vogelstein, The Johns Hopkins University, Baltimore, MD)[22]. The control adenovirus Ad.E1Δ, containing no transgene, was kindly donated by Dr. Karen Kozarsky (SmithKline Beecham Pharmaceutics, King of Prussia, PA). Large-scale production of recombinant adenoviruses was performed from infected HEK 293 cells as described previously[23].

For viral administration, mice of 2 mo-old were anesthetized by ether inhalation, the femoral vein was exposed, and 1x10e11 viral particles (in 0.1 mL of isotonic saline buffer) of control or recombinant adenoviruses were injected intravenously. An additional control group received 0.1 mL of saline buffer only. Animals were studied 12-24 h after adenoviral infection.

Gene expression analysis by semiquantitative RT-PCR

Five micrograms of total liver RNA from individual animals was reverse transcribed using random hexamer primers. Semiquantitative RT-PCR was performed in the presence of [α-32P]dCTP for *bax* and *mdm2* genes using primers based on mouse and rat cDNA sequences available in GeneBank databases (Table 1). After an initial 5 min 94℃ denaturation step, thermal cycling involved 28 cycles at 94℃ for 30 s, 52℃ for 30 s, and 72℃ for 30 s for *bax* mRNA analysis and 29 cycles at 94℃ for 1 min, 52℃ for 1 min, and 72℃ for 1 min for *mdm2* mRNA analysis.

Gene expression levels were compared to coamplification of 18S internal standards (Ambion, Austin, TX). PCR products were resolved by 2% agarose gel electrophoresis and transferred to a nylon filter.

Radiolabeled bands were quantified using an imaging system (Molecular Imager GS-525, BIO-RAD, Hercules, CA), with the results normalized to the signal generated by radiolabeled 18S PCR products.

MLN64 gene expression analysis by real-time PCR

Two micrograms of total RNA from each liver sample were pretreated with DNAse I (Invitrogen, Carlsbad, CA) to remove any contaminating genomic DNA, and then reverse-transcribed to cDNA with random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was performed with a Stratagene Mx3000P Real-Time PCR System and Brilliant SYBR Green QPCR Mater Mix (Stratagene, La Jolla, CA) using 50 ng cDNA per 20 μL reaction volume, a SYBR green master mix and *mln64*-specific primers (Table 1). Changes in gene expression for *mln64* were determined and normalized to 18S rRNA expression levels.

Immunoblotting analysis

For MLN64, total membrane extracts and 10 000 × *g* mitochondria/lysosome enriched-pellets from murine liver were prepared[24], and proteins were fractionated by 10% SDS-PAGE. After transfer to nitrocellulose, proteins were immunoblotted for MLN64 using anti-MLN64 antiserum[10] and a commercial antibody ab3478 (Abcam, Cambridge, UK). An anti-ε-COP (Coatomer Protein Complex Subunit Epsilon) antibody (obtained from Dr. Monty Krieger, Massachusetts Institute of Technology, Cambridge, MA) was used as a control for the membrane protein loading.

Liver homogenates were prepared for analyzing p53, p21, and Bax protein expressions[25]. For caspase-3 and-12 immunoblotting, liver homogenates were fractionated into total membranes and cytosol as described previously[23]. Proteins (50 μg/sample) were separated by 12% SDS-PAGE and immunoblotted using anti-caspase-12 (BD Biosciences Pharmingen, San Diego, CA), anti-caspase-3, anti-p53, anti-p21 and anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-albumin antibody was used for protein normalization.

Antibody binding to protein samples was visualized using enhanced chemiluminiscence and measured using an imaging system (Molecular Imager GS-525, BIO-RAD).

Bile, liver, and blood sampling

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After laparotomy, the cyst duct was ligated and a fistula was made in the common bile duct using a polyethylene catheter. Hepatic bile specimens were collected for 30 min, then plasma and liver samples were obtained as described previously[26,27].

Plasma, hepatic, and biliary biochemical analyses

Serum alkaline phosphatase (AP) and alanine aminotransferase (ALT) were measured by standard methods. Hepatic and biliary cholesterol as well as biliary phospholipids and bile acids were determined by standard protocols[28,29].

Hepatic immunofluorescence

Fresh-frozen liver tissues cryosectioned at 4-5 μm were fixed in acetone, rinsed three times in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 for 15 min, blocked overnight in 10% goat serum in PBS, and incubated for 2 h at 37℃ with the polyclonal antibody against MLN64 (1:500 dilution). The secondary antibody was fluorescein-isothiocyanate-conjugated goat antirabbit IgG (1:150 dilution). After washing in PBS, samples were mounted on coverslips using Fluoromount-G (EMS, Fort Washington, MD). Stained sections were examined by immunofluorescence microscopy.

Hepatic histology

Liver tissue was fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin, sectioned, and placed on glass slides. Hematoxylin and eosin staining was then performed according to standard procedures.

Hepatic TUNEL analysis

TUNEL (TdT-mediated dUTP nick end labeling) staining was performed on sections of liver samples using a commercially available kit (TUNEL Apoptosis Detection Kit, Upstate, Charlottesville, VA) according to the manufacturer's recommendations with minor modifications. Briefly, cryosections of fresh-frozen tissues were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. The tissue was permeabilized with 0.1% Triton X-100 and 0.1 mol/L sodium acetate for 2 min at 4℃, and then washed twice with PBS. Tissues were subjected to proteinase K treatment for 15 min. The DNA of apoptotic cells was enzymatically labeled with the terminal deoxynucleotidyl transferase for 1 h at 37℃ and visualized by staining with fluorescein.

Hepatic DNA fragmentation analysis

Liver tissue samples were lysed with hypotonic lysis buffer (50 mmol/L EDTA, 1% SDS, 50 mg/L proteinase K in 50 mmol/L Tris-HCl, pH 8) for 15 min on ice, and then incubated for 4 h at 37℃. DNA was extracted with an equal volume of phenol/chloroform followed by chloroform isoamylalcohol (24:1) extraction, the aqueous phase was collected, and DNA was precipitated by adding 150 mmol/L sodium acetate and two volumes of ethanol at -20℃ for 1 h. The precipitate was pelleted by centrifugation at 10 000 × *g*, and then washed in cold 70% ethanol. After centrifugation, each pellet was dissolved in autoclaved distilled water and electrophoresed on a 1.5% agarose gel containing 2 μg/mL ethidium bromide. DNA fragments were visualized in an ultraviolet transilluminator.

Cell culture and transfections, TUNEL staining, and Hoechst analysis

Chinese hamster ovary (CHO)-K1 cells were grown in Ham F-12 media supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin and maintained at 37℃, in an atmosphere of 5% CO_2, and saturated humidity. CHO cells were transfected using a lipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) with a pcDNA3.1 empty plasmid or a pcDNA3.1/MLN64 vector that was constructed by ligation of a cDNA HindIII fragment encoding mouse

MLN64 in pcDNA3.1 (Invitrogen, Carlsbad, CA). After 24 h cells were subjected to immunofluorescence, TUNEL analysis, and Hoechst staining.

For immunofluorescence, CHO cells were fixed in 4% paraformaldehyde in PBS for 1 h, permeabilized with 0.1% Triton X-100/0.1% sodium citrate in PBS for 2 min, blocked 20 min in 0.02% gelatin in PBS and incubated 2 h with anti-MLN64 ab3478 antibodies (Abcam, Cambridge, UK) at 1:1000 dilution, followed by incubation with a Alexa-594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, dilution 1:1000). TUNEL analysis was performed using a commercial kit following manufacturer's recommendations (Roche, Mannheim, Germany). For Hoechst 33258 staining, cells were incubated with 1:50 000 dilution of Hoechst 33258 (Polysciences, Warrington, Pennsylvania) for 15 min at 37℃. Fluorescence was analyzed and photographed under an appropriate microscope (Olympus BX51 TF, Tokyo, Japan).

Statistical analysis

Results are expressed as mean ± standard error values. The statistical significance of differences between the means of the experimental groups was evaluated using Student's *t*-test for unpaired data and with one-way analysis of variance (ANOVA) with a post-hoc Tukey multiple-comparison test (Prism 3.0, GraphPad). A difference was considered statistically significant when the probability value was $P < 0.05$.

RESULTS

Hepatic MLN64 expression and localization in recombinant MLN64 adenovirus-infected mice

To determine the effects of hepatic MLN64 overexpression, Ad.MLN64 and the control Ad.E1Δ were infected into C57BL/6 mice. Western blot analysis (Figure 1A) of mitochondria/lysosome enriched preparations demonstrated that MLN64 protein was increased several times in the livers of Ad.MLN64-treated C57BL/6 mice at 24 h after infection. Overexpressed MLN64 was detected as a predominant 50-kDa protein[16]. The increase in hepatic MLN64 protein levels induced by the infusion of Ad.MLN64 was time-dependent (Figure 1B), reaching its maximal effect after 24 h of infection, and decreasing to undetectable levels after 96 h. Thus, all the subsequent experiments were performed at 24 h after Ad.MLN64 infection.

The liver MLN64 staining was essentially intracellular in Ad.MLN64-infected mice, exhibiting an abundant punctate pattern (Figure 1C) consistent with MLN64 protein localization in vesicular compartments as reported previously in other cell types[8,10].

Liver damage and apoptosis in mice with adenovirus-mediated hepatic MLN64 overexpression

As described above, hepatic MLN64 expression was highly increased at 12-24 h after infection with Ad.MLN64, but it dramatically decreased 96 h thereafter (Figure 1). This short half-life for transgene expression is an unusual finding for adenovirus-mediated gene expression in the liver, given that both previous studies[30,31] and our own investigations have found substantial levels of protein expression for other genes using this type of adenovirus still at 2 wk after infection.

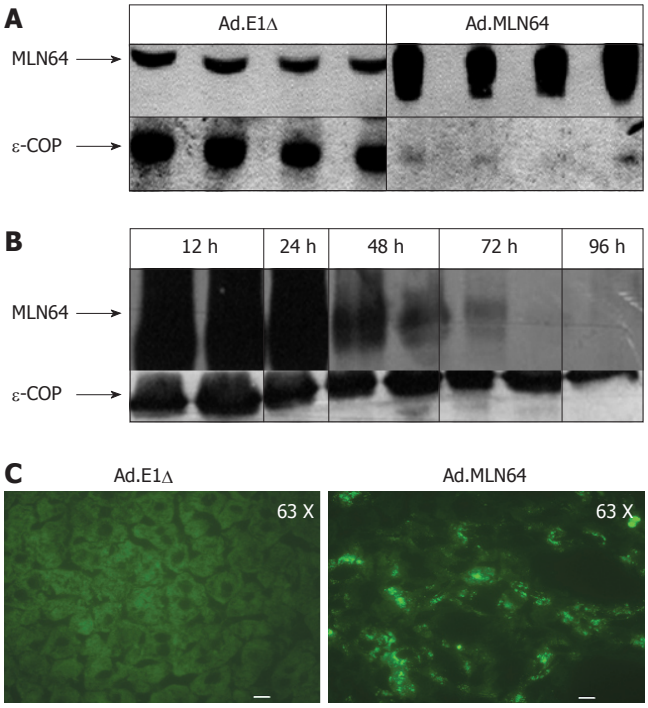

Figure 1 Effect of recombinant MLN64 adenovirus infection on MLN64 expression in mice livers. **A**: Immunoblot analysis of MLN64. At 24 h after infection, mitochondria/lysosome enriched extracts (50 μg protein/lane from Ad.E1Δ and 1 μg protein/lane from Ad.MLN64 mouse livers) were subjected to 10% SDS-PAGE and Western blotting with anti-MLN64 and anti-ε-COP antibodies; **B**: Time course of hepatic MLN64 expression after administration of recombinant adenovirus. At the indicated times (in hours) after infection, livers were collected and analyzed for MLN64 protein expression in total membrane extracts as described in (**A**); **C**: Immunofluorescence analysis of liver tissue 24 h after infection with control (Ad. E1Δ) or MLN64 (Ad.MLN64) recombinant adenovirus. Bar: 10 μm. Analysis were performed in four mice in each experimental group. Results are representative of three independent experiments.

To explore the potential mechanisms underlying the rapid decrease in MLN64 overexpression, we evaluated liver damage and apoptosis in MLN64-infected mice. The first evidence of hepatic damage in these animals was in the gross morphology of the liver, with the formation of yellowish-white zones suggesting the presence of hepatic necrosis at 24 h after infection (Figure 2A). Furthermore, serum ALT and AP were significantly elevated in mice infected with virus-encoding CMV-MLN64 compared with both noninfected mice and those infected with the control Ad.E1Δ virus (Table 2). Interestingly, we found a dose-effect response in serum ALT and AP levels utilizing lower doses of viral particles (results not shown). Histological examination of liver sections demonstrated the presence of multiple necrotic zones as well as numerous condensed and fragmented nuclei, which are characteristic of apoptosis (Figure 2B). DNA fragmentation analysis and TUNEL assays revealed the presence of a DNA "ladder" pattern and TUNEL-positive cells in livers of MLN64-overexpressing mice (Figure 3), confirming that the

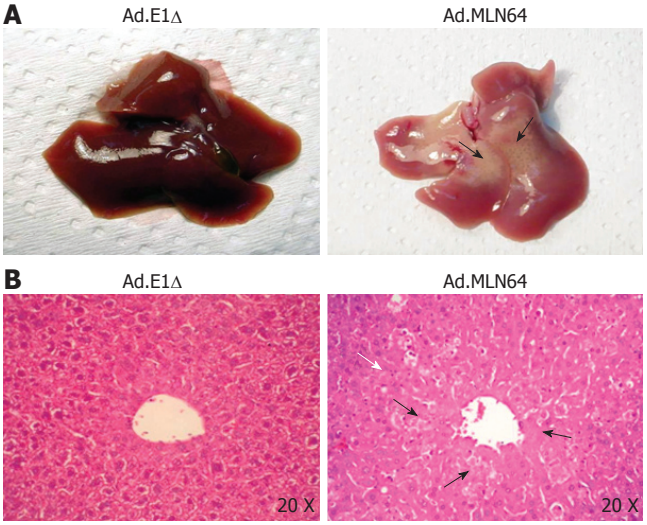

Figure 2 Effect of recombinant MLN64 adenovirus infection on liver morphology and histology in mice. **A**: Gross morphology of murine livers 24 h after infection with Ad.E1Δ or Ad.MLN64. Arrows indicate yellowish-white zones with liver necrosis in Ad.MLN64-infected mice; **B**: Liver sections from (**A**) were prepared for histology and stained with hematoxylin and eosin. Small arrows indicate necrotic zones, and the white arrow indicates an apoptotic zone. Analysis were performed in four mice in each experimental group. Results are representative of three independent experiments.

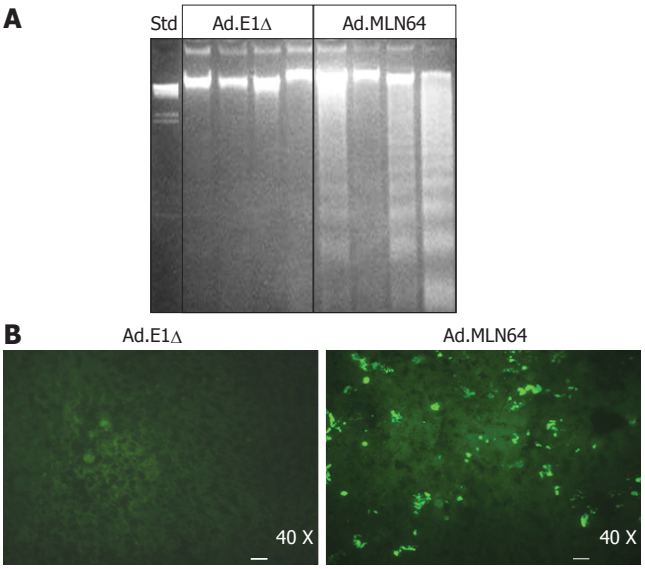

Figure 3 Effect of recombinant MLN64 adenovirus infection on liver apoptosis in mice. DNA fragmentation analysis and TUNEL assay were performed in murine livers 24 h after infection with Ad.E1Δ or Ad.MLN64 as biochemical markers of apoptosis. **A**: DNA fragmentation analysis of liver tissue by DNA agarose gel electrophoresis. DNA λ/Hind Ⅲ was used as the standard (Std); **B**: TUNEL assay. Substantially more apoptotic cells were detected in liver sections from Ad.MLN64-infected mice than in those from Ad.E1Δ-infected mice. Analysis were performed in four mice in each experimental group. Results are representative of three independent experiments.

Table 2 Effects of MLN64 recombinant adenoviral infection on serum alanine aminotransferase and alkaline phosphatase (mean ± SE)

Group	ALT U/L	AP U/L
No virus	14.00 ± 7.04	55.25 ± 6.44
Ad.E1Δ	39.67 ± 4.91	76.33 ± 4.42
Ad.MLN64	3780 ± 1010[a]	209.50 ± 20.17[a]

Measurements were performed in four mice in each experimental group. [a]$P < 0.05$ *vs* Ad. E1Δ-infected mice.

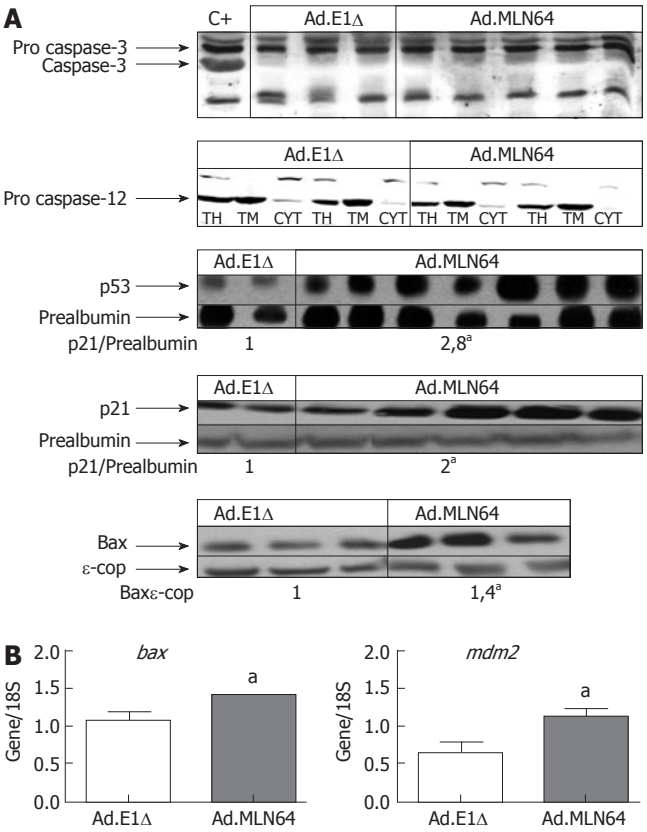

Figure 4 Effect of recombinant MLN64 adenovirus infection on hepatic expressions of various apoptosis-regulating genes in mice. **A**: For caspase-3 analysis, liver cytosolic fractions were prepared from Ad.MLN64-infected mice, and a liver cytosolic fraction from a CDCA-fed mouse was used as a positive control (C+). For caspase-12 analysis, liver homogenates (TH) from two mice in each group were fractionated into total membranes (TM) and cytosol (CYT). Total liver extracts were prepared for the analysis of p53, p21, and Bax expressions. Proteins were size fractionated by SDS-PAGE, immunoblotted with anti-caspase-3, anti-caspase-12, anti-p53, anti-p21, and anti-Bax antibodies, and subjected to densitometric analysis. The protein expression data are shown after normalization to the ε-COP or albumin signal. [a]$P < 0.05$; **B**: Relative quantitative RT-PCR in the presence of [α-^{32}P]dCTP was used to analyze the *bax* and *mdm2* RNA expressions. A 18S primer control was included in each sample. The products were resolved on a 1.5% agarose gel and transferred to a nylon filter, and the radiolabeled signals were quantified. Results are representative of three independent experiments. [a]$P < 0.05$.

apoptosis was induced by MLN64 overexpression.

The acute hepatocyte apoptosis/necrosis induced by MLN64 overexpression was fully reversible, with livers appeared healthy and normal at 1 wk after Ad.MLN64 infection, when no MLN64 expression was detected (data not shown). Remarkably, mortality was not increased in Ad.MLN-64-infected mice compared to controls.

To further clarify the mechanism of MLN64-induced apoptosis, we evaluated the hepatic expressions of various apoptosis-related genes (Figure 4). Western blot analysis in MLN64-overexpressing mice revealed a significant increase in the expressions of proapoptotic proteins p53,

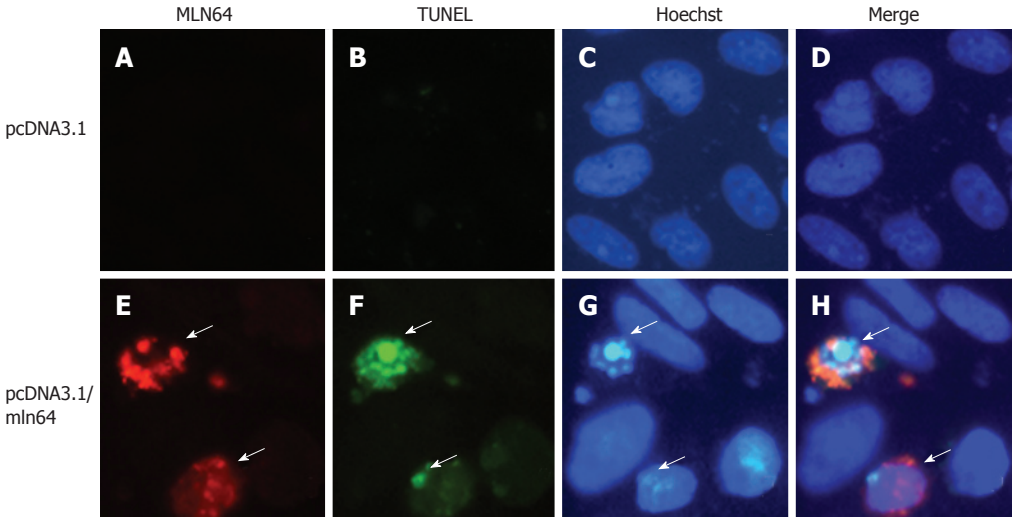

Figure 5 Effect of MLN64 over expression on apoptosis in CHO cells. CHO-K1 cells were transfected with a pcDNA3.1 empty or a pcDNA3.1/MLN64 vector, analyzed by MLN64 immunofluorescence (**A**, **E**), TUNEL (**B**, **F**), or Hoechst 33258 (**C**, **G**) staining, and visualized under fluorescence microscopy. Merge of panels **A**, **B** and **C** and **E**, **F** and **G** are shown in panels **D** and **H**. Arrows indicate MLN64 overexpressing cells.

Table 3 Effects of MLN64 recombinant adenoviral infection on hepatic cholesterol levels, biliary bile flow, and lipid secretion (mean ± SE)

Group	Liver weight	Hepatic cholesterol		Bile flow	Biliary lipid secretion		
		Unesterified (mg/g liver weight)	Ester (mg/g liver weight)	(μL/min/100 g body weight)	Cholesterol (nmol/min/100 g body weight)	Bile salts (nmol/min/100 g body weight)	Phospholipids (nmol/min/100 g body weight)
Ad.E1Δ	1.32 ± 0.04 (n = 11)	1.97 ± 0.08	0.20 ± 0.08	9.43 ± 0.76 (n = 8)	2.75 ± 0.33	254.1 ± 34.2	84.25 ± 5.5
Ad.MLN64	1.14 ± 0.04 (n = 16)[a]	2.38 ± 0.08[a]	0.21 ± 0.07	4.14 ± 0.61[a]	1.38 ± 0.19[a]	308.5 ± 45.4	54.64 ± 13.5

Measurements were performed in six mice in each experimental group, except for liver weight and bile flow as indicated. [a]$P < 0.05$ *vs* Ad.E1Δ-infected mice.

p21, and Bax, whereas the activated forms of caspase-3 and caspase-12 were not detected (Figure 4A). Semiquantitative PCR analysis revealed that *bax* and *mdm2* mRNA levels were increased in MLN64-overexpressing mice (Figure 4B).

Apoptosis in CHO cells with transient MLN64 overexpression

To determine if overexpression of MLN64 in cultured cells, by a system that does not use an adenovirus to overexpress the protein, directly results in increased apoptosis, we transiently transfected CHO-K1 cells and analyzed apoptosis by TUNEL and Hoechst staining. We observed a clear increase in TUNEL positive cells in MLN64-overexpressing cells (Figure 5). Furthermore, nuclear morphology assessed by Hoechst staining of these cells confirmed an ongoing apoptotic process with brightly stained nucleus and chromatin condensation (Figure 5). The percentage of MLN64 transfected CHO cells undergoing apoptosis was 32%, whereas no positive TUNEL cells were detected in control CHO transfected cells with pcDNA3.1. Similar results were obtained in HEK 293 cells with MLN64 transient overexpression (results not shown). These results support a potential role for MLN64 in determining cell death.

Hepatic cholesterol content in mice with adenovirus-mediated hepatic MLN64 overexpression

Since excess cellular free cholesterol is a potent inducer of cell apoptosis[32,33], and MLN64 overexpression in COS cells causes cholesterol accumulation[14], we determined the hepatic total and unesterified cholesterol levels in mice with adenovirus-mediated hepatic MLN64 overexpression. As indicated in Table 3, MLN64 overexpression increased the hepatic free-cholesterol content by 21% relative to control animals.

MLN64 expression in a murine model of liver damage induced by chenodeoxycholic acid

To begin to explore the pathophysiological relevance of our findings, we analyzed MLN64 liver expression in cholestatic hepatocyte injury induced by a diet high in CDCA, which is a well-characterized model of apoptosis-related liver damage induced by bile acids[34,35]. Figure 6 shows that MLN64 mRNA and protein levels increased significantly compared to control levels, demonstrating that MLN64 expression is indeed modulated in a liver-damage model involving apoptosis.

Biliary lipid secretion in mice with adenovirus-mediated hepatic MLN64 overexpression

Since the phenotype of MLN64-infected mice-including liver damage, apoptosis, and the increase in plasma AP levels-suggested that animals were in a cholestatic condition, we studied the effects of hepatic MLN64 overexpression on bile flow and biliary lipid secretion (Table 3). The total bile flow decreased by 56% in Ad.MLN64-infected mice. The biliary output of cholesterol decreased by approximately 46% and biliary phospholipid secretion showed a trend to decrease. Biliary

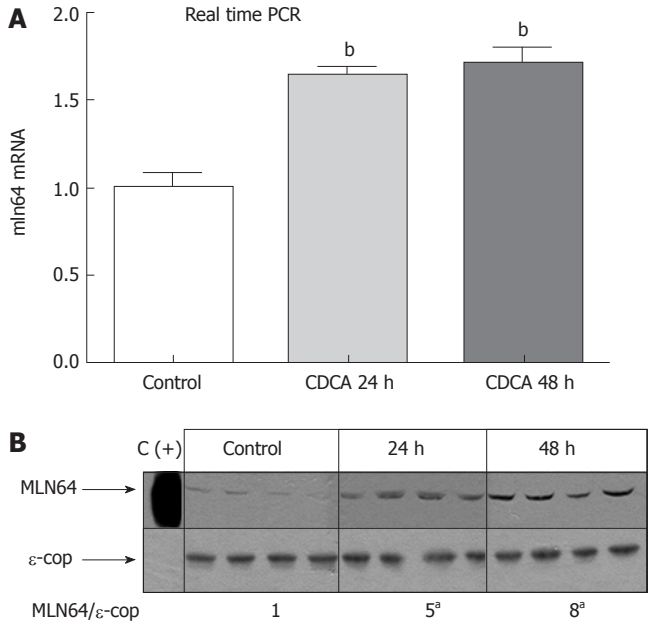

Figure 6 Effect of a 2% CDCA diet on hepatic MLN64 mRNA and protein levels in mice. Mice were fed with a 2% CDCA diet for 1 or 2 d. **A**: Real time PCR was used to analyze mln64 RNA expression. Each value represents the mean ± SE for mRNA values determined in CDCA-fed mice relative to those measured in chow diet-fed mice. There were 5 mice in each group. $^{b}P < 0.0001$; **B**: Immunoblot analysis of MLN64. Mitochondria-enriched liver extracts (50 μg protein/lane) were subjected to 10% SDS-PAGE and Western blotting with anti-MLN64 and anti-ε-COP antibodies. A liver membrane fraction from an Ad.MLN64-infected mouse was used as a positive control (C+). $^{a}P < 0.05$.

bile acid secretion remained within normal range, with a trend to increase. Consistent with the decrease in bile flow without changes in biliary bile acid output, biliary bile acid concentrations were increased twofold in Ad.MLN64-infected mice (71.07 ± 7.2 mmol/L in Ad.MLN64 mice *vs* 35.33 ± 7.4 mmol/L in Ad.E1Δ mice). The bile acid pool size and composition remained unchanged in Ad.MLN64-infected mice (data not shown).

DISCUSSION

This study demonstrates that adenovirus-mediated MLN64 overexpression in the mouse liver induces liver damage, most likely due to activation of the apoptosis cascade. Consistent with acute and reversible hepatocyte apoptosis induced by MLN64 overexpression, mortality was not increased and livers appeared healthy 7 d after Ad.MLN64 infection when no MLN64 overexpression was detected. In addition, MLN64 hepatic overexpression was associated with changes in hepatic cholesterol content, bile flow, and biliary bile acid concentration.

The precise function of MLN64 is unknown. The MENTAL region of MLN64, which contains four transmembrane domains, associates this protein with the membrane of endosomal compartments[10], and its C-terminal START domain makes it capable of mobilizing cholesterol[8,10]. Removal of the MLN64 N-terminal region increased steroidogenesis in COS-1 transfected cells[16] and enhanced the generation of steroid hormone by placental mitochondria in *in vitro* assays[8]. However, mice with targeted mutation of the MLN64 START domain did not exhibit a sterol-metabolism-related phenotype[15], questioning the physiological importance of the MLN64 START domain in cholesterol trafficking and metabolism.

The most significant finding of our study was the dramatic induction of liver damage in MLN64-infected mice, mainly through apoptosis as demonstrated by both TUNEL and DNA fragmentation analyses. We also detected an increase in ALT and AP plasma levels at 24 h after Ad.MLN64 infection, which is consistent with the current understanding of liver cell apoptosis; it is not a silent process and may also trigger liver inflammation and necrosis[36]. Adenovirus-mediated MLN64 overexpression was concentrated in some zones of the liver (results not shown). This was correlated with the visualization of hepatic damage in specific zones of the liver and suggests that acute and complete MLN64 down-regulation occurs mainly by apoptosis only in cells overexpressing MLN64. This response should induce liver regeneration in the injured zones and could explain that livers appear healthy 7 d after Ad.MLN64-infection.

We detected an increase in the hepatic levels of p53, p21, Bax, and Mdm2, suggesting that the apoptosis affects various signaling pathways. Surprisingly, active caspase-3 levels were not increased in MLN64-overexpressing livers. Caspase-3 is one of the central effector caspases when apoptosis is initiated by a variety of stimuli[37], and caspase-3 activation in liver has been described in several models of damage, such as iron deposition, steatohepatitis, and treatment with hepatocarcinogens[38,39]. The failure to detect caspase-3 activation in the present study could be due to a very low number of cells undergoing apoptosis or a low sensitivity of the caspase-3 antibody, or both. Alternatively, MLN64 overexpression may have induced apoptosis through non-caspase-3-mediated pathways.

The actual mechanism by which MLN64 mediates apoptosis is not clear and requires further study. Examination of the activation of other caspases, such as caspase 6, 7, 8, 9 and 10 should help elucidate is the observed apoptosis is caused by the intrinsic pathway or extrinsic pathway[37]. One attractive hypothesis for MLN64-induced apoptosis is that this protein stimulates the delivery of free cholesterol into the endoplasmic reticulum (ER) membrane, triggering protein unfolding[32]. Consistent with this idea, liver unesterified cholesterol levels were increased and hepatic LDL receptor expression, which is normally controlled by the cholesterol levels in the ER, was downregulated in MLN64-overexpressing mice (results not shown). However, caspase-12, a specific effector that is activated during the ER stress response[37], was not activated in our MLN64-overexpressing mice. Still, we can not totally exclude a role for the ER stress response in MLN64 overexpression-induced apoptosis given that is has been shown that caspase-12 and caspase 4 are not always required for caspase-dependent ER stress induced-apoptosis[40]. Another possibility is that the lysosomal pathway of apoptosis[41] was activated in livers of MLN64-overexpressing mice. In this pathway, which can be activated by death receptors, lipid mediators, and photodamage, lysosomal proteases such as Cathepsin b

(Ctsb) can be released by the lysosomes into the cytosol, where they contribute to the apoptotic cascade upstream of mitochondria[41]. MLN64 overexpression in the endolysosomal compartment may alter membrane structure and composition so as to favor the lysosomal release of proteases, which leads to apoptosis by activation of mitochondria-dependent apoptotic pathways without caspase-3 involvement. Indeed, it has been shown that MLN64 overexpression induces the formation of enlarged endosomes, an effect probably mediated by its MENTAL domain[42]. Interestingly, *in vitro* studies involving TNF-treated murine fibrosarcoma cells have shown that Ctsb is responsible for apoptosis-associated changes in the absence of caspase activity[43]. Further studies using Ctsb-/- mice and Ctsb inhibitors will help to ascertain the importance of lysosomal Ctsb release to this response and whether Ctsb inactivation attenuates the liver damage and apoptosis induced by MLN64 overexpression. Also, additional work is required to determine whether the increase in free-cholesterol levels and the MLN64 subcellular localization are important in the apoptotic response induced by MLN64 overexpression.

Our results using CHO cells with transient MLN64 overexpression supports a role for MLN64 in apoptosis. This cell culture system, which does not use the adenovirus to overexpress itself, is extremely useful in addressing concerns about the amount of adenovirus or adenoviral contaminants as the cause for the hepatotoxic effects. In addition, our results show that MLN64 effect on apoptosis is present in other cell types from diverse origin, such as CHO cells, which are derived from hamster ovary and not only in mouse hepatic-derived cells. We found that 30% of MLN64 overexpressing CHO were undergoing an apoptotic process. This results show that although there is a significant percentage of the population undergoing apoptosis, much higher that in cells that overexpress any protein, not all cells are suffering cell death. This may explain why previous overexpression studies of MLN64 in CHO, COS and Hela cells and primary rat hepatocytes have not seen an increase in cell death[8,14,19].

The (patho) physiological relevance of our findings is further supported by the results obtained in mice with CDCA-induced liver injury[34,35]. In fact, we found a significant increase in hepatic MLN64 protein and mRNA levels in the bile acid-induced liver injury model. This correlative evidence *in vivo* suggested a potential direct role on MLN64 expression in hepatocyte death due to CDCA administration.

MLN64 overexpression also increased the hepatic free-cholesterol content. This finding is consistent with a role for MLN64, proposed by Ren *et al*[19], in delivering cholesterol from lysosomes to the plasma membrane, where most of the cellular free cholesterol resides[44]. Another possibility is that cholesterol that accumulates in late endosomes primarily drives MLN64 protein to this subcellular compartment[14]. Further analyses are required to determine whether increased hepatic free cholesterol in MLN64-infected mice is localized in the plasma membrane or in intracellular compartments, and how this phenomenon is related to the apoptotic process.

In conclusion, we have shown that MLN64 overexpression induces apoptosis in the murine liver and alters cholesterol-metabolism-related parameters. Further studies are required to elucidate the physiological relevance of these findings.

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RAPID COMMUNICATION

Angiotensin-Ⅱ administration is useful for the detection of liver metastasis from pancreatic cancer during pharmacoangiographic computed tomography

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Abstract

AIM: To improve the preoperative diagnosis of liver metastasis from pancreatic cancer, we estimated computed tomography during arterial angiography (CTA) with/without administration of angiotensin-Ⅱ (AT-Ⅱ).

METHODS: Thirty-five patients with pancreatic cancer were examined in this study. After conventional CTA was performed, pharmacoangiographic CTA was performed with a 1-3 microgram/5 mL solution of angiotensin Ⅱ injected through a catheter into the celiac artery during spiral computed tomography. We prospectively analyzed the relative region of interest (ROI) ratio of tumor to liver with/without AT-Ⅱ.

RESULTS: In all patients, the relative ratio of each computed tomography (CT) number in the ROI was larger at pharmacoangiographic CT than at conventional angiographic CT. Administration of angiotensin-Ⅱ enhanced the metastatic liver tumor as compared with normal tissue. Intratumoral blood flow increased in all patients with malignant tumors due to the pressure effect of AT-Ⅱ. Furthermore, the metastatic lesions in the liver of three patients were represented by only pharmacoangiographic CT, not by conventional CT and conventional CT angiography. In even peripheral and central areas of metastatic liver tumor, the lesions were enhanced after administration of AT-Ⅱ.

CONCLUSION: These results support that high detection rate of liver metastasis revealed by pharmacoangiographic CT suggests the improvement of diagnosis on preoperative staging. Moreover, chemotherapy under AT-Ⅱ induced hypertension may have a better effect on the treatment of metastatic liver tumors.

Key words: Angiotensin Ⅱ; Phamacoangiographic CT; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer is a malignancy with a poor prognosis[1]. The cause of death in patients with advanced pancreatic cancer is primarily the local progression of cancer and distant metastases. At diagnosis pancreatic cancer is most commonly locally advanced and often accompanied by distant metastases to the liver and peritoneum[2]. In fact, liver metastasis is one of the major causes of cancer death after resection of pancreatic cancer. Foster reported that 73% of the patients who died of pancreatic cancer were found to have liver metastases at autopsy[3]. Therefore, liver metastasis appears to be an important prognostic factor. Ultrasonography and CT are the first line investigations to diagnose and localize the primary tumor and to detect metastatic lesions of pancreatic cancer[4,5]. However, some patients come to laparotomy with liver metastases not suspected or detected during these conventional preoperative examinations. To overcome this situation, some preoperative diagnostic procedures have been attempted. Computed tomography during arterial portography (CTAP) has been reported to be the most sensitive preoperative imaging modality for detecting and determining the location of hepatic lesions[6,7]. However, these results were reached mainly for the evaluation of hepatocellular carcinoma and metastatic tumor from colorectal cancer. Hence, angiotensin-Ⅱ (AT-Ⅱ) is the most potent vasoconstrictive agent. Ekelund et al reported

the diagnostic value of phramacoangiography using AT-Ⅱ[8]. Elevation of the arterial blood pressure by systemic infusion of AT resulted in a 5.7 fold selective increase in the blood flow of the tumor tissue without increasing the blood flow in normal tissue[9,10]. Sasaki *et al*[11] investigated the distribution of the hepatic blood flow using a short-lived radioisotope and demonstrated a 3.3 times increase in the tumor/non-tumor (T/N) ratio of the blood flow induced by intra-arterial infusion of AT-Ⅱ. They also observed that intra-arterial infusion of AT-Ⅱ increased the T/N ratio more than intravenous infusion did, with a smaller rise in the peripheral blood pressure. To our knowledge, there is no study of metastatic liver tumor from pancreatic cancer by means of CT angiography with/without administration of AT-Ⅱ. We examined the efficacy of pharmacoangiographic CT with AT-Ⅱ in patients with metastatic liver tumor from pancreatic cancer with/without administration of AT-Ⅱ.

MATERIALS AND METHODS

Subjects

Between May 2002 and December 2005, we performed dynamic CT during celiac arteriography (hepatic arteriography) administrating with/without AT-Ⅱ in 35 patients (10 men, 25 women) with liver metastasis from pancreatic cancer. Mean age was 64.3 years, with a range 43-83 years.

Imaging technique

The celiac trunk catheter placement was done by using Seldinger's approach through the right femoral artery with a 5.0-Fr or 3.2-Fr catheter. Celiac arteriography was performed before CT for the evaluation of the tumor feeding arteries. For small arteries, the coaxial technique was used by placing a 2.7 Fr microcatheter (Renegade Hi Fro catheter, Target Therapeutics, San Jose, CA, USA or Micro Ferret, Cook, Bloomington, IN, USA) in the appropriate feeding artery, depending on tumor location.

CT arteriography was started 3 s after the initiation of a transcatheter hepatic arterial injection of 30 mL of nonionic contrast material (Omnipaque 100 mgI/mL, Daiichi, Osaka, Japan) through a catheter, using an automated power injector (Auto Enhance A-50; Nemoto Kyorindo. Tokyo). Injection rate ranged from 1.5 to 2.0 mL/s depending on the catheter tip location. CT imaging was performed on a helical CT scanner (HiSpeed Advantage; GE Medical Systems, Milwaukee, Wis) with the following technical parameters: 120 kVp, 200 mA, 1-second scan speed, 24-s scanning time, 6-s delay, 4-6 mm/s velocity of table feed, 3-5 mm section thickness, 12-cm field of view, 5-mm increment of reconstructed axial images and a standard body reconstruction algorithm.

After about 10 min. from conventional angiographic CT, AT-Ⅱ was infused into the catheter at a rate of 5 μg in 5 mL normal saline for 10 s. Two min after administration of AT-Ⅱ, repeat CT arteriography was carried out during the injection of contrast material with the same dose, injection rate, and technical parameters as at the conventional angiographic CT.

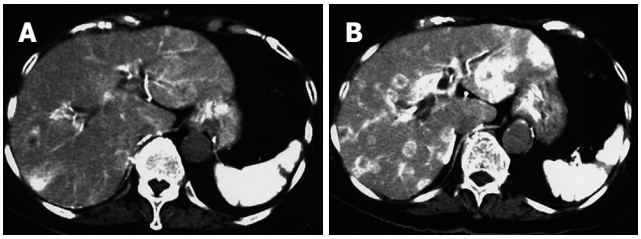

Figure 1 A 78 years old woman with liver metastasis from pancreatic cancer. **A**: Conventional angiographic CT; **B**: Pharmacoangiographic CT with angiotensin Ⅱ. The tumor to liver contrast is increased, and well demarcated low density area in the liver is now highly indicative of liver metastasis.

The mean attenuation values of regions of interest (ROIs) in the tumor and in the liver parenchyma were measured on the angiographic CT images obtained with and without AT-Ⅱ.

Region of interest measurements of the normal liver tissue and central area of metastatic liver tumor were obtained at each time point. All ROIs were approximately 1 cm^2. Attenuation (in Housefield Units) and enhancement (attenuation after injection minus attenuation before injection) were plotted as a function of time for each dose regimen and as a function of dose at each time point.

Radiologists evaluated the images independently; discrepancies in their interpretations were solved by consensus.

The contrast of the tumor to liver parenchyma in angiographic CT with/without AT-Ⅱ infusion respectively were compared using the Wilcoxon paired test.

All patients gave their informed consent after being fully informed on this study. The study was approved by the Ethical Committee of our hospital and was conducted in accordance with the principles of the Declaration of Helsinki.

RESULTS

To evaluate the effect of AT-Ⅱ on angiographic CT images, we divided the liver into two areas (normal parenchyma and central area of metastatic liver tumor). We calculated the number of enhanced areas on the images from the studies with and without AT-Ⅱ.

For instance, a 78 year old woman with liver metastasis from pancreatic cancer was examined with dynamic CT during celiac arteriography administrating with/without AT-Ⅱ as described. Phamacoangiographic CT showed marked multiple liver metastasis enhancement (Figure 1).

The attenuation value of the tumor was significantly increased after injection of AT-Ⅱ at angiographic CT. The mean values of the tumor contrast at angiographic CT with and without AT-Ⅱ were respectively 122.1 HU and 90.3 HU ($P < 0.01$) (Figure 2).

Furthermore, even central necrosis was well enhanced after injection of AT-Ⅱ at pharmacoangiographic CT.

Although the attenuation value of tumors was also increased on images obtained after the injection of angiotensin Ⅱ, the tumor to liver contrast was significantly greater at pharmacoangiographic CT (Figure 3).

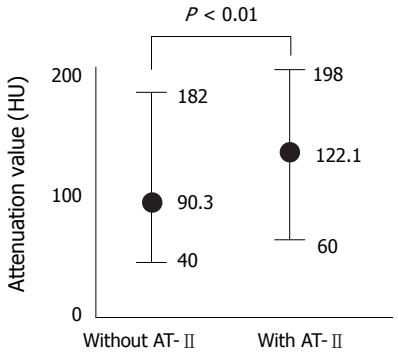

Figure 2 Comparison of the attenuation value of the enhanced liver metastasis in angiographic CT with and without angiotensin Ⅱ (AT-Ⅱ).

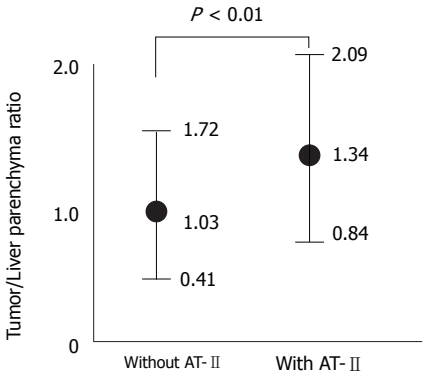

Figure 3 Tumor to liver parenchyma contrast in angiographic CT with and without angiotensin Ⅱ (AT-Ⅱ) in 35 patients with liver metastasis due to pancreatic cancer.

Pharmacoangiographic CT showed marked enhancement of the tumor surrounded by poorly enhanced parenchyma, although digital subtraction angiography revealed no tumor stain.

Side effects

As expected, the hepatic arterial infusion of AT-Ⅱ induced a modest rise in systemic blood pressure (an increase in the systolic pressure of up to 40 mmHg) which reached a peak at the end of the 100 s infusion, then gradually declined. No rebound hypotension was observed. These effects were accompanied by transient elevation of mean arterial pressure, but no change in pulse rate.

DISCUSSION

The prognosis for pancreatic cancer is poor. Surgery is considered the only curative therapy. However, the 5 year survival rate after resection of pancreatic cancer is still very low, even when radical surgery is performed[12,13]. Most patients with pancreatic cancer have distant metastases at the time of diagnosis, particularly involving the liver. Even when small lesions (< 2 cm) can be detected, most of them are locally advanced and microscopically invasive. Some patients come to laparotomy with liver metastasis not suspected or detected during the conventional preoperative examinations. In 70% of patients who undergo pancreatectomy, occult liver metastasis that may already have existed at the time of surgery are one of the most common sites of treatment failure[14]. Patients with certain types of metastatic liver tumors such as colorectal cancer who undergo surgical resection of the tumor have improved long term survival rates compared with similar patients who do not undergo resection[15]. However, resection is useless and harmful for most of the patients with metastatic liver tumors from pancreatic cancer. Although Fortner[16] has attempted to resect such advanced tumors by using regional pancreatectomy, they failed to improve patient survival. The exact number of metastatic lesions is irrelevant in cases of pancreatic cancer, as opposed to metastatic colon cancer.

To improve the survival rate of pancreatic cancer, it is important to diagnose its resectability before operation. Furthermore, to improve the value of preoperative diagnosis in patients with pancreatic cancer in terms of evaluation of resectability, a modality other than conventional examination methods is needed. Therefore, we examined pharmacokinetic CTA.

Intratumoral blood vessels are immature, lacking both smooth muscle cells and immunoreactive nerves[17]. Therefore, tumor vessels are unable to react to vasoconstricting agents[18,19].

AT-Ⅱ causes arteriolar constriction in normal blood vessels. It is a powerful vasoconstrictor which has been shown to alter the distribution of blood flow in favor of intrahepatic tumor perfusion during short (3-4 min) intra-arterial infusions of the compound[11]. Increased tumor blood flow following AT-Ⅱ infusion may increase the exposure of tumor to chemotherapeutic agents, and CT angiography may provide useful information about the potential of AT-Ⅱ and other vasoconstrictors to enhance targeting precision.

It is reported that there is a temporary increase in the relative arterial perfusion of human hepatic tumors during an infusion of AT-Ⅱ[11]. Furthermore, it was reported that the addition of AT-Ⅱ to hepatic artery infusional chemotherapy, significantly improves the tumor/liver ratio of drug uptake in an experimental model of hepatic metastasis[20]. However, to our knowledge, there is no study of metastatic liver tumor from pancreatic cancer by means of CT angiography with/without administration of AT-Ⅱ.

We quantitatively evaluated computed tomography during arterial angiography (CTA) with/without administration of AT-Ⅱ by means of pharmacoangiographic CT in patients with metastatic pancreatic cancer for the preoperative evaluation of liver metastasis from pancreatic cancer.

The attenuation value of the liver metastasis was significantly increased in pharmacoangiographic CT. Furthermore, even central necrosis was well enhanced after injection of AT-Ⅱ at pharmacoangiographic CT. The tumor to liver contrast was also significantly greater at pharmacoangiographic CT.

Pharmacoangiographic CT by AT-Ⅱ showed marked enhancement of the tumor surrounded by poorly enhanced parenchyma, although digital subtraction angiography revealed no tumor stain.

Because the injection of AT-Ⅱ into the celiac artery

results in increased blood flow in the intrahepatic arteries, the tumor to liver contrast was increased significantly at angiographic CT. As shown in this study, prolonged enhancement of the tumor blood flow as compared with the liver suggests the usefulness of intra-arterial infusion of AT-Ⅱ.

This finding described above was not recognized in the metastatic liver tumor from the cancer other than pancreas (data not shown). This result, therefore, may be a specific finding in metastatic liver tumor from pancreatic cancer.

Thus, CTA with/without administration of AT-Ⅱ is useful for the preoperative evaluation of liver metastases from pancreatic cancer.

In terms of adverse effects, there were some accompanying symptoms such as a sense of chest oppression and headache when the blood pressure was elevated, but they were not so severe and the administration of AT-Ⅱ could be performed in nearly all the cases. But, careful attention must be paid to control blood pressure values to avoid excess elevation during the administration of AT-Ⅱ.

AT-Ⅱ administration may induce not only the high detection rate of liver metastasis from pancreatic cancer, but also the selective increase in tumor blood flow in drug delivery such as chemotherapy.

In conclusion, AT-Ⅱ administration is useful for the detection of liver metastasis from pancreatic cancer during pharmacoangiographic computed tomography. This pharmacoangiographic CT is a useful technique for decisions in preoperative staging in pancreatic cancer.

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RAPID COMMUNICATION

Comparison of three different recombinant hepatitis B vaccines: GeneVac-B, Engerix B and Shanvac B in high risk infants born to HBsAg positive mothers in India

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Abstract

AIM: To evaluate a low cost Indian recombinant hepatitis B vaccine GeneVac-B® for its immunogenicity and safety in comparison to Engerix B® and Shanvac B® vaccine in high risk newborn infants born to hepatitis B surface antigen (HBsAg) positive mothers.

METHODS: A total of 158 infants were enrolled in the study. Fifty eight infants were enrolled in the GeneVac-B® group while 50 each were included for Engerix B® and Shanvac B® groups. A three-dose regimen of vaccination; at birth (within 24 h of birth), 1st mo and 6 mo. were adopted with 10 μg dosage administered uniformly in all the three groups. Clinical and immunological parameters were assessed for safety and immunogenicity of the vaccines, in all the enrolled infants.

RESULTS: Successful follow up until seven months of age was achieved in 83% (48/58) for GeneVac-B®, 76% (38/50) and 64% (32/50) for Engerix B® and Shanvac B® groups respectively. 100% seroconversion and seroprotection was achieved in all the three groups of infants. The geometric mean titers of anti-HBs one month after the completion of three dose of vaccination were 90.5, 80.9 and 72.5 mIU/mL in GeneVac-B®, Engerix B® and Shanvac B® vaccine group respectively. Furthermore the level of anti-HBs increases with age of babies who were born to HBsAg positive mothers. The GMT values of anti-HBs were 226.7, 193.9 and 173.6 mIU/mL respectively in GeneVac-B®, Engerix B® and Shanvac B® groups one year after the completion of the three doses of vaccine. No systemic reactions were reported in infants during the entire vaccination process of GeneVac-B® and the other two vaccines. Clinical safety parameters remained within the normal limits throughout the study period.

CONCLUSION: The study concludes that there is no significant difference between the three recombinant hepatitis B vaccines. Administration of these vaccines within 24 h of birth to babies, born to HBsAg positive mothers will reduce the incidence of HBV infection.

Key words: GeneVac-B; Maternal screening; High risk infants; Infant vaccination

Velu V, Nandakumar S, Shanmugam S, Jadhav SS, Kulkarni PS, Thyagarajan SP. Comparison of three different recombinant hepatitis B vaccines: GeneVac-B, Engerix B and Shanvac B in high risk infants born to HBsAg positive mothers in India. *World J Gastroenterol* 2007; 13(22): 3084-3089

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INTRODUCTION

Hepatitis B is a global communicable disease with an estimated 400 million chronically infected patients[1,2]. Mother to child transmission occurs often, either in-utero or through exposure to blood or blood contaminated fluids at or around the time of birth. Such perinatal transmission is believed to account for 35% to 50% of hepatitis B carriers[3]. The risk of perinatal transmission is associated with the hepatitis B envelope antigen (HBeAg) status of the mother. The chance of a child becoming chronically infected with hepatitis B virus, when a mother is positive for both hepatitis B surface antigen (HBsAg) and envelope antigen is around 70%-90%[4,5]. However, if a mother is positive for the surface antigen but negative for

the envelop antigen, the risk of transmission is significantly lower[6-9].

Screening for HBV among pregnant women is not routine in India, as hepatitis B immunoglobulin (HBIG) is not affordable and hence prevention of hepatitis B carriage from perinatal transmission must rely on vaccine alone. Universal neonatal vaccination is effective and has been shown to favorably alter the clinical course of hepatitis B in regions where disease is endemic[10]. Repeated injections over months are required to mount an effective antibody response with vaccination. Hepatitis B immunoglobulin has high levels of antibody to hepatitis B surface antigen. The immunoglobulin is immediately effective and seems protective for several months, after which it wanes[11,12]. Prevention of HBV transmission from HBsAg positive mothers to the children born to them is effectively achieved by administrating hepatitis B vaccine to their babies starting with the first dose at birth.

A recombinant hepatitis B vaccine, GeneVac-B®, a low cost hepatitis B vaccine[13] is manufactured by the Serum Institute of India Ltd., Pune, India. This vaccine is registered in India, and several hundred thousand doses are in use in India and abroad. The immunogenicity and safety of GeneVac-B® was proved in healthy adults [14], adolescents[15-17] and infants[18,19]. The aim of the present study was to asses and compare the immuogenicity, safety and efficacy of GeneVac-B® (Group-1), with two different commercially available vaccines, Engerix B® (Group-Ⅱ) Smithkline Beecham biologicals, Belgium and an Indian vaccine, Shanvac B® (Group-Ⅲ) Shantha Biotechniques Ltd, Hyderabad, India, in infants who were born to hepatitis B surface antigen (HBsAg) positive mothers, with 3 doses of vaccines at birth (within 24 h of birth), 1 mo and 6 mo of age, without passive prophylaxis at birth.

MATERIALS AND METHODS

Ethical review

The protocol of this study was approved by the Institutional review committee of Dr. ALM Post graduate Institute for basic Medical Sciences, University of Madras and the study design was prepared and monitored by National institute of Epidemiology, Indian Council of Medical Research, Chennai. Mothers were informed about the study and prognosis of the hepatitis B viral infection and benefits of vaccination to their babies. The written informed consent was obtained from all mothers prior to their registration in the study.

Vaccines

GeneVac-B vaccine consists of the purified surface antigen (Ag) of HBV obtained from the genetically engineered *Hansenula polymorpha* yeast cells expressing the surface antigen gene of the virus. It does not contain any material of human or animal origin. Each pediatric dose of 0.5 mL contains 10 μg of surface Antigen adsorbed on ≤ 0.40 mg of aluminum hydroxide, with ≤ 0.025 mg thimerosal added as a preservative. While Engerix-B used *Saccharomyces cerevisiae* as a host system, Shanvac B utilized *Pichia pestoris* as the expression system, the absorbent and the preservative being the same for all the three vaccines. The vaccines were stored at 4℃. Use and storage of the vaccine were under the supervision of responsible medical and research staff participating in the study.

Design of the study among infants born to HBsAg positive mothers

A total of 3000 healthy asymptomatic pregnant women attending the following hospitals in and around Chennai were studied: Institute of Obstetrics and Gynecology, Egmore, Chennai-600008; Kasthuribai Gandhi Hosptial, Triplicane, Chennai-600005; Department of Obstetrics and Gynecology, Rajamuthial Medical College and Hospital, Chidambaram. Among the 3000 pregnant women 5.9% (178/3000) was positive for HBsAg. All the enrolled mothers and their infants were subjected for the screening of HBsAg, anti-HBs, anti-HB core antigen. Mothers positive for HBsAg were also tested for the HBeAg. Babies born to these HBsAg positive women with acute febrile illness; any other infection; conditions associated with immunosuppression due to disease or therapy; seropositive for HBsAg; HBeAg and/or anti-HBs antibody; and participation in another clinical trial were excluded from the study. Thus, out of the 178 mothers and baby pairs, only 158 were included in the study.

One hundred and fifty eight infants were randomly recruited into 3 groups, 58 infants were administered with the recombinant hepatitis B vaccine, GeneVac-B® (group Ⅰ) and 50 infants each with the commercially available recombinant vaccines Engerix B® (group Ⅱ) and Shanvac B vaccine (group Ⅲ). All the infants were administered with 10 μg of hepatitis B surface antigen per 0.5 mL of dose. The vaccines were administered intramuscularly on the lateral aspect of the thigh within 24 h of birth, at birth as first dose, second dose at one month after birth and the third dose at 6 mo after birth. The follow-up visits were scheduled according to the vaccine administration and also at 3 mo intervals until the infants were 12 mo old. Strict adherence to the follow-up schedule was maintained; detailed information was collected using the structured proforma at every follow up. All the mother- baby pairs were asked to return within 3 d of the target date and those who did not were visited at their homes by the field visit official consisting of medical and research staff. The infants were bled using a scalp vein bleeding needle from the cubital vein at 0 mo (if they were more than 2 kg), 1, 2, 7, 9 and 12 mo. 3 mL of blood samples were collected from all infants prior to vaccination. The serum was separated and stored at -70℃ until tested.

Methodology

Detailed information was collected using the structured proforma at the period of antenatal check-up, at the time of delivery and on follow-up of mother and child. At each well-child visit, blood samples were collected from infants and information was obtained on illnesses, and physical examination findings. All the mother and child pairs were tested for HBsAg, anti-HBs, anti-HBC by using commercially available (BIORAD) elisa kits respectively. They were assayed for quantitative levels of anti-HBs

using Monolisa anti-HBs (BIORAD) 3.0 commercial kits. The anti-HBs standards were supplied by M/s. Sanofi Pasteur were used to develop the calibrated linear graph by the software installed in the ELISA Reader-Biotech Model ELx 800. The titer of anti-HBs was expressed as milli international units per milliliter (mIU/mL). Seroconversion and seroprotection rates were defined as anti HBs titer of < 10 mIU/mL and ≥ 10 mIU/mL respectively. Seroconversion and seroprotection rates after administration of the three vaccine doses were calculated. The anti-HBs concentrations were log transformed, and the antilog of the mean log values was calculated for the geometric mean titers (GMTs). Potential differences between the vaccines were statistically analyzed by Tukey's multiple comparison tests.

For reactogenicity assessment, subjects were physically examined during all their visits for vaccination. Parents were asked to report any adverse event assumed to be causally associated with vaccination until the total follow-up period of 7 mo ended. In these special circumstances, the child was examined thoroughly and the details were recorded. Infant's body temperature was recorded by measuring the oral temperature using a standard mercury thermometer. Fever was considered as mild when the temperature ranges 37.0℃ to 38.0℃ and moderate when it exceeds 39.0℃.

RESULTS

A total of 3000 pregnant women visiting the above hospitals were screened for HBsAg. Among the 3000 pregnant women only 178 (5.9%) were positive for HBsAg. Out of 178 HBsAg positive pregnant women, 158 mothers and infants pairs were enrolled in the study after fulfilling the study criteria, while the remaining HBsAg positive mothers were excluded from the study due to the various personal reasons. In addition among the 158 HBsAg positive mothers 28 (17.7%) were positive for HBeAg. No infants were positive for HBsAg, anti-HBs or anti HBC IgM during prevaccination visit and all the babies received the respective vaccines. After the three doses of vaccination, we could follow-up only 118 infants, while the remaining 40 (25.3%) infants were lost in follow up due to various reasons. Of the 118 successfully followed up infants, 48 babies were administered with GeneVac-B®, 38 with Engerix B® and 32 with Shanvac B® vaccines. It could be seen that the seroconversion and seroprotection on completion of the vaccination schedule of 0, 1 and 6 mo were 100% in all the three groups of infants. After seven months, the GMT values of anti-HBs titers were 90.3 mIU/mL with GeneVac-B®, 77.5 mIU/mL with Engerix-B® and 61.9 mIU/mL with Shanvac-B®. Notably the difference in anti-HBs level of GeneVac-B® was not statistically significant ($P > 0.05$) when compared with either Engerix B or Shanvac B groups. Thus the study validates that all the three studied recombinant hepatitis B vaccines had elicited protective levels of specific immunogenicity in the vaccinated infants.

The adverse effects of the three vaccine groups in our study population are given in Table 1. The most common systemic reaction effects in the infants were mild to moderate fever; excessive crying, rash, and irritability. However all the effects reported were transient and resolved without any further sequelae. None of the infants were hospitalized due to vaccine-associated adverse effects. Post vaccination safety profile was similar for the three test groups under study.

Table 1 Adverse effects reported by infant vaccinees in GeneVac-B, Engerix B and Shanvac B groups *n* (%)

Symptoms	GeneVac-B (48)	Engerix B (38)	Shanvac B (32)
Mild fever	4 (8.3)	3 (7.8)	3 (9.3)
Moderate fever	3 (6.2)	2 (5.2)	2 (6.2)
Excessive crying	1 (2)	1 (2.6)	1 (3.1)
Irritability	2 (4.1)	1 (2.6)	1 (3.1)
Local swelling	2 (4.1)	3 (6.8)	2 (6.2)
Local erythema	2 (4.1)	2 (5.2)	2 (6.2)
Induration	1 (2)	1 (2.6)	1 (3.1)
Rash	1 (2)	nil	1 (3.1)

Table 2 Immunogenicity data of GeneVac-B, Engerix B and Shanvac B in babies born to HBsAg positive mothers

Parameters	1st dose	2nd dose	3rd dose	After one year
	Group- Ⅰ - GeneVac-B			
No. of infants	58	48	48	35
Seroconversion	57.10%	94.20%	100%	100%
Seroprotection	22.80%	82.80%	100%	100%
GMT (mIu/mL)	11.6	35.4	93.5	226.7
	Group-Ⅱ - Engerix B			
No. of infants	50	43	38	21
Seroconversion	57.10%	90.00%	100%	100%
Seroprotection	19.00%	71.40%	100%	100%
GMT (mIu/mL)	8.4	24.4	80.9	197.9
	Group-Ⅲ Shanvac B			
No. of infants	50	45	32	12
Seroconversion	50%	91.60%	100%	100%
Seroprotection	16.60%	66.60%	100%	100%
GMT (mIu/mL)	8	18.6	72.55	173.6

Level of anti-HBs titer increases with age in vaccinated babies born to HBsAg positive mothers

Totally, 118 infants were followed up after full course of vaccination and among them only 68 were followed up to 12 mo. Among the 68 babies, 35 were administered with GeneVac-B®, 21 with Engerix B® and 12 with Shanvac B® vaccine. After the completion of 12 mo post vaccination at 0, 1 and 6 mo schedule, 100% of seroconversion and seroprotection were observed in all the three groups of vaccinees. The GMT values of anti-HBs titers were 226.7 mIU/mL with GeneVac-B®, 197.9 mIU/mL with Engerix B® and 173.6 mIU/mL with Shanvac B® vaccinees. No differences were found in the anti-HBs levels ($P > 0.05$) between Engerix B®, Shanvac B® and GeneVac-B®. Children who were followed up till 12th mo had high antibody response when compared to anti-HBs response on 7th mo follow up (Table 2).

Protective efficacy of the vaccines administered in infants

During the 9th mo follow up, tests for HBsAg, anti-HBs and anti-HBc were performed in all the babies. Infants in all the three groups had 100% of seroprotection (data not shown). Although all the mothers were HBsAg carriers, none of their children were HBsAg positive. However 7 infants, 3 from GeneVac-B® group and 2 each from Engerix B® and Shanvac B® group were positive for anti-HBc IgM. This test became negative in 4 of 7 who were followed for 12 mo, thus 1 in each vaccine group were positive for anti-HBc, however the percentages of infants with Protective anti-HBs concentrations did not decrease in any of the vaccine groups.

DISCUSSION

Hepatitis B virus vaccines provide highly effective protection against acute and chronic hepatitis B infection and the chronic carrier's status[20,21]. Immunization with hepatitis B vaccine for infants, children, adolescents and high-risk adults has been recommended by WHO and other international organizations[22,23]. Previous studies have shown that the risk of chronic HBV infection is higher in a younger age group and thus it is inversely correlated with age [24]. In South East Asia, chronic hepatitis B is often caused by maternal-infant transmission during the perinatal period[4] and this fact is reinstated in studies conducted in Egypt[25], Saudi Arabia[26] and Sub-Saharan Africa[27]. In endemic countries, effectiveness of universal vaccination has already been proven to reduce hepatitis B carriage leading to a decline of Hepatocellular Carcinoma in children[28]. Evidence is mounting to support the universal infant vaccination to control HBV-related diseases and is the best means of controlling disease in countries with intermediate or high levels of HBV endemicity.

In the present study, the prevalence of HBsAg among the pregnant women enrolled in the study was 5.9% which confirms that the HBV infection is endemic in Indian population. Prevalence of HBeAg positive mothers in our study was 17.7%, however the reported HBeAg positive rates among HBsAg-positive pregnant women in India varies between 8% and 47%; most studies show positive rates towards the lower end of this range[29-31]. Without prophylaxis, a very large proportion of infants born to HBV positive carrier mothers may become carriers of HBV[30,31]. Infection in this high risk setting is prevented most effectively by prenatal screening to identify carrier mothers[32,33], with administration of hepatitis B immunoglobulin at birth along with a course of vaccines to achieve both immediate and long lasting immunity to HBV. Many countries such as India lack the resources to implement such programs and must depend on vaccine alone to prevent the development of chronic HBV carriage in these babies. It is important that regimen of vaccination chosen for the use in a nationwide program should be highly immunogenic and cost effective. Previous studies about infants in several countries revealed that there were wide variations in the immunogenicity between different hepatitis B vaccines. Furthermore, the response of new born infants to vaccine varies and hence, it is important to evaluate the efficacy and immunogenicity of different vaccines that can widely be adopted for infants in India.

In this study, we found no significant difference in the immunogenicity between the three groups of recombinant vaccines after the third dose of vaccination. Most infants who received recombinant vaccines acquired the antibody levels to hepatitis surface antigen above 10 mIU/mL. Comparison of the geometric mean titers of anti-HBs levels achieved by the three vaccinees reveals that GeneVac-B® has achieved slightly higher anti-HBs titers compared to the other two vaccines. In addition this finding is similar to the results obtained in HBV vaccine along with DPT in babies born to HBsAg negative mothers in India with GeneVac-B® and Engerix B®[18]. Moreover in this study 97.5% of the vaccinated babies born to HBsAg positive mothers who were immunized with the three groups of vaccines at birth controlled the infection up to one year which is not often seen in babies born to HBsAg positive mothers. Our results indicate that administration of hepatitis B vaccines with in 24 h of birth prevents the occurrence of hepatitis B virus in the newborn infants who were born to HBsAg positive mothers. However, long-term follow up is needed to know the preventive efficacy of these vaccines.

This is the first study in India to test the effectiveness of the three different commercially available recombinant hepatitis B vaccines in babies born to HBsAg positive mothers without HBIG administration. Several studies report that the plasma and recombinant hepatitis B vaccines alone without HBIG can be quite effective in preventing chronic HBV infection among high risk infants of HBeAg-positive carrier mothers, with efficacy estimates ranging from 65%-95%. Here in our study, though 17.7% of the mothers were HBeAg positive, we have not seen any case of infection in the infants during our follow up period of 12 mo. This result clearly indicates that the vaccines administered immediately (24 h) after the births in babies born to HBsAg positive mothers are highly effective in high risk infants even without the coadministration of HBIG.

The safety and tolerability of hepatitis B vaccines under study has been extensively studied by several investigators including studies conducted in adults on GeneVac-B®, Engerix B® and Shanvac B® in our laboratory[14]. In summary, mild adverse effects as mentioned below seem to occur: (1) temperature greater than 37.7℃ in 1%-6%; (2) erythema in 3%; (3) swelling in 3%; and (4) rash in 3%, the observations made in the present study show slightly higher mild fever in 9.3%-7.8% of the infants and moderate fever was reported in 2%-3%. However, in the present study no other generalized symptoms were observed. The study has clearly shown that, all the three vaccines were well tolerated without significant difference in vaccine associated adverse effects.

In an effort to further reduce the cost involved in HBV vaccination which facilitate universal immunization against hepatitis B in all countries, studies are being conducted to find out whether a two dose schedule can replace the existing recommendation of three dose hepatitis B

vaccination regimens. These studies have also looked at the logistics of vaccine delivery given at varying intervals of time[34-36]. All these studies have shown that the two dose regimen of recombinant hepatitis B vaccine is highly immunogenic and induces effective immunological memory like the three dose regimen. If a similar study is also conducted in countries like India, the results may facilitate the policy planners to adopt the programme of universal hepatitis B immunization expediously with significant reduction in vaccine cost and the vaccine compliance rate also increase to the desired level. However, out of three vaccines compared in the present study, GeneVac-B® is less expensive hepatitis B vaccine available in the Indian market[13,19] which can be widely considered in India to prevent hepatitis B infection.

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COMMENTS

Background

Hepatitis B (HB) is a serious public health problem throughout the world and is responsible for more than 600 000 deaths every year. In high-incidence areas, perinatal transmission of hepatitis B virus (HBV) from carrier mothers to newborns appears to be the most important factor for the high prevalence of HBV infection. Around 70% to 90% of infants become chronically infected with HBV when a mother is positive for both HBsAg and HBeAg. It may be beneficial to administer vaccines to these babies at birth to prevent HBV incidence.

Research frontiers

The current recommendation for hepatitis B prophylaxis in babies born to HBsAg positive mothers is to co-administer vaccine and hepatitis B immunoglobulin (HBIG). Several studies have assessed the immunogenicity of vaccines along with HBIG in babies of known HBsAg positive mothers. However, screening for HBV among pregnant women is not a routine in India and HBIG is not affordable to most of the people. Universal neonatal vaccination is effective and has been shown to favorably alter the clinical course of HB in regions where the disease is endemic. To better understand the response of the vaccine alone in babies born to HBsAg positive mothers, we studied the immunogenicity and safety of three different vaccines without the co-administration of HBIG in this population.

Innovations and breakthroughs

Several studies have assessed the immunogenicity of hepatitis B vaccines along with the administration of HBIG in babies born to known HBsAg positive mothers. On the other hand, there is not much data on the immunogenicity of vaccine alone in this population. However, because of the two above-mentioned constraints, which limit the use of HBIG, we thought that it is valuable to assess the immunogenicity of the recombinant vaccine alone in these babies.

Applications

The results of this study demonstrate that, in the absence of the HBIG, babies born to HBsAg positive mothers can be administered the available hepatitis B vaccines and they mount a high immune response. This may prevent the occurrence of the HBV infection to a certain level. However, long term follow-up studies are needed for determining the clinical efficacy of the vaccines alone in this population.

Terminology

Tukey's multiple comparison tests: It is one of the several tests that can be used to determine which means amongst a set of means differ from the test.

Peer review

The authors compared three hepatitis B vaccines in terms of efficacy and safety. They demonstrated GeneVac-B as effective as and more cost-effective than other two vaccines. This is an important and useful finding.

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RAPID COMMUNICATION

Evaluation of diagnostic findings and scoring systems in outcome prediction in acute pancreatitis

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Abstract

AIM: To determine factors related to disease severity, mortality and morbidity in acute pancreatitis.

METHODS: One hundred and ninety-nine consecutive patients were admitted with the diagnosis of acute pancreatitis (AP) in a 5-year period (1998-2002). In a prospective design, demographic data, etiology, mean hospital admission time, clinical, radiological, biochemical findings, treatment modalities, mortality and morbidity were recorded. Endocrine insufficiency was investigated with oral glucose tolerance test. The relations between these parameters, scoring systems (Ranson, Imrie and APACHE Ⅱ) and patients' outcome were determined by using invariable tests and the receiver operating characteristics curve.

RESULTS: One hundred patients were men and 99 were women; the mean age was 55 years. Biliary pancreatitis was the most common form, followed by idiopathic pancreatitis (53% and 26%, respectively). Sixty-three patients had severe pancreatitis and 136 had mild disease. Respiratory rate > 20/min, pulse rate > 90/min, increased C-reactive protein (CRP), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) levels, organ necrosis > 30% on computed tomography (CT) and leukocytosis were associated with severe disease. The rate of glucose intolerance, morbidity and mortality were 24.1%, 24.8% and 13.6%, respectively. CRP > 142 mg/L, BUN > 22 mg/dL, LDH > 667 U/L, base excess > -5, CT severity index > 3 and APACHE score > 8 were related to morbidity and mortality.

CONCLUSION: APACHE Ⅱ score, LDH, base excess and CT severity index have prognostic value and CRP is a reliable marker for predicting both mortality and morbidity.

INTRODUCTION

Although acute pancreatitis (AP) has been recognized for more than a century, no definitive treatment has been developed. The recent improvements in outcome are brought about by the progress in intensive care and supportive treatment. Although the incidence varies between countries, it is on the rise[1-4].

Improvements in diagnostic techniques and standardization in diagnosis and treatment have provided better understanding of the disease and many centers reported successful results[4-8]. The etiology of AP is heterogeneous and determined by local and social factors. Gallstones are the leading causes in many centers. Idiopathic cases comprise 10%-20% of the cases but this ratio varies with respect to the diagnostic capabilities of the center[3,4].

Although the majority of the patients are successfully managed by medical treatment, complications develop in 15%-20% of the cases and cause significant risk of mortality. Reliable scoring systems, radiological evaluation and laboratory markers are required for identifying high-risk patients at an early stage in order to take prophylactic measures. Numerous scoring systems and laboratory parameters have been used to predict the severity and mortality: Ranson, Imrie (Glasgow), Goris and APACHE Ⅱ scores, contrast-enhanced abdominal computed tomography (CET), C-reactive protein[4,8,9]. The APACHE Ⅱ score and the CRP level have been reported to be useful markers. Although there is a general consensus on the value of CRP, conflicting opinions have been expressed on the APACHE Ⅱ score[10].

In this study from a tertiary referral center, we aimed to present the characteristics of the AP cases and to identify clinical, radiological and laboratory parameters as

well as scoring systems that are associated with treatment outcome.

MATERIALS AND METHODS

Patients

The data of the patients who were treated for AP at the Ondokuz Mayıs University Medical School, Department of General Surgery, between 1998 and 2002 (5-year period) were recorded prospectively in prepared forms. AP was diagnosed by history, physical examination, laboratory and radiological findings (ultrasonography and contrast-enhanced computed tomography (CECT) which was taken in the first week after admission in some cases but not all). Amylase and lipase levels higher than three times the upper level of the normal range were considered significant. In patients with findings of acute abdomen, the diagnosis was made by laparotomy. The CECT findings were graded according to the Balthazar-Ranson classification and a CT-severity index was determined[6]. Patients with gallstones on ultrasonography were accepted as cases of biliary pancreatitis; patients consuming large amounts of alcohol were considered as having alcoholic pancreatitis; in patients with hyperlipidemia (triglyceride level more than 1000 mg/dL), this was accepted as the etiological factor. Patients with undetermined etiology were considered to be idiopathic cases. Patients with APACHE Ⅱ scores ⩾ 8 were diagnosed as having severe AP. If the patients were getting worse clinically and CECT findings demonstrated infected necrosis they were accepted as severe. Treatment algorithm was illustrated in Figure 1. Cholecystectomy and/or ERCP were performed before discharge in patients with biliary pancreatitis. Surgery was performed in patients with clinical deterioration and those with infected necrosis, which was diagnosed by fine needle aspiration under CT guidance. Antibiotic prophylaxis was conducted in patients with severe AP (carbapenems or a quinolone). The antibiotics were changed according to culture results. Oral glucose tolerance test (OGTT) was performed to evaluate endocrine function at one year after the onset of AP in patients with no history of diabetes mellitus.

Demographic data, etiology, time of admission after onset of symptoms, disease severity, clinical and laboratory findings including blood urea nitrogen (BUN), creatinine, glucose, calcium, alkaline phosphatase (ALP), bilirubin, aspartate amino transferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), albumin, base excess (BE), CRP, α-1 antitrypsin, and treatment methods were recorded and their associations with mortality and morbidity were investigated. Also, the associations of clinical, laboratory and radiological findings with disease severity were analyzed

Statistical analysis

Statistical evaluation was performed with SPSS 13.0 for Windows. The associations of clinical, radiological and laboratory findings with disease severity were investigated by univariate analysis (chi-square, Mann-Whitney-U and Fisher's exact test). After identification of radiologic findings, laboratory findings and disease scores (Ranson, Imrie and APACHE Ⅱ) significantly associated with mortality and morbidity, their diagnostic or predictive values were determined by ROC (Receiver Operating Characteristic) analysis.

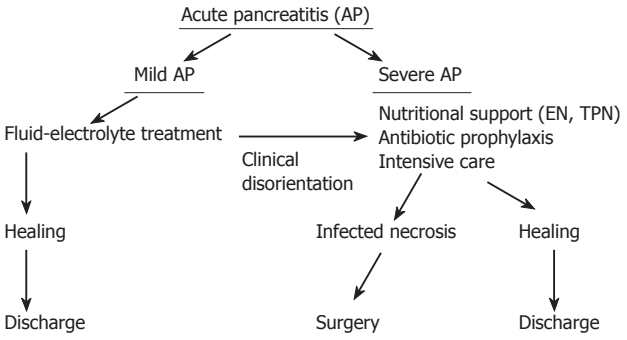

Figure 1 Treatment algorithm of the patients.

Table 1 Etiology and disease severity of the patients

Etiology	Disease severity		
	Mild (*n*)	Severe (*n*)	Total (*n*, %)
Biliary	71	34	105 (52.7)
Idiopathic	37	14	51 (25.6)
Alcohol	14	-	14 (7)
Hyperlipidemia	4	5	9 (4.5)
Miscellaneous	10	10	20 (10)
Drug			7
Infection			5
Trauma			5
Hypercalcemia			2
Others			1

There was no significant correlation between the etiology and disease severity.

RESULTS

Epidemiology

During the study period of 5 years, 199 patients with AP were hospitalized; 99 were women and 100 were men; the mean age was 55.1 ± 1.1 years (range: 16-92). Overall, 136 patients (68%) had mild AP and 63 (32%) had severe AP. Mean interval between onset of symptoms and admission was 48 ± 3 h (range: 1-300) and it had no association with mortality or morbidity.

Biliary pancreatitis was the most common form, followed by idiopathic pancreatitis (53% and 26%, respectively) (Table 1). Seven patients were using thiazide diuretics. The infectious agents were leptospirosis in 2 patients and various viruses in 3. Traumatic AP was due to blunt trauma in 3 patients and coronary bypass operation in 2. One patient had obstruction of the afferent loop after Billroth Ⅱ gastrectomy. Biliary pancreatitis was more frequent in women than in men (59.59% and 45.45%, $P < 0.002$). With the introduction of endoscopic retrograde cholangiopancreatography (ERCP), the frequency of idiopathic pancreatitis decreased from 31.5% at the beginning of the study to 20% at the end of the study.

Table 2 Relations between clinical, radiological and laboratory findings and disease severity

Parameters	MAP	SAP	*P*
RR > 20	21.7%	48.1%	< 0.001
PR > 90	36%	65%	< 0.001
Necrosis ratio in CT > 30%	4. 1%	27. 1%	< 0.001
CRP (mg/L)	116 ± 8	171 ± 15	< 0.002
LDH (U/L)	570 ± 31	959 ± 104	< 0.05
AST (U/L) × N	4.7 ± 1. 3	4.75 ± 0. 7	< 0.05
Leukocytosis (× 10^3/mm^3)	13 ± 0.4	16 ± 0.7	< 0.05

RR: Respiratory rate; PR: pulse rate; CRP: Serum C-reactive protein; LDH: lactate dehydrogenase; AST: Aspartate aminotransferase; CT: Contrast-enhanced abdominal computed tomography.

Table 3 Disease severity and CECT findings in 172 radiologically examined patients *n* (%)

CECT findings	Disease severity	
	MAP	SAP
Normal (non-diagnostic)	20 (16.4)	3 (6.3)
Edema in pancreas	62 (50.8)	18 (37.5)
Pancreatic necrosis < 30%	33 (27)	14 (27.1)
30% < Pancreatic necrosis > 50%	5 (4.1)	13 (27.1)[b]
Pancreatic necrosis > 50%	2 (1.6)	0

[b]$P < 0.001$ *vs* patients with mild AP. CECT: Contrast-enhanced abdominal computerized tomography; MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

Factors related to disease severity

Tachypnea (respiratory rate > 20 at the initial examination), a heart rate > 90 and pancreatic necrosis more than 30% were found to be associated with disease severity. CRP, LDH, AST and leukocyte levels were significantly higher in severe AP (Table 2). The bilirubin level was higher in biliary pancreatitis in comparison with other forms ($P < 0.05$, Z-test). In 13 patients, although the amylase level was 3 times higher than the upper limit of the normal range, the lipase level was normal. Disease severity and etiology showed no statistically significant association with amylase, pancreatic amylase and lipase levels.

Factors associated with morbidity

CT showed varying degrees of necrosis in 56.3% of the patients with severe AP (Table 3). CT findings were unremarkable (non-diagnostic) in 16% of the mild pancreatitis cases and 6% of the severe cases. Extensive necrosis more than 30% was significantly associated with disease severity, early complication and mortality rates ($P < 0.001$, $P < 0.05$ and $P < 0.004$, respectively, Table 2). The early complication rate was significantly higher in the severe AP group in comparison with mild AP (67% *vs* 8%, $P < 0.001$). The overall early complication rate was 26%: abscess in 11 patients, multiorgan dysfunction syndrome (MODS) in 9, pseudocysts in 8, ARDS in 3 patients and upper gastrointestinal bleeding in 2 (33 patients in total). Fourteen of these patients died. Factors associated with morbidity are shown in Table 4.

At the end of 1 year, OGTT was performed in 112 patients. Twenty-seven of these patients (24%) had impaired glucose tolerance. In 13 (12%) of these patients, diabetes developed. The impaired glucose tolerance was not associated with necrosis or disease severity.

Suspicion of necrosis led to fine needle aspiration and culture in 20 patients; cultures grew bacteria in 13; *E. coli* ($n = 6$) was the most common bacterium. Eighty-two per cent of the cases (163 patients) were treated conservatively and 18% (36 patients) underwent surgery. Seven underwent laparotomy for acute abdomen and AP was diagnosed by operative findings. Necrosectomy + closed lavage techniques were performed in 23 patients and "open abdomen" and planned re-laparotomy in 6. Nutritional support was not significantly associated with mortality or morbidity.

Table 4 Factors related to morbidity and mortality

Factors	Morbidity			Mortality		
	Cut-off level	Positive LR	AUC	Cut-off level	Positive LR	AUC
APACHE-II	8.5	5	0.72	8.5	5	0.88
BE	-5	3.36	0.69	-5	4.63	0.84
CT-INDEX	2	2.04	0.67	3	4.3	0.68
BUN (mg/dL)	22	2.23	0.70	23	2.85	0.70
CRP (mg/L)	142	2.03	0.72	160	2.03	0.82
LDH (U/L)	667	4.07	0.82	667	2.79	0.82

The AUC (Area Under Curve) value near to 1.00 means having high prediction rate of the morbidity and mortality. Positive LR (likelihood ratio) is also correlated with high prediction rate of the morbidity and mortality. CRP: Serum C-reactive protein; LDH: Lactate dehydrogenase; BE: Base excess.

Factors associated with mortality

Mortality was 37.5% in severe AP, 2.3% in mild AP and 13.6% (27 cases) overall. The mean base excess was -7.6 in patients who died and -1.9 in those who survived ($P < 0.05$). Among Ranson, Imrie and APACHE Ⅱ scores, the APACHE Ⅱ score was the best predictor of mortality. Mean APACHE Ⅱ score was 5.7 ± 0.3 in patients who survived and 15 ± 1.7 in those who died ($P < 0.05$). CT severity index, LDH, BUN, CRP and base excess were the other parameters associated with mortality (Table 4).

DISCUSSION

Although the mortality due to AP has decreased markedly in recent years, it is still a life-threatening disease. The demographic characteristics of AP are similar in many series; most patients are in the 50-60 year age group. In most series published in the English literature, gallstones are the leading cause, followed by alcohol. Although the reported figures vary, the frequencies of metabolic and infectious causes in the present series are higher than those reported[4,8,11,12]. Idiopathic AP includes cases with unelucidated etiology. The frequency is lower in centers that perform extensive investigations and usually biliary causes are revealed. Accordingly, with the introduction of ERCP, the frequency has decreased from 30% to 20% in our center.

The increased frequencies of tachypnea, tachycardia and leukocytosis which are components of the systemic inflammatory response syndrome (SIRS) are expected findings. Also, AST level was significantly higher in severe AP than in mild AP. AST, which is a component of various scoring systems, is a marker of serious liver damage[6,10]. Higher levels of bilirubin in biliary AP in comparison with other forms are not surprising. Although many studies include ALT and ALP elevations with hyperbilirubinemia[2,6,13], our results are not in accordance. It was reported that, lipase was more specific and sensitive than amylase[5,6]. Thirteen patients with AP in the present study had higher than 3 times normal amylase level but normal lipase levels. Our view is that lipase measurement does not contribute to the diagnosis. Conversely, the diagnosis of AP should be approached with caution in patients with increased lipase but normal amylase[14].

Contrast-enhanced CT has been used for a long time for diagnosis and prediction of severity of the disease[2,5,6]. In some series, the frequency of organ failure increases with more extensive necrosis[15]. In the present study, extensive necrosis more than 30% was associated with increased severity and mortality. Mertele and Balthazar reported similar results in their series[16,17]. The restricted power in our study prohibited the detailed evaluation of these findings. CT was non-diagnostic in 6% of the severe AP patients and only 63% of the severe AP patients had necrosis on CECT in our study. The majority of the patients with necrosis had mild AP according to scoring systems. This may be explained by the possibility that radiological findings do not always reflect disease severity. Because AP is a mediator disease, pancreas necrosis is not mandatory for cytokine effects.

Disease severity was evaluated by APACHE Ⅱ scoring system instead of other scoring systems like Ranson's and CT scan scoring in this study. The need of CECT was decided by clinical behavior of patients and not all patients underwent CECT. Atlanta criteria overlook the amount and location of necrosis on CECT[7]. However, Kemppainen *et al*[18] showed that, the outcome was favorable for patients with necrosis restricted to the distal part of the pancreas. CECT may yield negative findings in 20%-30% of the mild AP patients. If the CT staging is required, the CT severity index, as prepared by Balthazar should be used[19]. The CT index that reflects the extent of necrosis as well as peripancreatic or extrapancreatic inflammation is valuable in predicting morbidity and mortality in this study and the literature[2,5,6,16]. One of the complications of AP, peripancreatic abscess, may develop in the absence of pancreatic necrosis. CT not only shows necrosis but also guides the fine-needle aspiration for culture (FNAC). As in the present series, the diagnostic value of FNAC varies between 60% and 90%[19,20].

Surgical treatment was performed in 18% (36) of the cases. Necrosectomy and continuous closed lavage were the techniques we preferred mostly. Although this technique and planned relaparotomy do not appear to differ significantly with respect to mortality and morbidity, less invasive procedures are usually preferred[21-25]. The best strategy is probably the one with which the center feels most comfortable. Because there is a large difference between the numbers of patients who underwent each treatment (necrosectomy + closed lavage versus planned re-laparotomy), comparison was not made. Nine of the 29 patients (31%) who underwent surgery for AP died; this is slightly higher than the 20%-25% rate in the literature[21-23]. Although favorable results with percutaneous drainage have been reported with a limited number of patients, this approach has not received general acceptance. The reason for the failure is recurrent obstruction of the catheter by the necrotic debris[26].

Glucose intolerance was detected in 24% of the AP cases. In two series with a smaller number of patients, the frequency was 25%-35%[27,28]. All patients in those series were necrotizing pancreatitis, which may account for the higher value in those series. Diabetes mellitus may be due to inflammation and fibrosis that destroys parts of the gland.

The overall mortality rate (13.6%) was slightly higher than the previous series, and the mortality of severe AP was higher than the literature[8,9,25]. Fourteen of the 33 patients who had early complications died. Deaths were mostly due to MODS. We think that, the mortality rate was also dependent on the suitability of the hospitals' intensive care unit (ICU) for these kinds of cases. Although our center was a regional tertiary care center, the ICU bed availability might not be possible at that time. This factor might be the reason for our high mortality rate. The deterioration parameters such as base excess and BUN reflect that morbidity and mortality are due to distant organ injury and tissue perfusion impairment, that is to say, due to systemic damage. ROC analysis showed that these are useful parameters for predicting mortality (Table 4).

The CT index, in conjunction with the APACHE Ⅱ score and the CRP value are important determinants of mortality and morbidity. CRP is a practical and inexpensive parameter that has been proved in other studies also[29]. Of the Ranson, Imrie and APACHE Ⅱ scoring systems, APACHE Ⅱ was the most reliable in the present study. The role of systemic complications in mortality and morbidity decreased the usefulness of pathology-specific scoring systems such as Ranson and Imrie scores. One potential weakness of the APACHE Ⅱ is that patients older than 65 years have very high scores and there is a possibility of a false-positive score in that age group[30]. In the present study, age had no association with disease severity, morbidity or mortality. A multiorgan system score has been developed to supersede the APACHE Ⅱ and Ranson scores, but the number of cases in that report is small[10].

In conclusion, AP is a condition with high morbidity and mortality and may cause endocrine dysfunction in the long run. Base excess, CRP, CT index and the APACHE Ⅱ score are useful in prediction of the course.

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RAPID COMMUNICATION

Results of percutaneous sclerotherapy and surgical treatment in patients with symptomatic simple liver cysts and polycystic liver disease

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Abstract

AIM: To evaluate the results of the treatment of simple liver cysts (solitary and multiple) and polycystic liver disease (PLD) using percutaneous sclerotherapy and/or surgical procedures in a single tertiary referral centre.

METHODS: Retrospective analysis of 54 patients referred for evaluation and possible treatment of simple liver cysts (solitary and multiple) and PLD, from January 1997 to July 2006.

RESULTS: Simple liver cysts were treated in 41 pts (76%) with a mean size of 12.6 cm. The most common reason for referral was abdominal pain or discomfort (85%). Percutaneous sclerotherapy was performed as initial treatment in 30 pts, showing cyst recurrence in 6 pts (20%). Surgical treatment was initially performed in 11 pts with cyst recurrence in 3 pts (27%). PLD was treated in 13 pts (24%) with a mean size of the dominant cyst of 13 cm. Percutaneous sclerotherapy for PLD was performed in 9 pts with recurrence in 7 pts (77.8%). Surgical treatment for PLD was undertaken in 4 pts (30.8%) with recurrence in all. Eventually, 2 pts with PLD in the presence of polycystic kidney disease underwent liver- and kidney transplantation because of deterioration of liver and kidney function.

CONCLUSION: The majority of patients with simple liver cysts and PLD are referred for progressive abdominal pain. As initial treatment, percutaneous sclerotherapy is appropriate. Surgical deroofing is indicated in case of cyst recurrence after percutaneous sclerotherapy. However, the results of percutaneous sclerotherapy and surgical treatment for PLD are disappointing. Partial liver resection is indicated when there is suspicion of a pre-malignant lesion.

Key words: Simple liver cyst; Polycystic liver disease; Percutaneous sclerotherapy; Deroofing; Complications

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INTRODUCTION

Liver cysts comprise a heterogeneous group of lesions classified as congenital or acquired[1]. Nowadays, most cysts are detected as an incidental finding when imaging of the liver is performed for abdominal complaints[2]. In most cases, liver cysts are of the simple type and are asymptomatic without clinical significance. Nevertheless, a minority attains large size and may cause progressive abdominal pain and/or discomfort. Although the diagnosis is readily made by radiological imaging, the mode of treatment is controversial. Nowadays, treatment usually consists of percutaneous aspiration of the cyst followed by instillation of a sclerosant (sclerotherapy)[3], or surgical treatment, either laparoscopically[4] or during laparotomy, in case of recurrence after radiological intervention. Only few published studies have focused on the outcome of treatment of simple liver cysts and PLD (including adult polycystic liver disease and adult polycystic kidney disease) after percutaneous or surgical interventions.

The aim of this study was to assess the outcome of patients with simple liver cysts and polycystic liver disease treated with percutaneous sclerotherapy or by surgical intervention, and to propose a treatment algorithm for these lesions.

MATERIALS AND METHODS

Between January 1997 and July 2006, a total of 54 consecutive patients were referred for evaluation and treatment of simple liver cysts (including solitary and multiple cysts) or polycystic liver disease, to the Department of Radiology, Gastroenterology and Hepatology and/or Surgery, Academic Medical Center, Amsterdam, The Netherlands. The study group consisted of 49 women (90.7%) and 5 males (9.3%) with a mean age of 56.3 ± 13.6 years (range 21-81). Medical records were reviewed for demographic features, presenting symptoms or indications for further analysis, in addition to size and location of treated cysts. The liver cysts were classified into two groups: simple (solitary or multiple) liver cysts or polycystic liver disease (PLD). The latter included PLD in the presence of polycystic kidney disease and autosomal dominant PLD). The following items were recorded: mode of treatment consisting of ultrasound-guided percutaneous sclerotherapy or surgical treatment, complications, duration of hospital stay and clinical outcome. Simple liver cysts typically presented on ultrasound (US) as anechoic, unilocular, sharply demarcated lesions with imperceptible walls showing posterior acoustic enhancement[5]. On CT, a simple liver cyst appeared as a well-demarcated lesion with fluid density without enhancement after contrast administration. Polycystic liver disease typically appears as multiple homogeneous lesions with fluid density without wall or content enhancement after contrast administration[6].

Techniques

Percutaneous sclerotherapy was performed under local anesthesia. The cyst was aspirated by needle puncture under US guidance and examined for bile content to exclude communication with the biliary system. A pigtail catheter was then inserted for complete drainage aspiration and 95% ethanol or tetracycline was injected to destruct the epithelial lining of the cyst wall. The volume of the aspirated cyst fluid was recorded and only partially replaced by ethanol or tetracycline. The injected solution was left inside the cyst for approximately 2 h before drainage. The patients were observed for 24 h after percutaneous sclerotherapy.

Laparoscopic deroofing was performed under general anesthesia with the patient in a supine position. After insertion of the Veress needle just below the umbilicus, a CO_2 pneumoperitoneum was installed and three trocars were inserted. The cyst was aspirated and deroofed with excision of the walls near the liver tissue by an electro-surgical hook knife[7].

Statistical analysis was performed using SPSS® (SPSS 12.0.1, Chicago, Illinois, USA). Continuous variables were expressed as mean ± SD and differences were analysed using the Mann-Whitney U test. Categorical variables were analysed using Pearson's χ^2 test or Fisher exact test (when a table had a cell with an expected frequency of less than 5). $P < 0.05$ was considered significant.

RESULTS

Simple liver cysts

Simple liver cysts were diagnosed in 76% (41/54) of patients. The mean age at initial diagnosis was 57.5 yrs. The most common reason for referral was abdominal complaints in 35 patients (85%), consisting of pain in the right upper quadrant or epigastrium, discomfort or feeling of abdominal distension. Three of these patients presented with an acute onset of abdominal pain which could be attributed to intracystic bleeding as the appearance on US or CT was suggestive of a bleed within the cyst. Two patients were analysed because of jaundice and cholestasis, respectively, with abdominal imaging studies showing internal septations and a nonhomogeneous appearance. In 2 of the 41 patients (5%), suspicion of a malignant lesion (because of prior surgery for colorectal cancer) was the indication for further analysis. One patient presented with fever due to infection of the cyst. One patient with a prior medical history of a simple liver cyst on imaging was referred for analysis on the suspicion of intracystic bleeding in the absence of abdominal complaints.

Abdominal US was performed in all patients, additional contrast enhanced CT in 23 (56%) and MRI in two patients (Table 1). In case of an uncomplicated, simple liver cyst, abdominal imaging showed the characteristic features as described above (methods section) (Figures 1 and 2). Patients with intracystic bleeding as complication mainly showed a nonhomogeneous appearance of the lesion or internal echogenic material, with septations within the cyst (Figure 3). In most of these patients, the diagnosis of a hepatobiliary cystadenoma was considered but rejected because they all were known with simple liver cysts on

Table 1 Characteristics of 54 patients with simple liver cysts and PLD mean ± SD

	Simple liver cyst	PLD	Total
n (male:female)	41 (4:37)	13 (1:12)	54 (5:49)
Age (yr)	57.5 ± 14.9	52.8 ± 7.8	56.3 ± 13.6
(range)	(21-81)	(40-67)	(21-81)
Mean lesion size (cm)	12.6 ± 6.3	13 ± 4.8	12.7 ± 5.6
(range)	(3.5-25)	(8-21)	(3.5-25)
Diagnostic imaging (US:CT:ERCP:MRI)	41:23:2:2	13:11:1:0	54:34:3:2
Location of treated cyst (right:left:bilateral)	14:09:18	07:02:04	21:11:22

PLD: polycystic Liver Disease; US: ultrasound; CT: computed tomography; ERCP: endoscopic retrograde cholangiography; MRI: magnetic resonance imaging.

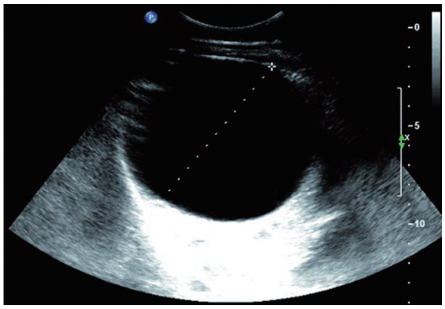

Figure 1 Abdominal ultrasound of a solitary simple liver cyst showing a well-defined anechoic unilocular fluid-filled lesion with posterior acoustic enhancement.

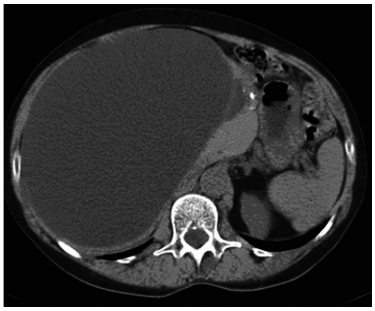

Figure 2 Abdominal computed tomography of a large solitary simple liver cyst showing a well-demarcated lesion with fluid density and without enhancement after contrast administration.

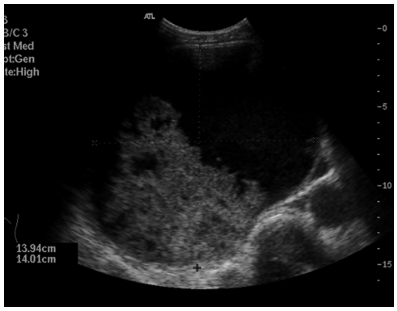

Figure 3 Abdominal ultrasound of a complicated liver cyst showing a well defined hypoechogenic lesion with solid appearing blood clot contents.

Table 2 Treatment and outcome during follow-up of 54 patients with simple liver cysts and PLD

	Simple liver cyst (n = 41)		PLD (n = 13)		Total (n = 54)	
	Surgical	Percutaneous sclerotherapy	Surgical	Percutaneous sclerotherapy	Surgical	Percutaneous sclerotherapy
Treatment[1]	11 (27)	30 (73)	4 (31)	9 (69)	15 (28)	39 (72)
Cyst recurrence	3 (27)	6 (20)	4 (100)	7 (78)	7 (47)	13 (33)
Additonal treatment after recurrence	2 (18)	1 (3)	3 (75)	4 (44)	5 (33)	5 (13)

PLD: polycystic liver disease. [1]Percentages of total are given in parentheses.

previous imaging studies. In two patients, endoscopic retrograde cholangiography (ERC) was used to assess possible communication with the intrahepatic biliary system. The mean size of the cysts was 12.6 cm. The cysts requiring treatment were located centrally in 18 patients (44%) with extension into the left and right liver lobes. In 14 patients (34%), the cyst was located in the right liver lobe. The mean follow-up time, from initial presentation elsewhere or in our center to initial treatment, was 31.5 mo (range 1-156 mo).

Percutaneous sclerotherapy was applied in 30 patients (73%). Of these, 2 patients had undergone percutaneous aspirations elsewhere to determine whether abdominal symptoms could be attributed to the cyst. Although the complaints had completely resolved after the intervention, recurrence after approximately 6 weeks with concomitant recurrence of abdominal pain occurred.

Four patients had unsuccessful, previous percutaneous sclerotherapy elsewhere with the larger cysts showing recurrence within 6 to 9 mo along with abdominal complaints. In the present series, the mean aspirated volume of the cyst was 2223 ± 1772 mL (range 50-5000). The mean volume of the sclerosant used for cyst ablation was 179 ± 95.8 mL (range 25-350 mL). In all these patients, an immediate decrease in size was demonstrated after percutaneous sclerotherapy on ultrasound examination. Complications were encountered in 2 patients (6.7%), including an intracystic bleed which did not require further treatment and one infection of the treated cyst which required re-admittance for intravenous antibiotic treatment. During follow-up, cyst recurrence was seen on US in 6 of the 30 patients (20%) within 4 mo. Of these, only 1 patient (1/30; 3.3%) showed concomitant recurrence of symptoms and in this patient, percutaneous sclerotherapy was eventually repeated twice. In the remaining 5 patients, relief of abdominal symptoms was attained despite small cyst recurrence, be it that the recurrent cysts were all significantly smaller than before treatment. These patients were discharged from further follow-up when no further increase in size of the recurrent cyst was detected. The mean hospital stay in patients after percutaneous sclerotherapy for a simple liver cyst was 3.4 d (median 2; range 2-23). The mean time of follow-up after treatment was 15 mo (2-35 mo).

Of all 41 patients, surgical treatment was performed in 11 patients (27%) with a mean duration of operation of 132.5 min (range 70-340). Of these patients, 4 patients had had previous treatment for relief of abdominal complaints, including prior percutaneous sclerotherapy in a hospital elsewhere in 3 patients and laparoscopic deroofing in 1 patient. Cyst recurrence in these patients was seen within 5 mo after the initial procedure. Surgical procedures included cyst wall deroofing and omentoplasty in 8 patients, of which 5 had laparoscopic procedures and 3 had an open approach. Two patients underwent laparotomy and enucleation of the cyst and 1 patient underwent local excision of a cyst near the common hepatic duct. Reasons for an open procedure were a superior or posterior location of the cyst in the liver or the fact that a malignancy could not be ruled out. A postoperative complication occurred in 1 patient (1/11; 9%) consisting of bile leakage requiring percutaneous drainage of a bile collection.

Cyst recurrence after surgical treatment was seen in three patients (3/11; 27%). Additional percutaneous sclerotherapy was carried out in 2 of these patients because of concomitant progressive abdominal complaints (2/11; 18.2%). Eventually, complete regression of these cysts was achieved (Table 2). The mean hospital stay in patients after surgical treatment for a simple liver cyst was 13.9 d (median 8; range 4-35).

In patients with simple liver cysts (including solitary and multiple cysts), no significant differences in recurrence rate were observed after surgical treatment compared to recurrence after percutaneous sclerotherapy [27.3% (3/11) *vs* 20% (6/30), respectively; P = 0.680]. However, additional treatment was required in 18.2% (2/11) of

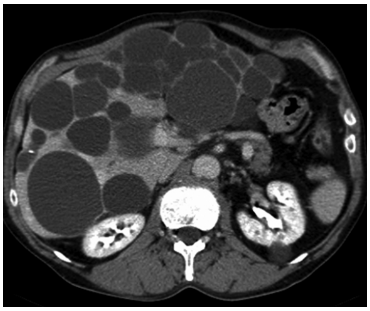

Figure 4 Abdominal computed tomography of a patient with PLD showing multiple cysts throughout the liver.

patients with simple liver cysts after surgical treatment compared to 3.3% (1/30) of simple liver cysts after percutaneous sclerotherapy ($P = 0.170$).

Polycystic liver disease

Thirteen patients (13/54; 24%) were diagnosed with PLD and all experienced progressive abdominal complaints. Abdominal US was performed in all patients. Eleven patients underwent abdominal CT (11/13; 85%) showing multiple unilocular cysts throughout the liver (Figure 4). In two patients, the imaging findings were suggestive of an intracystic bleeding and the diagnosis was confirmed by percutaneous drainage of dark brown, haemorrhagic fluid. The mean size of the largest dominant cyst was 13 cm and these larger cysts were located in the right liver lobe in 7 patients (54%) (Table 1).

In PLD, percutaneous sclerotherapy was carried out in 9 patients (69%). Only 3 patients had previous percutaneous drainage of a dominant cyst. Nevertheless, these cysts recurred within 4 mo. The maximum volume of intracystic fluid drained in this series was 5200 ml. No complications were seen after percutaneous sclerotherapy. Cyst recurrence was seen in 7 patients (77.8%). Of these, 4 patients required repeat percutaneous sclerotherapy because of progressive abdominal pain.

Surgical treatment was undertaken in 4 patients (30.8%) with PLD, and the mean duration of operation was 110 ± 47 min (60-170 min). All these patients had previous treatment in their medical history; three patients had undergone several attempts at percutaneous sclerotherapy and laparoscopic deroofing, and 1 patient had undergone percutaneous aspiration only.

In these patients, cyst recurrence occurred within one year after the various procedures. The surgical procedures included laparoscopic deroofing in 2 patients and laparotomy with deroofing in another 2 patients. Postoperative complications were seen in 2 patients, consisting of intracystic bleeding in one patient and bile leakage from a peripheral bile duct requiring biliary stenting in the other patient. Cyst recurrence after surgical treatment was seen in all patients after surgical treatment for PLD (Table 2). Eventually, liver transplantation was required in 2 patients because of deterioration of liver and kidney function in one patient, and failed percutaneous sclerotherapy in the other patient.

DISCUSSION

The series described in this study, represents a selected group of patients who underwent treatment because of symptomatic large liver cysts. These patients mainly presented with abdominal symptoms (88%) and showed a mean cyst size of 13 cm. The large size of the cysts in this population is due to referral bias, because patients with abdominal complaints and large liver cysts are more likely to be referred for further diagnosis and/or treatment. Most of our patients (76%) were diagnosed with simple liver cysts. The precise incidence is difficult to determine, but is reported to be around 2% to 5%, according to ultrasound studies[1,8]. A predominance in women is found with a mean age between 50 and 60 years, which is in accordance with our results[9].

Abdominal US or CT are the first choice of imaging for abdominal pain and are highly accurate for simple liver cysts[10]. Complications such as intracystic bleeding, rupture to the peritoneal cavity or intracystic infection may give rise to diagnostic problems because of their unusual appearance. Intracystic bleeding was responsible for abdominal complaints in five (15%) patients with liver cysts in our series. In one patient with a preoperative suspicion of a hepatobiliary cystadenoma, intraoperative frozen section of the cyst wall showed features of a simple cyst without ovarian stroma, and deroofing revealed hemorrhagic intracystic fluid. In this particular case, a simple cyst was misdiagnosed as cystadenoma[11]. The precise etiology of bleeding in a simple cyst remains unclear, but rupture of blood vessels inside the cyst wall due to rapid enlargement is thought to be a likely mechanism[12]. In such cases, abdominal multiphase contrast enhanced computed tomography (CT) may be required to further characterize the nature of the lesion. CT or MRI imaging is also useful for exact localization and establishing the relationship of the cyst with surrounding vascular structures, when surgery is considered.

PLD is a rare clinical entity characterized by multiple simple cysts throughout the liver with variable size. Approximately 80% of PLD occurs in patients older than 60 years[2,13], as was also found in our patients. This entity may be associated with polycystic kidney disease or autosomal dominant PLD. Therefore, the kidneys and the pancreas should be assessed during imaging studies to determine whether these organs are affected too. Patients with PLD usually lack symptoms and are diagnosed during physical examination or incidentally, when imaging studies are performed for other reasons. Routine liver function tests usually are normal but may show elevated cholestatid parameters due to external compression of the bile ducts[14].

Although most patients with liver cysts are asymptomatic, a minority develops symptoms due to enlargement. As mentioned above, treatment should be considered only for progressive abdominal pain, or when complications have occurred. Also in case of suspicion on a hepatobiliary cystadenoma, surgical resection should be performed. Complete evaluation including upper GI endoscopy must be undertaken to exclude other causes of abdominal symptoms before symptoms may be attributed to the cyst. When the diagnosis has been established, several options ranging from no intervention to surgical treatment can be considered. Percutaneous sclerotherapy is first choice treatment because

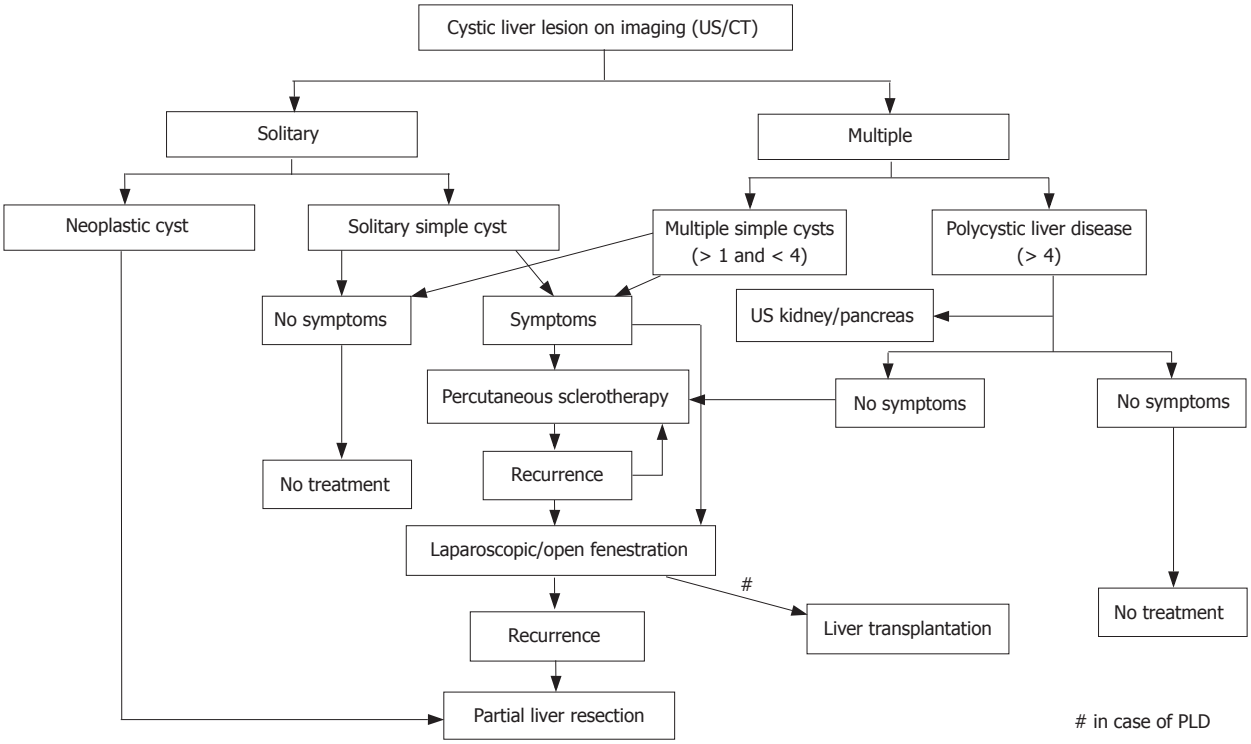

Figure 5 Proposed algorythm in the management of patients with cystic liver lesions. US indicates ultrasound; CT: computed tomography; PLD: polycystic liver disease.

of its minimally invasive character and safety of the technique.

Percutaneous drainage of the cyst only results in temporary relief of abdominal pain and may be used as a trial treatment, to determine whether abdominal symptoms are attributable to the cyst[15]. For definitive treatment, concomitant instillation of tetracycline or ethanol in the cyst is applied to ablate the epithelial lining of the cyst which reduces recurrence remarkably[3,16]. Nevertheless, in our series, recurrence of the cyst after single percutaneous sclerotherapy of simple liver cysts (including solitary and multiple cysts) was seen in 20% of patients, as is consistent with other reports[3]. This failure rate may be explained by instilling an insufficient amount of sclerosant or by insufficient exposure of the cyst lining to the sclerosant, especially in large cysts. Of note is that percutaneous sclerotherapy for PLD is unsuccessful in the short-term, as was reflected by a recurrence of 77.8% within a few months in this series. Percutaneous sclerotherapy in PLD is indicated to assess whether symptoms are attributable to a dominant cyst or to bridge the patient to liver transplantation (Figure 5).

The second choice of treatment after percutaneous sclerotherapy has failed, comprises cyst wall deroofing[17]. This technique is indicated when a cyst recurs after percutaneous sclerotherapy with concomitant increase of abdominal complaints. The technique is based on removal of part of the cyst wall, usually the part lying external to the liver surface, allowing free drainage of intracystic fluid into the peritoneal cavity. Alternatively, enucleation of the cyst or a formal partial liver resection may be an option. Obviously, enucleation is the more parenchyma sparing method while a relatively avascular dissection plane exisits between the cyst wall and the surrounding liver parenchyma.

In previous reports, laparoscopic approach of cysts located in segments Ⅵ, Ⅶ and Ⅷ were considered a contraindication for this non-invasive procedure[15,18]. However, according to a recent study, location of the cyst should not be a contraindication anymore for a laparoscopic procedure[4]. During the surgical procedure, the cyst content should be aspirated to determine whether communication with the intrahepatic biliary system is present. This may prevent bile leakage from cut bile ducts running from the parenchyma into the cyst wall. As mentioned above, complicated liver cysts after intracystic bleeding may show internal septations and/or wall nodules on imaging studies, and therefore, resection is advised if a neoplastic cyst cannot be ruled out.

In conclusion, the majority of patients with simple liver cysts or PLD are referred for progressive abdominal pain. As initial treatment, percutaneous sclerotherapy is appropriate and may determine whether the symptoms are attributable to the cyst. Surgical deroofing of the cyst wall, either laparoscopically or during laparotomy, is indicated when percutaneous treatment has failed. However, the results of percutaneous sclerotherapy and surgical treatment for PLD are disappointing. In case of suspicion of a complicated cyst or malignant lesion, complete excision of the cyst or partial liver resection is indicated.

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RAPID COMMUNICATION

Anal plugs and retrograde colonic irrigation are helpful in fecal incontinence or constipation

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Abstract

AIM: To evaluate the feasibility, clinical effect and predicting factors for favorable outcome of treatment with anal plugs in fecal incontinence and retrograde colonic irrigation (RCI) in patients with fecal incontinence or constipation.

METHODS: Patients who received treatment with an anal plug or RCI between 1980 and 2005 were investigated with a questionnaire.

RESULTS: Of the 201 patients (93 adults, 108 children), 101 (50%) responded. Adults: anal plugs (8), five stopped immediately, one stopped after 20 mo and two used it for 12-15 mo. RCI (40, 28 fecal incontinence, 12 constipation), 63% are still using it (mean 8.5 years), 88% was satisfied. Younger adults (< 40 years) were more satisfied with RCI (94 % *vs* 65%, P = 0.05). Children: anal plugs (7), 5 used it on demand for an average of 2.5 years with satisfactory results, one stopped immediately and one after 5 years. RCI (26 fecal incontinence, 22 constipation), 90% are still using it (mean time 6.8 years) and felt satisfied. Children tend to be more satisfied (P = 0.001). Besides age, no predictive factors for success were found. There was no difference in the outcome between patients with fecal incontinence or constipation.

CONCLUSION: RCI is more often applied than anal plugs and is helpful in patients with fecal incontinence or constipation, especially for younger patients. Anal plugs can be used incidentally for fecal incontinence, especially in children.

Key words: Fecal incontinence; Constipation; Retrograde colonic irrigation; Anal plug

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INTRODUCTION

Fecal incontinence is a devastating complaint from patients and affects their quality of life. The prevalence of fecal incontinence is estimated to be around 5% in the general population, being higher in women than in men and up to 40% in nursing homes[1,2].

The first step in treatment of all forms of fecal incontinence is to regulate defecation with a fiber enriched diet, fiber supplementation and physiotherapy or biofeedback of the pelvic floor. When this fails, a sphincter repair is performed in patients with sphincter defect. Surgical treatments such as gracilis plasty, sacral neuromodulation (SNS), artificial sphincter or eventually a stoma are other options[3]. However, these techniques are not always successful, carry a substantial morbidity and are not generally available. Especially in non-Western countries, surgical options are very scarce. Another possibility is the use of an anal plug. This is a device consisting of compressed foam in cone shape used to close off the anus. Some patients reach continence but pelvic floor function is needed to support the plug. A recent Cochrane review showed some effects of a short-term usage, but little is known about long-term possibilities[4]. Although no research has been done in non-Western countries, they are used frequently and available even on market.

An alternative is retrograde colonic irrigation (RCI). RCI in the morning can diminish the chance of unwanted fecal loss during the day. The patient installs a rectal tube connected with a bag with 0.5-1 liter warm tap water and let this pour in, while sitting on a toilet. This "super-enema" will reach higher than the rectum and cleans the left hemicolon. A recent study about RCI reports a success rate of 41%[5].

Chronic idiopathic constipation is a common complaint in adults with a frequency of around 2%-7% in the general population, increasing up to 20% in nursery homes[6,7]. In children, a frequency of 17% has been reported[8].

Management of chronic constipation includes increasing fluid and dietary fiber intake, laxatives and increasing physical activity. Physiotherapy or biofeedback is the next step in both patients with or without slow transit or anismus[9,10].

In patients with constipation, RCI is also used to clean the bowel. One study reported an effective rate of 65%[5].

Although there are indications in the literature and clinical experience about the improvement of defecation disorders with RCI and the anal plugs, little is known about the long-term outcome.

The aim of the present study is to evaluate the feasibility, effectiveness and predicting factors for a favorable long-term outcome with the use of an anal plug or RCI for fecal incontinence and constipation.

MATERIALS AND METHODS

Patients

The database of the enterostomal therapists were searched for patients with fecal incontinence or constipation who received treatment with an anal plug or retrograde colonic irrigation (RCI) between 1980 and 2005. Fecal incontinence was defined according to the Vaizey criteria[11] and all patients scored higher than 12. Constipation was defined according to the Rome II criteria[12]. None of the patients responded to medical treatment or biofeedback.

Firstly, the general practitioner was approached for permission to contact the patients. The patients or their parents (in children, age < 18 years) were asked to answer questions in a questionnaire about their defecation problems (incontinence or constipation), urologic problems, medication, surgical procedures, the actual treatment used, frequency of use of anal plugs or RCI, the effect of the treatment for their complaints and quality of life (scale 1-5: 1 excellent and 5 very poor, and 1-3 satisfactory), and side effects of the treatment or procedure.

The study was approved by the Medical Ethical Committee of the VU University Medical Center.

Anal plugs

The Conveen® anal plug (Figures 1 and 2) was used (Coloplast, Amersfoort, The Netherlands). This is a disposable (for single use) polyethylene plug. It consists of compressed foam in a conus shape, with a removal cord at one side. It is introduced in the anus with the cord hanging out. After introduction, the plug will extend within 30 s to its maximum size, thus closing off the anus. Proper instruction about introduction and removal (after 12 h or before defecation) of the plug was given. Costs of one plug are approximately €4.

RCI

For RCI, most patients used the Iryflex® (Braun Medical BV, Oss, The Netherlands) and occasionally the hand RCI from Braun or Coloplast (Figure 3). Patients were instructed about the proper use of the system. The Iryflex® pump with a reservoir was filled with hand warm tap water, the connecting tubes were prefilled with water to avoid air insufflation, and the cone was inserted. Then the pump was activated at a preset speed.

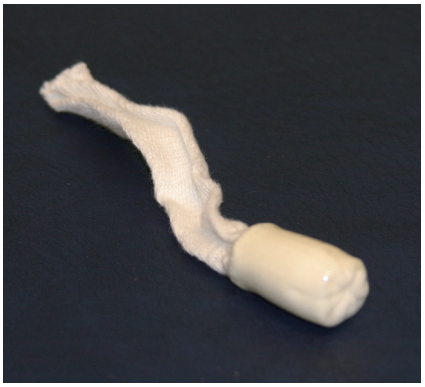

Figure 1 Wrapped anal plug.

Figure 2 Unwrapped anal plug.

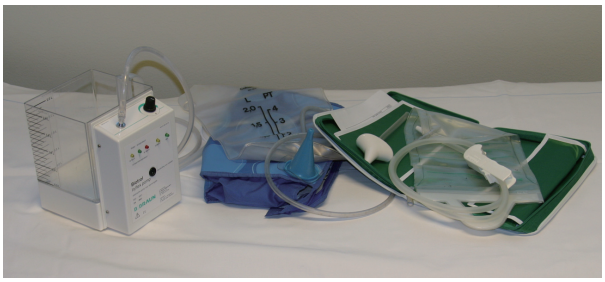

Figure 3 Rectal cleasing system.

The hand system device consisted of an irrigation bag, a tube and a cone tip. The irrigation bag was hung at shoulder height and filled with 500-1000 mL of hand warm tap water. The tube was prefilled with water to avoid air insufflation. Then the lubricated cone was inserted and irrigation started. The speed is manually regulated by a clamp. The procedure was performed before or two hours after breakfast preferably. The patient was instructed to wait for the urge to defecate before removing the cone. Next evacuation of the fluid and feces could take place.

The Iryflex® pump and the tubes were replaced every six mo. The hand systems should also be replaced every 6 mo.

All patients had one visit for instruction and a second one when necessary. In addition, there was an open access for telephone consultation. Written instruction was also provided.

Statistical analysis

Results were described as medians and range. Differences among groups were assessed with a Chi-square test or

Table 1 Patients treated with anal plugs or RCI and response to questionaire *n* (%)

	Anal plugs		RCI		Total	
	n	Response	*n*	Response	*n*	Response
Adults	14	8 (57)	79	40 (51)	93	46 (49)
Children (< 18 yr)	16	7 (44)	92	48 (52)	108	55 (51)
Total	30	15 (50)	171	88 (51)	201	101 (50)

RCI: retrograde colonic irrigation.

Fischer's exact test when appropriate. $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

From a total of 201 patients, 101 (50%) questionaires were obtained (Table 1). Sixteen cases refused the general practioners and 84 patients did not respond. The database showed no differences between responders and non-responders in underlying disorders, age or sex.

Adults

Anal plugs: Of the 14 patients with fecal incontinence who were prescribed an anal plug, 8 patients including 6 women (median age 57 years, range 37-77) returned the questionnaire. The causes of fecal incontinence were multiple sclerosis (5), dystrophia myotonica (1), haemangioblastoma (1) and idiopathic (1). Five patients had urological problems. Four patients complained of an impact on their social life. Of the 8 patients, 5 patients stopped using the plug immediately, one patient stopped after 20 mo and two patients used the anal plugs for 12-15 mo and are still using it satisfactorily. Patients used the plug generally on demand. Major side effects of the anal plugs were displacement of the plug and leakage during diarrhea.

Retrograde colonic irrigation: Of the 79 patients including 32 women, 40 (median age 42 years, range 19-90) returned the questionnaire. There were 28 patients with fecal incontinence and 12 patients with constipation. Their demographics are shown in Table 2. No significant difference was found between patients with fecal incontinence or constipation.

Twenty-five patients (63%) are still using the irrigation (Table 2). The mean time of using RCI was 8.5 (range 2.5-18) years. Of the 25 patients, 8 (32%) irrigated daily, 9 (36%) 3 times a week and 8 (32%) twice or less a week. Overall, 29 patients (73%) were satisfied with the therapy. From the 25 actual users, 22 (88%) were satisfied about the therapy. There was a tendency of more actual use and satisfaction among patients with fecal incontinence, but this was not statistically significant (Table 2). Younger adults (< 40 years) were more satisfied with RCI, 94% *vs* 65% ($P = 0.05$).

Major side effects were abdominal cramps (37.5%) and the cumbersome procedure (30%) which is time consuming and difficult to take outside home. The patients who discontinued were less satisfied about the therapy (43% *vs* 88%, $P < 0.003$) and (85% > 40 *vs* 32% > 40, $P < 0.005$). Of the 25 patients who used RCI, 17 (68%) were < 40 years of age. Gender did not influence the discontinuation of the therapy. The major reason for discontinuation was the side effects.

Table 2 Demographics and actual use of retrograde colonic irrigation in adult patients *n* (%)

	Fecal incontinence	Constipation	*P*
Total	28	12	
Age (yr)	42	46	
Female /male	23/5	9/3	
Urological problems	15 (54)	6 (50)	
Gynaecological problems	3 (11)	3 (25)	
Neurological disease	11 (39)	3 (25)	
- Spinal bifida	4	2	
- Multiple sclerosis	2	1	
- Spinal injury	3	0	
Anal atresia	2	0	
Impact complaints social life	16 (64)	8 (67)	
Actual using RCI	20 (71)	5 (42)	0.09
Therapy satisfaction all patients	23 (82)	6 (50)	0.056
Therapy satisfaction among actual users	19 (95)	3 (60)	0.7

No significant differences between patients with fecal incontinence and constipation were found.

Children

Anal plugs: Of the16 patients with fecal incontinence using an anal plug, 7 patients including 3 females (median age 10 years, range 7-16) returned the questionnaire. The causes of fecal incontinence of the respondents were spina bifida (6) and anal atresia (1). All underwent surgical procedure(s) and 6 had urological problems. Five patients reported an impact on their social life.

Five patients felt satisfied, and used the plug generally only on demand once a week before swimming or social events. Two patients stopped using the plug immediately and after five years. The others used the tampon for an average of 2.5 years. A major side effect was displacement of the plug.

Retrograde colonic irrigation: Of the 92 patients, 48 and/or their parents including 22 females (median age 12 years, range 4-19) returned the questionnaire. There were 26 patients with fecal incontinence and 22 with constipation. Their diagnoses were spina bifida (29), anorectal malformation (7), Hirschsprungs disease (4), idiopathic constipation (5) and miscellaneous disorders (3). No difference between patients with fecal incontinence or constipation was found in demographics (Table 3).

Among all patients, 44 (90%) underwent surgical procedures and 39 (81%) had urological problems. Twenty-nine (60%) of the patients complained that the use of the rectal cleansing device had an impact on their social life.

Forty-three (90%) continued the RCI on a regular basis and were satisfied about the therapy. The mean time of using RCI was 6.8 years. A few patients complained that the device was difficult to take outside home, that the insertion piece was too big and sometimes an adjustable toilet seat was needed. No difference was found in the therapeutic outcome in patients with fecal incontinence and constipation.

Table 3 Demographics and use of retrograde colonic irrigation in children *n* (%)

	Fecal incontinence	Constipation
Total	26	22
Age (yr)	11	13
Female /male	14/12	12/10
Urological problems	22 (85)	17 (77)
Any surgery	25 (96)	19 (86)
Neurological disease	11 (96)	3 (25)
- Spina bifida	19	10
- Hirsschprungs disease	1	3
- Anorectal malformation	4	3
- Idiopathic constipation	0	5
- Miscellaneous disorders	2	1
Impact complaints social life	15 (58)	14 (64)
Actual using RCI	23 (88)	20 (91)
Therapy satisfaction all patients	24 (92)	21 (88)
Therapy satisfaction among actual users	23 (100)	20 (100)

No significant differences between patients with fecal incontinence and constipation were found.

When compared with adults, children tend to be more satisfied ($P = 0.001$). No other factors were found that predicted a successful treatment.

DISCUSSION

This study shows the long-term benefit of anal plugs in selected patients with fecal incontinence and RCI for patients with fecal incontinence or constipation, both in children and adults. The small number of patients lies in a major limitation. The response of 50% is due to the fact that some general practitioners were not permitted to approach the patients and the time span was 25 years. In spite of this, some conclusions can be made.

Our study found that anal plugs are not often prescribed. Five of the 8 (62%) adults and 2 of the 7 (14%) children stopped their use immediately due to local irritation and displacement. The seven patients who continued generally used it once a week during social events, the children mainly during swimming. The term of use however, is not very long, the longest being 5 years. Children seem to more appreciate the plug, possibly due to their swimming lessons.

Little information is found in the literature about the use of anal plugs[4,13-18]. A Cochrane review from 2005[4] looking at randomized and quasi-randomized controlled trials only found four studies, from which only two were published[13,14]. In all published anal plug studies, the evaluation was only made during the study and lasted not more than 2-3 wk. Early dropout varied between 20%-65% and was less in children[13]. Continence was achieved in about 80%-90% in those who continued the therapy. The maximum time of having the plug in place was 12 h[15,18]. Size of the plug did not seem to matter much[13]. Only patients with anal atresia sometimes needed smaller plugs[14]. A study in children comparing two different types of plugs showed that plug loss was less and overall satisfaction was greater with poly-urethane plugs than poly-vinyl alcohol plugs[14].

Major side effects in all the studies are local irritation, leakage and plug loss. These local side effects prevent its daily use. No predicting factors for a successful outcome were reported in any of the studies. Our study is the first evaluating the long- term use of anal plugs and shows that in selected patients intermittent use up to 5 years is possible and especially in children is worthwhile trying.

RCI was applied in much more patients, and continuation and satisfaction were much higher, both in adults (65% and 88%) and children (90% and 100%). The average time of use was 8.5 years, ranging from 2.5 years to 18 years in adults. This indicates that RCI should be considered in patients with fecal incontinence or evacuation disorders, even when taking the response rate of the questionnaire into account. Children coped especially well with the procedure and were more pleased with the results. Besides younger age, no predictive factors were found for a successful treatment. In adults RCI was tend to be more successful in patients with fecal incontinence than in patients with constipation. The time consuming procedure and irrigation related problems are the major side effects and are often the reason for discontinuation.

A Dutch group[5] evaluated 169 (60%) of 267 patients who were offered RCI. The overall continuation was 45%. The dropout was higher in patients with fecal incontinence or soiling. Patients with evacuation disorders continued all, although not all were satisfied. The time consuming caused the discontinuation of the procedure. The median frequency of RCI was once daily. The average observation period was 4.5 years with a maximum of 13 years.

Two other groups[19-23] reported their results with RCI. A Japanese group[19] introduced RCI first successfully in 10 patients with evacuation after a lower anterior resection with defecation problems. A Danish group reported in 25 adults and 10 children, a 42% improvement in patients with fecal incontinence and 8% in patients with constipation[20]. In 21 neurological patients of the same group, these figures were 73% for fecal incontinence and 40% with constipation, respectively[21]. The lower efficacy of RCI in constipated patients was thought to be due to an overstretched, less sensitive bowel wall[22].

Irrigation requires a lot of self-motivation and consumes valuable time. Good instruction and feedback are mandatory. The exact mechanism behind RCI is not known. The effect of water is obviously partly due to a wash-out effect. In addition, a large amount of water generates mass movements[24].

Another cleansing treatment is antegrade colonic irrigation through an appendicostoma, a tapered ileum or a continent colonic conduit as an alternative for patients with defecation disorders[25-27]. Although results have been reported to be better, RCI requires no surgical intervention and has minimal side effects.

Although the response rate of 50% was not very high and responses in children represent in younger children also the impression from the parents, it shows that some patients do benefit from the therapy and it is worthwhile trying. Unlike surgical procedures, both anal plugs and RCI cause no harm. However, motivation does play an important role.

Side effects and the cumbersome procedure with RCI are the main drawbacks with these therapies. In many European countries, these therapies have found their way and are accepted. Prospective studies including evaluation of anorectal function[28] might help predict favorable outcome and to focus on subgroups for these therapies.

In conclusion, although the response in our study was limited, these data do give an insight in the long-term use of anal plugs and RCI in patients with fecal incontinence and chronic constipation both in adults and children. This is the longest observation period ever reported.

Anal plugs are not often prescribed and few patients will continue their use. Generally they are used on demand during social events. Children seem to be more pleased with the therapy. The longest period of use reported was 5 years. Local side effects limited its use.

RCI is more often applied, continued longer and can be helpful in many, especially younger patients. Continuation varies between 63%-90% with a satisfaction of around 90%. The longest reported use was 18 years. The abdominal cramps and cumbersome procedure are a major drawback.

Patients should at least be offered a try-out with these two therapies to improve their complaints and quality of life. These treatments can be helpful, especially in non-Western countries where unfortunately less (surgical) options are available.

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RAPID COMMUNICATION

Selective sphincteroplasty of the papilla in cases at risk due to atypical anatomy

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Abstract

AIM: To analyze the indications, efficacy and safety of sphincteroplasty in our centre.

METHODS: A retrospective study of sphincteroplasty in 53 cases of papilla at high risk was performed in 2004-2006. The procedure consisted of duodenoscopy with Olympus TJF 145 Videoduodenoscope, approach to the biliary tract using a catheter with a guidewire, and dilatation of the papilla with a dilatation balloon catheter using a syringe with a manometer for control of the filling pressure.

RESULTS: The indications included intradiverticular papilla in 26 patients (49%), stenosis of a previous sphincterotomy in 19 patients (35.8%), small size of the papilla in 4 patients (7.5%), Billroth Ⅱ gastrectomy in 3 patients (5.6%), and coagulopathy in one patient (1.9%). The efficacy was 97.8%, with all the calculi extracted from the common bile duct in 84.4% of the patients, even though 21 of the patients (39.6%) had calculi with a diameter equal to or greater than 10 mm. Seven patients (13.2%) presented complications: haemorrhage in 1 patient (1.9%) and mild pancreatitis in 6 patients (11.3%). The mean hospital stay in case of complications was of 3 ± 0.63 d.

CONCLUSION: Sphincteroplasty is highly effective, with a complication rate similar to that of sphincterotomy, furthermore, the complications are of low clinical importance. The use of the 10 mm balloon makes it possible to extract calculi with a diameter of over 15 mm and to extract more than 3 calculi without increasing the rate of complications and reduces the need to resort to lithotripsy or rescue sphincterotomy.

Key words: Sphincteroplasty; Hydrostatic dilatation of the papilla; Choledocholithiasis; Sphincterotomy; Function of the sphincter of Oddi; Acute pancreatitis; Intradiverticular papilla

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INTRODUCTION

The prevalence of cholelithiasis is 10%-15% in adult population. Calculi are detectable in the biliary tract in 17% of patients with symptomatic cholelithiasis[1]. The introduction of laparoscopic cholecystectomy has led to an increase in the demand for endoscopic treatment of choledocholithiasis.

Currently, the standard treatment for calculi located in the biliary tree is endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy. This procedure is not exempt from risks and the incidence of complications is of 6%-10%, with a mortality of 1%[2]. The early complications occurring after ERCP include acute pancreatitis, haemorrhage and perforation. Sphincterotomy also causes a permanent loss of function of the sphincter of Oddi, thus exposing the biliary tree to reflux of the duodenal contents. This leads to bacterial colonisation and chronic inflammation of the biliary tree, which, hypothetically, could increase the incidence of primary choledocholithiasis and tumours of biliary origin. Although this is not a significant problem in elderly patients, it is the cause for concern in younger individuals. For this reason, Staritz, in 1983, proposed the hydrostatic dilation of the papilla as an alternative that would enable the removal of calculi without the risks of sphincterotomy[3].

At that time, it was hypothesised that the dilation of the papilla could become the treatment of choice in younger individuals as it transitorily increases the diameter of the papillary orifice, allowing the extraction of calculi while preserving the architecture and function

of the sphincter. However, Disario[4] showed that a high frequency of complications could lead to hydrostatic dilation of papilla falling into disuse as the treatment of choice in choledocholithiasis. Currently, it is considered as an alternative to sphincterotomy in certain cases at a high potential risk, principally of haemorrhage or perforation.

The objective of the present study was to analyse the indications, efficacy and safety of hydrostatic dilation of the papilla and to compare them with the same variables for conventional sphincterotomy in our centre.

MATERIALS AND METHODS

A retrospective study of 461 consecutive ERCPs was performed in our centre between February 2004 and March 2006. Sphincterotomy was performed in 231 (50%) and hydrostatic dilation of the papilla in 53 patients (11.5%). This study was to focus on these latter cases.

In all cases, ERCP and dilation of the papilla procedure consisted of signing of the informed consent form for anaesthesia and ERCP, antibiotic prophylaxis with amoxicillin-clavulanic acid (ciprofloxacin in cases of allergy) at the time of starting the endoscopy, monitoring and deep sedation with propofol supervised by an anaesthetist, Olympus TJF 145 Videoduodenoscope, approach to the biliary tract using a catheter ("XL cannula", Microvasive Rapid Exchange, taper tip) with a 0.035/260 cm Jagwire Stiff Shaft guide wire.

A cholangiography was performed after introduction of the catheter into the biliary tract. If pathological material observed justified therapeutic action, the catheter was withdrawn, leaving the guide wire in place. Dilatation of the papilla was performed with a Hurricane Rx Microvasive biliary dilation balloon catheter (180 cm long with a balloon length of 4 cm and diameter of 6 or 10 mm) using a syringe with the "Breeze TM RX inflation device" manometer (Boston Scientific) for control of the filling pressure. The dilation balloon catheter was advanced over the guide wire and until the mid-portion of the balloon was situated in the region of the biliary sphincter. After positioning, a diluted contrast (50% contrast plus 50% saline) was introduced under endoscopic and fluoroscopic control to maintain the correct position until a pressure of 11 atmospheres was reached in the case of the 6 mm balloon and 8 atmospheres in the 10 mm balloon. The pressure was maintained until the notch in the balloon was gradually observed to disappear, after which the balloon was maintained inflated for a further 60 s.

Extraction of the possible calculi was then attempted using a balloon catheter (Extractor RX retrieval balloon, Boston Scientific). A dormier basket (Boston Scientific) was occasionally used and a mechanical lithotripsy basket was also available in case it was required.

Finally, removal of all the calculi and pathological biliary material from the common bile duct was confirmed by an occlusion cholangiography.

The patient remained in the day hospital after the procedure. Depending on the clinical course and the blood amylase level, the patient was either discharged 4-8 h after the procedure or was admitted.

Table 1 Indication of hydrostatic balloon dilation

Indication	*n*	%
Intradiverticular papilla	26	49
Stenosis of previous sphincterotomy	19	35.8
Papilla of small size	4	7.5
Billroth II gastrectomy	3	5.6
Coagulopathy	1	1.9

The size of the calculi was calculated by the ratio between the diameters of the calculus and the tip of the endoscope, both measured on the X-ray image and corrected according to the true diameter of the endoscope[5].

The complications were evaluated in accordance with the consensus document published by Cotton *et al*[6] in 1991.

Statistical analysis

The qualitative variables were described by absolute numbers and percentages. Quantitative data are expressed as mean ± SD. Comparison of the qualitative variables was performed using the odds ratio (OR) and the 95% confidence interval (CI).

RESULTS

A total of 461 ERCPs were performed in February 2004-March 2006. Endoscopic sphincterotomy was performed in 231 patients (50%) and hydrostatic dilation in 53 patients (11.5%).

Our study focused on these 53 patients, 22 males (41.5%) and 31 females (58.5%). The mean age was 73 ± 12.9 years. The most common indication for endoscopic balloon dilation (Table 1) was intradiverticular papilla. In only one case the indication was a septic shock with a secondary coagulopathy (platelets: 39 000, international normalized ratio: 2.1) but a coagulopathy was detected in two further cases: a cirrhotic patient in the group of intradiverticular papilla and a patient on acenocoumarol anticoagulation in the stenosis group.

Of the patients treated with dilatation, 24 (45.3%) did not undergo a cholecystectomy, and 29 (54.7%) were previously cholecystectomized. Of the non-cholecystectomised patients, one underwent laparoscopic cholecystectomy with hydrostatic dilation (Endolap).

In 42 cases (79.2%), calculi were detected on cholangiography. In 44 cases (83%), biliary sludge or calculi were obtained after the dilatation and clearance using a balloon catheter and lavage. The cholangiography gave a false positive result in one case and false negative results in three cases.

The extraction was complete, with total clearance of the biliary tract in 37 of the 44 patients (84%). Although 21 patients (39.6%) presented calculi with a diameter equal to or greater than 10 mm (Table 2), the calculi were over 15 mm in diameter in 10 cases (19%). In one of the cases, the dilatation was not sufficient to achieve extraction of the calculi and a rescue sphincterotomy was performed.

Partial extraction was achieved in 7 patients (16%).

Table 2 Classification of patients according to the presence, size and number of calculi

Biliary material	*n*	%
Normal common bile duct	8	15.1
Biliary sludge / microcalculi / calculi ⩽ 10 mm	24	45.3
Calculi > 10 mm and ⩽ 15 mm	11	20.7
Calculi > 15 mm	10	18.9
Total number of calculi > 10 mm	21	39.6
Total number of cases with 3 or more calculi	14	26.4
Number of cases with calculi > 10 mm or with 3 or more calculi	25	47.2

In 6 cases, despite the extraction of calculi and biliary sludge, one or more residual calculi were observed on occlusion cholangiography, proceeding to the insertion of a 7 cm 10 Fr polyethylene stent. The remaining case was a patient with a Billroth Ⅱ gastrectomy and 19 calculi in the common bile duct, 7 of which had a diameter greater than 12 mm. After the extraction of multiple calculi, an occlusion cholangiography was performed, showing the identified images as air bubbles. The patient was readmitted 11 d later for cholangitis and, in view of the technical difficulty experienced in the previous exploration, it was decided to treat the patient surgically. Choledochoduodenostomy was performed and residual choledocholithiasis was found.

A polyethylene stent was inserted in another 5 cases. In 4 cases, despite evidence of complete clearance of the common bile duct after the extraction manoeuvres, it was decided to ensure biliary drainage by the insertion of a stent because their tendency to recur, stenosis of the previous sphincterotomy with multiple episodes of cholangitis or choledocholithiasis. In the remaining case, the patient presented residual choledocholithiasis with a biliary fistula after laparoscopic cholecystectomy. The papilla was intradiverticular and balloon dilation was therefore performed, with extraction of the calculi and insertion of a plastic stent.

Overall, 43 of 44 patients (97.8%) with bile duct stones had successful bile duct clearance or drainage (endoprosthesis).

Acute pancreatitis was the most common complication, being mild in 5 cases and moderate in 1 case. In our series, no relationship was found between post-dilatation pancreatitis and the size or number of the calculi (Table 3). The incidence of reactive pancreatitis was 11.3% in balloon dilation and 5.2% in sphincterotomy, with an OR of 2.33 (95% CI: 0. 83-7.54).

Late haemorrhage occurred in one (1.9%) of our patients. This was a patient on acenocoumarol anti-coagulation who presented with late haemorrhage five days after the dilatation with melaena and a fall in the haemoglobin (2 g/dL). Early upper GI endoscopy did not detect a potentially haemorrhagic lesion and blood residues. The other two patients with coagulopathy did not present any complication. None of the other complications was serious.

During the same period of time, complications were detected in 21 (9.2%) of the cases treated with sphincterotomy: acute pancreatitis in 12 cases (5.2%), haemorrhage in 5 cases (2.2%), cholangitis in 3 cases (1.3%), and perforation in 1 case (0.4%). Although they were not homogeneous populations, as the high-risk patients were preferentially treated with hydrostatic balloon dilation, we compared the incidence of different complications in the two groups and found no statistically significant differences. However, in view of the frequency of events and the size of the sample, we recognised that our series had an insufficient statistical power.

DISCUSSION

Currently, the standard treatment of choledocholithiasis is endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy. However, this technique is not exempt from risks such as haemorrhage, perforation and pancreatitis in an early phase and complications derived from the loss of function of the sphincter of Oddi in a late phase. The incidence of early complications after sphincterotomy is 6%-10%[2,6], with a mortality of 1% (Table 4). The incidence of complications in our series was similar to that found in the literature.

The morbidity due to endoscopic balloon dilation of the papilla varies between 17% and 21% in randomised, controlled studies and between 7% and 19% in the non-controlled studies. The morbidity is due principally to pancreatitis which, in some cases, can be severe (3% to 6.8%) and even lead to death[4,7]. A meta-analysis[8] of randomized, controlled studies comparing sphincterotomy whith hydrostatic dilation of the papilla was published recently, and included a total of 1106 patients, which showed that papillary dilation is a theoretically attractive option in young patients as it preserves sphincter function, although the high rate of reactive pancreatitis limits its use in selected cases. Paradoxically, the risk of pancreatitis is higher in young patients who would theoretically benefit most from the preservation of sphincter function.

The results of the meta-analysis showed no haemorrhage after papillary dilation, thus favouring this procedure in patients with a coagulopathy and in those who require re-initiation of anticoagulation within a period of 72 h. Platelet or whole blood transfusions could prevent early haemorrhage but do not protect against late bleeding, which is responsible for 50% of the cases of haemorrhage[9,10].

Currently, hydrostatic balloon dilation is considered an alternative in patients with coagulation disorders and in cases of atypical anatomy, such as an intradiverticular papilla, a Billroth Ⅱ gastrectomy or Roux-en-Y anastomosis and in stenosis of the papilla due to scarring of a previous sphincterotomy.

Cirrhotic patients represent another higher-risk population which could benefit from dilatation. Sphincterotomy in these patients has a mortality of 6.4%-25% in different series, whilst dilatation did not cause any complications in one series of 9 cirrhotic patients, of whom 6 presented a coagulopathy[11-13]. Three patients in our series presented a coagulopathy: one patient on acenocumarol anticoagulation who developed melaena 5 d after the procedure, one with hepatic cirrhosis and the other with cholangitis, septic shock and a consumption

Table 3 Analysis of the patients with post-dilatation pancreatitis and possible risk factors

Case	Diameter largest calculus	Total number of calculi	Diameter balloon (mm)	Indication	Hospital stay (d)
1	20 mm	19	10	Billroth Ⅱ	4
2	Microcalculi	Several	10	Intradiverticular	3
3	14.5 mm	3	10	Intradiverticular	3
4	Microcalculi	Several	10	Stenosis	3
5	10.5 mm	Several	10	Intradiverticular	2
6	0	0	10	Intradiverticular	3

Table 4 Complications of sphincterotomy in the literature[2,6]

Complication	Percentage (%)	Surgery (%)	Death
Pancreatitis	1-7	6.3	10-10.9 (0.2% of the total)
Haemorrhage	2.5	22	13 (0.6% of the total)
Perforation	1.3	27	16 (0.2% of the total)
Sepsis	1.7		

coagulopathy, who presented no complications.

Stenosis of the papilla after sphincterotomy is not a rare event, with a prevalence ranging 3.4%-13%[14,15]. In these cases, sphincterotomy appeared to be associated with a higher rate of perforation and pancreatitis[6], and papillary balloon dilation was thus proposed as an alternative. Stenosis of the papilla due to scarring from a previous sphincterotomy was the indication for dilatation in 21 of the cases in our series. Complications arose in two of these cases (9.5%): one presenting mild pancreatitis and the other a late haemorrhage (the patient mentioned above was on treatment with acenocumarol).

The majority of authors consider that sphincterotomy is associated with a higher morbidity and mortality in patients with a Billroth Ⅱ gastrectomy. However, controversy exists on this matter[5,16]. In a randomised study, hydrostatic dilation was not found to be associated with a higher morbidity in patients with a Billroth Ⅱ gastrectomy than in patients with a normal anatomy of the papilla and was also not associated with an increase in the need for mechanical lithotripsy. In our series, dilatation had no complications in 3 patients with a Billroth Ⅱ gastrectomy.

Balloon dilation is highly effective, with extraction of the calculi and complete clearance of the biliary tract achieved in 80%-100% of cases, comparable to the success rate after sphincterotomy (96%). Some authors have proposed a rescue sphincterotomy when it is not possible to extract all the calculi after dilatation[17].

On the other hand, some authors[18] performed dilatation of the papilla with large diameter balloons (12-20 mm in diameter) in patients with retained calculi after sphincterotomy, whether due to their large size or to narrowing of the distal common bile duct, but they could not extract the calculi (11% of cases). However, the efficacy of mechanical lithotripsy was 80%-98%.

The most frequent complication of papillary dilation is pancreatitis, with an incidence ranging 4%-35%, depending on the series. During dilatation, trauma is applied circumferentially to the sphincter and, therefore, partially in the direction of the pancreatic duct, causing transmural inflammation and intramucosal haemorrhage of the sphincter. The traction exerted on the calculi in the attempts to extract them[17] also gives rise to additional trauma to the sphincter.

Hydrostatic dilatation of the papilla with an 8 mm balloon enables extraction of calculi in almost all cases with a diameter of less than 10 mm[19]. However, mechanical lithotripsy is required in 50% of cases when there are more than 3 calculi or when the calculi have a diameter greater than 10 mm. Mechanical lithotripsy makes the procedure more laborious, and its manipulation can increase the incidence of pancreatitis[20]. Furthermore, additional sphincterotomy or repetition of ERCP is required in 15% to 30% of the patients[9]. For this reason, some authors[8] do not favour balloon dilation in patients with calculi of these characteristics.

Vlavianos[21] used a 10 mm balloon and lithotripsy was only required in 6.8% of cases, despite the total clearance of calculi with a diameter greater than 10 mm was achieved in the biliary tract of 70.7% of cases.

In the present study, although 39.6% of the patients presented calculi with a diameter greater than 10 mm, and more than 3 calculi were extracted in 26.4% of cases, mechanical lithotripsy was not required in any case and rescue sphincterotomy was only required in one case in order to extract the calculus (Table 2). The incidence of pancreatitis was not related either to the number of calculi or to their size. We do not know whether these results are due to the use of the 10 mm hydrostatic balloon, as the previously mentioned data come from studies in which an 8 mm or smaller diameter balloon was used[22].

A recent study in Japan[23] showed that the effect of balloon dilation is associated with the temporary insertion of a stent into the pancreatic duct. A tendency to reduce the rate of pancreatitis was observed, though this did not reach statistical significance due to the low incidence of pancreatitis in the control group (6%).

Another advantage of dilatation derives from its capacity of preserving sphincter function. The loss of function of the sphincter of Oddi after sphincterotomy is permanent and exposes the biliary tree to reflux of the duodenal contents, producing bacterial colonisation and chronic inflammation of the biliary tree. This may be a cause for concern in younger patients as it can increase the incidence of primary choledocholithiasis caused by deconjugation of the bilirubin by bacterial enzymes, and the number of tumours of biliary origin. Acamada[24] detected primary cancer of the biliary tree detected in 7.4% of patients undergoing transduodenal surgical destruction of the muscle fibres of the sphincter of Oddi after a mean follow-up of 18 (10-22) years. In the majority of these

cases, bacterial contamination of the biliary tract was also observed. However, studies on series of sphincterotomy with long-term follow-up have not detected serious complications despite the permanent abolition of the function of the sphincter of Oddi[25]. The incidence of late complications after sphincterotomy was 24% in one study with a mean follow-up of 15 years[26].

Dilatation of the papilla preserves the function of the biliary sphincter in the majority of cases, thus preventing chronic reflux of the duodenal contents into the biliary system. Studies have shown that the pressure in the common bile duct, the basal pressure of the sphincter of Oddi, the peak pressure and the contractile frequency are significantly reduced one week after dilatation of the papilla[19,27,28]. Two studies of the histology of the papilla after dilatation, one performed in pigs[29] and the other in humans[30], demonstrated no disruption of the smooth muscle or distortion of the architecture, although inflammation and mild or moderate fibrosis was observed in the majority of cases.

In conclusion, our results indicate that balloon dilation of the papilla is highly effective, the complication rate is comparable to that of sphincterotomy, and the clinical importance of the complications is low. The use of a 10 mm balloon enables extraction of multiple calculi with a size greater than 15 mm with no increase in the complication rate and reduces the need for lithotripsy or sphincterotomy.

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COMMENTS

Background

Endoscopic sphincterotomy was introduced in 1973 as a nonsurgical procedure enabling removal of bile duct stones and has become the treatment of choice for their extraction. This is a most dangerous technique performed by endoscopists with an early complication rate of 6%-10% and a mortality of 1%. Sphincterotomy also causes a permanent loss of function of the sphincter, thus exposing the biliary tree to bacterial colonisation and chronic inflammation. In 1983, Staritz proposed the endoscopic balloon dilation as a safe and effective alternative to sphincterotomy with the hope of avoiding these short and long-term complications by preserving the function of the sphincter. However, the high frequency of complications can lead to papillary dilation, thus falling into disuse as the treatment of choice in choledocholithiasis. A meta-analysis of randomized, controlled studies comparing sphincterotomy whith balloon dilation of the papilla concluded that in young patients, the dilation is a theoretically attractive option as it preserves sphincter function, although the high rate of reactive pancreatitis wouldt limit its use in selected cases. Currently, it is considered an alternative to sphincterotomy in certain cases at a high potential risk, principally of haemorrhage or perforation.

Research frontiers

Nowadays, the safety and efficacy of larger endoscopic balloons for great size lithiasis extraction are under investigation. Some authors have adapted the balloon diameter to the distal choledocal diameter or to the lithiasis size. A very interesting investigation field is the study of drugs and procedures with the capacity of reducing the incidence of acute pancreatitis after hydrostatic dilatation of the papilla. Another interesting field of research is the study of the incidence of late complications and stone recurrence after endoscopic balloon dilation.

Innovations and breakthroughs

The use of a 10 mm balloon enables extraction of multiple calculi with a size greater than 15 mm with no increase in the complication rate and reduces the need for lithotripsy or sphincterotomy.

Applications

Papillary dilation is the treatment of choice in patients with coagulopathy and a local anatomy that makes dangerous a sphincterotomy (Billroth Ⅱ gastrectomy, periampullary diverticula) independently of the size and the number of calculi.

Terminology

Patients with papilla at high risk: patients in whom the local anatomy makes a sphincterotomy impossible or dangerous (e.g., patients with periampullary diverticula or undergoing Billroth Ⅱ gastrectomy). Occlusion cholangiography: occlusion cholangiography is done after the balloon catheter is inflated and withdrawn from the papilla (to avoid contrast leak). Endolap: single-stage treatment with laparoscopic cholecystectomy and intraoperative ERCP.

Peer review

In the current paper, the authors report their experience with endoscopic balloon dilation of the sphincter of Oddi for the treatment of bile duct stones versus endoscopic sphincterotomy. Their results indicate that balloon dilation of the papilla is highly effective, and the complication rate is comparable to that of sphincterotomy. The paper is of interest.

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RAPID COMMUNICATION

Diagnosis of pancreatic tumors by endoscopic ultrasound-guided fine-needle aspiration

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Abstract

AIM: To evaluate the diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) for pancreatic solid tumors larger or smaller than 3 cm, and cystic lesions.

METHODS: From January/1997 to December/2006, 611 patients with pancreatic tumors were subjected to EUS-FNA. The final diagnosis was obtained either by surgery (356 cases) or after a mean clinical follow-up of 11.8 mo in the remaining patients.

RESULTS: There were 405 solid tumors, 189 cystic lesions and 17 mixed. Pancreatic specimens for cytological assessment were successfully obtained by EUS-FNA in 595 (97.4%) cases. There were 352 (57.6%) malignancies and 259 (42.4%) benign tumors. Among the malignancies, pancreatic adenocarcinomas accounted for 67% of the lesions. Overall, the sensitivity, specificity, positive and negative predictive values, and accuracy of EUS-FNA were, respectively, 78.4%, 99.2%, 99.3%, 77.2% and 87.2%. Specifically for solid tumors, the same parameters for neoplasms larger and smaller than 3 cm were, respectively, 78.8% *vs* 82.4%, 100% *vs* 98.4%, 100% *vs* 99%, 54.8% *vs* 74.1% and 83.1% *vs* 87.8%. For cystic lesions, the values were, respectively, 72.2%, 99.3%, 97.5%, 91% and 92.2%.

CONCLUSION: EUS-FNA can be used to sample pancreatic tumors in most patients. Even though the negative predictive value is inadequate for large solid tumors, the results are rather good for small solid tumors, especially concerning the sensitivity, negative predictive value and diagnostic accuracy. Among all pancreatic lesions, EUS-FNA for cystic lesions can reveal the best negative predictive value and diagnostic accuracy, both higher than 90%.

Key words: Diagnosis; Endoscopic ultrasound; Fine needle-aspiration biopsy; Pancreas cancer; Pancreatic disease; Sampling

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INTRODUCTION

Endoscopic ultrasound (EUS) is the best diagnostic tool for locoregional staging of pancreatic tumors[1-3]. However, similar to other imaging methods, EUS cannot differentiate easily a pancreatic malignancy from an inflammatory process[3-5]. Cytological brushes from the main pancreatic duct performed during endoscopic retrograde pancreatography, as well as surgical biopsies are additional procedures to improve the diagnostic yield, although both methods have neither a good sensitivity nor a better diagnostic accuracy for pancreatic neoplasms[6-9]. To overcome these drawbacks, EUS-guided fine needle aspiration (EUS-FNA) of pancreatic tumors is a very good choice, with its safety, feasibility and high diagnostic accuracy confirmed by many studies[10,11].

We conducted this study to evaluate the diagnostic accuracy of EUS-FNA for pancreatic solid tumors larger or smaller than 3 cm, and cystic lesions.

MATERIALS AND METHODS

From January/1997 to December/2006, 1043 patients with pancreatic tumors were subjected to EUS-FNA in a single referral center. Four hundred thirty-two cases were lost to follow-up. Six hundred eleven (58.6%) patients were available for retrospective review. The final diagnosis was based on surgical findings (n = 356) or by a mean clinical follow-up of 11.8 (range: 2 to 32) mo (n = 255). Procedures were carried out by the same endosonographer (JCA) using either Pentax linear echoendoscopes FG

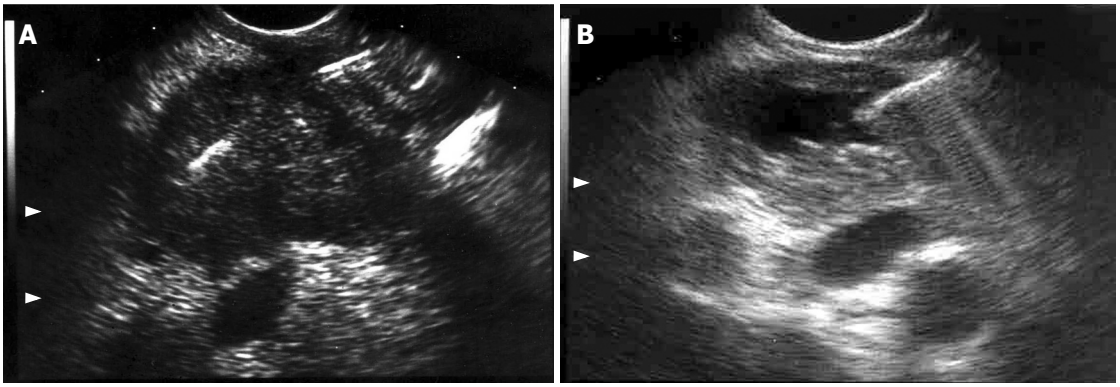

Figure 1 EUS images during FNA of (**A**) solid pancreatic tumor (colon carcinoma metastasis), and (**B**) cystic lesion (cystadenocarcinoma), both confirmed by the cytopathological assessment. Note the needle inside both lesions.

32-UA, FG 36-UX and FG-38UX (Pentax Precision Instruments, Inc., Orangeburg, NY) with a HITACHI 405 or 515 EUB ultrasound platform, or Olympus UCT-160 (Olympus Optical Corp. Ltd., Tokyo, Japan) with a UC-60 ultrasound platform (Suzy-Olympus Optical Corp. Ltd., Tokyo, Japan). EUS-FNA was performed by using a 22-gauge, 8-cm shot gun aspiration needle (NA-11J-KB, Olympus Optical Co., Tokyo, Japan), under conscious sedation with propofol and cardiorespiratory monitoring. Patients were left on their left side position after overnight fast before the procedure. Antibiotic prophylaxis was given during the procedure for puncturing all cystic lesions. Passage was transduodenal for lesions in the head and uncinate process of the pancreas and transgastric through the lesser sac for lesions in the body and tail (Figure 1).

Cytopathological assessment

Aspirated samples were evaluated either by means of cytological smears or cell blocks. All cytological samples were interpreted by one of the two experienced cytopathologists (FV and GCS). The number of passes of the needle until satisfactory specimens were obtained was documented in each case. Briefly, once aspirated, the material was expelled onto slides, and two smears were made, followed by fixation in buffered formalin, and staining with Papanicolaou and Wright-Giemsa stains. Specimens for cell blocking were fixed in buffered formalin, submitted to centrifugation, and immersed in liquid agarose. Once solidified, the agar cone with the cells in the top was embedded in paraffin to be handled as a routine tissue block. Thin 3-mm sections from paraffin-embedded cell blocks were cut, mounted on glass slides, and stained with haematoxylin and eosin. Immunocytochemical stains were carried out by the avidin-biotin peroxidase method. On review of the slides, cellularity, presence of loosely cohesive aggregates or single tumor cells, quality and quantity of cytoplasm, nuclear pleomorphism, chromatin patterns, nucleus to cytoplasm ratio and necrosis were systematically analysed in each case.

Statistical analysis

The significance level was 5% for all statistical procedures. Numerical variables were expressed as mean ± SD and comparative analysis between them was performed by Student's *t*-test. All categorical data were analysed by chi-square test with Yates correction and Fischer's exact test. Concerning the diagnosis obtained by EUS-FNA, sensitivity, specificity, positive and negative predictive values, and accuracy were calculated with a 2 × 2 table. For statistical procedures, lesions in the head, uncinate process and pancreatic neck were grouped together; and the same was done for masses invading both body and tail, which were grouped as body lesions.

Table 1 Diagnostic evaluation before EUS

Imaging modalities	*n*	%
Only CT	289	47.3
CT + MRI	190	31.1
CT + ERCP	120	19.6
CT + MRI + ERCP	12	2.0

CT: Computed tomography; MRI: Magnetic resonance imaging; ERCP: Endoscopic retrograde colangiopancreatography.

RESULTS

Three hundred fourteen (51.4%) patients were females, and their mean age was 57.8 (range: 11-89) years.

The main reasons to perform EUS-FNA in pancreatic tumors were suspicion of solid malignant neoplasia (44.2%), cystic collections (28.3%) and to obtain the differential diagnosis between pancreatic cancer and focal chronic pancreatitis (11.6%). Other indications are delineated on Figure 2. In addition to the endosonographic evaluation, as well as computed tomography, some patients were previously submitted to other diagnostic approaches (Table 1).

Four hundred ten (67.1%) tumors were located in the pancreatic head, 161 (26.3%) in the body, 40 (6.6%) in the tail (Table 2). Passage was transduodenal for 410 (67%) tumors, and transgastric for 201 (33%) cases. The general mean size of the tumors was 3.4 cm (range: 0.4-14.4), and lesions less than 3 cm accounted for 43% of the cases. The average size of solid tumors larger and smaller than 3 cm, was, respectively, 4.3 cm (3-10.3 cm) and 1.8 cm (0.4-2.9 cm). The mean size of cystic lesions was 3.7 cm (0.4-14.4).

EUS diagnosed 405 (66.3%) solid tumors, 189 (30.9%) cystic collections and 17 (2.8%) mixed pattern lesions. Malignant or pre-malignant disease was detected in 352 (57.6%) cases. Adenocarcinoma was diagnosed in 236 (38.6%) cases. The remaining diagnoses are depicted in Table 3. Aspiration samples were successfully collected in

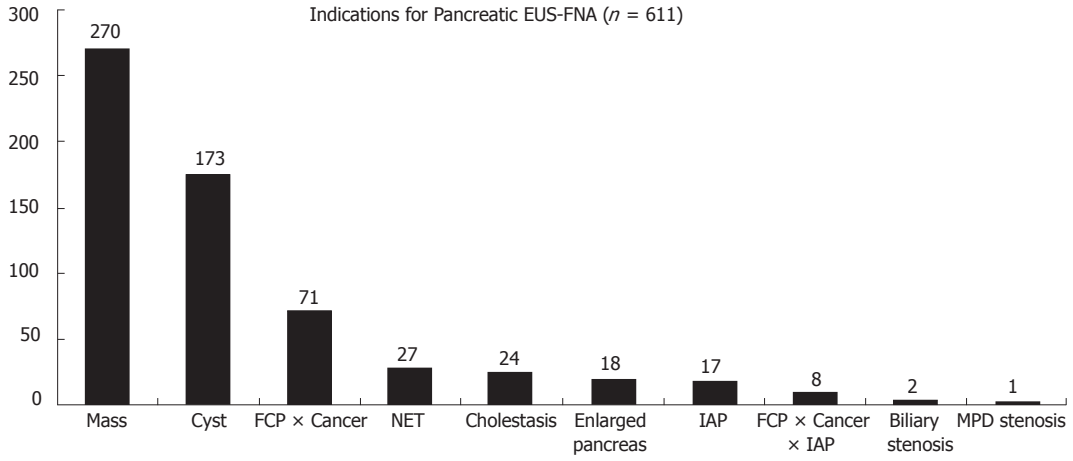

Figure 2 Indications for pancreatic EUS-FNA. FCP: focal chronic pancreatitis; NET: neuroendocrine tumor; IAP: idiopathic acute pancreatitis; MPD: main pancreatic duct.

Table 2 Location of the pancreatic tumors in patients submitted to EUS-FNA

	Head[1]	Body[2]	Tail	Total
Solid tumors ⩾ 3 cm	169	42	14	225
Solid tumors < 3 cm	119	52	9	180
Cystic lesions	122	67	17	206
Total	410	161	40	611

[1]Lesions in the head, uncinate process and pancreatic neck were grouped together. [2]Tumors invading body and tail were grouped as body lesions.

Table 3 Diagnoses of pancreatic lesions obtained by surgery and clinical follow-up (*n* = 611)

Tumour	Type	*n*
Solid (405)	Adenocarcinoma	233
	Focal Chronic Pancreatitis	87
	Neuroendocrine tumor	46
	Metastasis	13
	Lymphnode	9
	Splenosis	4
	Lymphoma	4
	Autoimmune pancreatitis	4
	Adenoma	2
	Sarcoma	2
	Blastomycosis	1
Cystic (189)	Pseudocyst	84
	Serous cystadenoma	42
	Mucinous cystadenoma	18
	IPMT	18
	Abscess	12
	PanIN	8
	Chronic Pancreatitis	4
	Tuberculosis	2
	Neuroendocrine tumor	1
Mixed (17)	Cystadenocarcinoma	8
	Adenocarcinoma	3
	Frantz tumor	3
	IPMT	1
	Metastasis	1
	Neuroendocrine tumor	1

IPMT: Intraductal papillary mucinous tumor; PanIN: Pancreatic intraepithelial neoplasia.

595 (97.4%) patients after an average of 2.2 (range:1-4) passes. The puncture of the tumor was not possible in 2 cases, and in 14 cases the amount of the aspirated specimens was not adequate for cytological assessment, even after multiple passes of the needle. All these cases were diagnosed after surgical resection.

In total, there was agreement between the cytological diagnoses of malignancy with those from surgery or clinical follow-up in 269 of 352 (76.4%) patients. On the other hand, the cytopathology correctly classified 257 of 259 (99.2%) non-neoplastic cases as a benign condition.

In an intention-to-treat analysis, EUS-FNA confirmed the final diagnosis in 526 of 611 (86%) cases. Overall, the sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy of EUS-FNA were, respectively, 78.4%, 99.2%, 99.3%, 77.2% and 87.2% (Table 4).

In regard to the value of the method for solid lesions smaller or larger than 3 cm, and cystic lesions, our results were rather good for small solid tumors, especially concerning the sensitivity, negative predictive value and diagnostic accuracy. For large solid tumors, although the positive predictive value was 100%, the negative predictive value was somewhat disappointing, reaching a rate lower than 55%. Furthermore, among all pancreatic lesions, EUS-FNA for cystic lesions revealed the best negative predictive value and diagnostic accuracy of this series, both higher than 90% (Table 4).

Five patients developed FNA-related minor complications (fever in 2 after puncturing of cystic lesions, acute pancreatitis in 2 and haemorrhage in 1) managed clinically for 48 h. One case developed a severe abdominal pain due to bile peritoneum after puncturing a serous cystadenoma. Only conservative management in an intensive care unit was offered, with no surgery, and the patient was discharged after 20 d.

DISCUSSION

Histological diagnosis of pancreatic tumors can influence the choice of the best therapeutic approach[10,12]. CT- or US-guided percutaneous puncture of the pancreatic tumors is usually more difficult to undertake due to the retroperitoneal situation of the pancreas[13-15]. Specimens from pancreatic cancer can also be collected by means of laparoscopy[2,6], brushing or forceps and needle aspiration biopsies during ERCP[7], as well as directly from the pancreatic juice. The sensitivity of the biopsy from the

Table 4 Sensitivity, specificity, positive and negative predictive values and accuracy of EUS-FNA in the diagnosis of pancreatic tumors

	General (n = 611)		Solid tumors ⩾ 3 cm (n = 225)		Solid tumors < 3 cm (n = 180)		Cystic lesions (n = 206)	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Sensitivity	78.4	74.1-82.8	78.8	72.8-84.8	82.4	75.5-89.2	72.2	60.3-84.2
Specificity	99.2	98.1-100	100	100-100	98.4	95.2-100	99.3	98.1-100
PPV	99.3	98.2-100	100	100-100	99.0	97-100	97.5	92.7-100
NPV	77.2	72.6-81.7	54.8	44.1-65.4	74.1	64.5-83.6	91.0	86.6-95.3
Accuracy	87.2	84.5-89.9	83.1	78.2-88	87.8	83-92.6	92.2	88.6-95.9

pancreatic duct is similar to that obtained by needle aspiration biopsy and intraductal brushing during ERCP, ranging from 40% to 67%[7-9].

The low sensitivity of all these sampling methods, in addition to more complex and laborious techniques, has increased the interest in the use of EUS for pancreatic tumors. However, the images obtained by endoscopic ultrasonography are not able to differentiate precisely malignancy from inflammation[4]. Wiersema *et al*[5] reported a sensitivity of 76% to detect pancreatic cancer, and Palazzo *et al*[4] demonstrated a specificity of only 73% for diagnosing inflammatory processes. In fact, in an attempt to improve the diagnostic yield of pancreatic lesions by means of cytopathological assessment[10], EUS-FNA has been used more frequently, and constitutes a very useful method for locoregional staging of pancreatic cancer, making EUS possible to sample almost everything detected during the diagnostic procedure[1,16].

Our diagnostic accuracy by EUS-FNA was 86.6%, which is in accordance with the reported experience, ranging from 84% to 95%[1,10,11,17]. Sampling was successful in 98.4% of the cases, with a mean number of punctures for every pancreatic lesion lower than that reported by other authors without an on-site cytopathologist[10,18]. It was not possible to obtain cytological specimens in 16 cases, either due to extremely hard lesions, in which insertion of the needle into the mass was not possible for more than 1 cm (1 pancreatic carcinoma and 1 nodule of focal chronic pancreatitis), or due to scarce material obtained even after multiple passes of the needle, half of them for lesions further than 3 cm from the probe. Although a subject of much discussion, in this particular group of patients we believe on-site cytopathologist might enhance the diagnostic yield, as proposed by Chang *et al*[10]. In addition, another way to improve the sampling might be the use of larger needles. Nevertheless, 19-gauge needles could also increase the occurrence of complications, which should be evaluated in randomized clinical trials[19].

In an attempt to improve the diagnostic yield, new methods are available to guide EUS-FNA of the pancreas, such as elastography and contrast-enhanced ultrasound. Giovannini *et al*[20] evaluated the tissue elasticity of pancreatic malignancies during ultrasound examination, and reported that its sensitivity is 100% and specificity is 67%, respectively. In addition, echo-enhanced ultrasound is a newly available imaging modality for the evaluation of pancreatic lesions. Based on the characteristic vascularization patterns of different tumors, experience has demonstrated that the sensitivity and specificity of the method in diagnosing pancreatic masses are greater than 85% and 90%, respectively[21]. However, even with these recent advances, histology is still the standard of reference. At this point, a combined evaluation of cytological smears and cell blocks can guarantee the maximum utilization of the material aspirated from pancreatic tumors, thus providing more accurate and clinically useful findings[22,23].

In our referral center, the general sensitivity for detection of pancreatic neoplasms by CT or US is respectively, 89% and 76% (data not shown). The literature reports a sensitivity of 100% to detect pancreatic tumors bigger than 3 cm, higher than that obtained by CT or US, and similar to the findings from ERCP[2,3,10,24]. Nonetheless, for small tumors, EUS-FNA presents a better sensitivity in relation to CT or ERCP[4,10,25], which might be a great advantage, as tumors less than 3 cm accounted for 43% of our patients. Regarding the value of EUS-FNA for solid tumors, our results were rather good for tumors smaller than 3 cm, as reported in the literature[26-28]. For larger tumors, even with an excellent positive predictive value, the negative predictive value was lower than 55%. Furthermore, the negative predictive value and diagnostic accuracy of EUS-FNA for cystic lesions were higher than 90%, which are consistent with other studies[29,30].

EUS-FNA has the following advantages over CT- or US-guided FNA: the shorter distance between the gut wall and tumor, the real time visualization of the needle, and the Doppler scanning to avoid punctures of the adjacent blood vessels[10,11]. Potential disadvantages could be the sedation and seeding of the needle tract by malignant cells, which have been reported with the percutaneous approach as well[31,32]. However, except for body-tail lesions[10,11], tumors located in the pancreatic head should be punctured through the duodenum, which is resected with the probable site of malignant seeding in case of surgery. Besides, the sensitivity of percutaneous CT- or US-guided FNA for pancreatic tumors ranges from 45% to 100%, with a specificity being close to 100%[13,14]. However, a pitfall of this technique is the difficulty for identifying and positioning the lesion properly to be punctured[13-15]. In our experience, EUS could identify all lesions previously detected by other imaging methods and insertion of the needle was possible in 99.6% of the cases.

FNA-related complications occurred in 1.1% of the patients, with the minor complications managed clinically. Most likely our lower number of passes of the needle could explain our low rate of complications. In fact, two factors have led to the occurrence of complications: the puncture of cystic lesions and the passage of the needle through large areas of normal parenchyma to reach tumors less than 2 cm. On the other hand, only

one case developed a life-threatening complication, a bile peritoneum which could be successfully treated with intensive care support. None of these cases required surgical intervention. Our experience is in line with the literature about this issue, in which the complication rate ranges from 0.5% to 5%[17,33]. Besides, CT-guided FNA presents a complication rate close to 1%[14,15], similar to that obtained in our series.

EUS-FNA proved to be a safe and successful procedure for sampling pancreatic lesions in most patients. The cytopathological assessment confirmed the final diagnosis in almost 90% of the cases. As a result of an impressive specificity and positive predictive value, a malignant cytopathology guarantees the presence of cancer. The sensitivity, negative predictive value and diagnostic accuracy are pretty good for small solid tumors. EUS-FNA for cystic lesions can reveal the best negative predictive value and diagnostic accuracy.

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RAPID COMMUNICATION

Specific serum immunoglobulin G to *H pylori* and CagA in healthy children and adults (south-east of Iran)

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Abstract

AIM: To evaluate the serologic IgG response to *H pylori* and CagA across age groups and in healthy children and adults.

METHODS: Totally, 386 children aged 1-15 years and 200 adults aged 20-60 years, were enrolled to study. The serum samples of participant were tested for presence of anti-*H pylori* and anti-CagA IgG by using ELISA method.

RESULTS: The seroprevalence of *H pylori* in adults was significantly higher than that observed in children (67.5% *vs* 46.6%; $P < 0.000003$). In children, the seropositivity rate in males (51.9%) was significantly ($P < 0.05$) higher than that observed in females (41.7%). The prevalence of serum anti-CagA antibody was 72.8% and 67.4% in infected children and adults, respectively. The mean titer of serum anti-CagA antibodies was significantly higher among children in comparison to adults (64.1 Uarb/mL *vs* 30.7; $P < 0.03$). In infected children and adults the prevalence of serum anti-CagA antibody was higher in males compared to females (78.4% *vs* 66.3%; $P = 0.07$ and 75.6% *vs* 54.71%; $P < 0.04$, respectively). The age-specific prevalence of anti-*H pylori* and anti-CagA antibody (in infected subjects) was 37.6% and 59.57% at age 1-5 years, 46.9% and 75% at age 6-10 years, 54.9% and 79.45% at age 11-15, 59.01% and 83.33% at age 20-30 years, 66.6% and 60.52% at age 31-40 years, 73.46% and 63.88% at age 41-50 years and 75.75% and 60% at age 51-60 years with mean titer of anti-CagA antibody of 75.94, 63.32, 57.11, 52.06, 23.62, 21.52 and 21.80 Uarb/mL, respectively. There was significant difference between mean serum anti-CagA antibody in age subgroups ($P < 0.001$).

CONCLUSION: These results showed that anti-*H pylori* and anti-CagA antibodies were common in the children and adults. The *H pylori*-specific antibodies influenced by age and sex of subjects. Moreover, it seems that males are more susceptible to infection with $CagA^+$ strains compared to females. The seroprevalence of anti-CagA antibody was increased with age, up to 30 years and then decreased. It was also found that the magnitude of the IgG response to CagA decreased with advanced age.

Key words: Seroprevalence; *H pylori*; Adults; Children; CagA; Iran

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INTRODUCTION

Epidemiologic studies have clearly demonstrated a major etiologic role of *H pylori* for several gastroduoedenal diseases, including gastric ulcer, duodenal ulcer, gastric MALT lymphoma, and distal gastric cancer[1]. The prevalence of *H pylori* infection varies worldwide, but higher colonization rates are seen in developing countries compared to developed countries[2]. The infection is usually acquired during childhood, although expression of disease does not occur in most cases until adulthood. There has been evidence for both transient and persistent colonization in children[3], while colonization of adults with *H pylori* almost always persists[4].

H pylori strains are genetically diverse. *H pylori* strains may be divided into at least two subgroups based on the expression (type Ⅰ) or nonexpression (type Ⅱ) of CagA and the vacuolating cytotoxin. The cytotoxin-associated gene A (CagA) has been identified as a possible marker of virulence of *H pylori*[5]. In our previous study an association was observed between infection with $CagA^+$ *H pylori* strain and peptic ulcer[6]. Moreover, we observed higher levels of serum inflammatory cytokine IL-18 in *H pylori*-infected subjects, especially in individuals infected with $CagA^+$ strains[7].

There is no study regarding the immune response to *H pylori* and CagA antigen across age groups in the same population. This study conducted for the first time to evaluate the serologic immunoglobulin G (IgG) response to *H pylori* and its virulence factor, CagA protein,

across age groups and in children and adults with same population and similar socioeconomic levels.

MATERIALS AND METHODS

Subjects

From August 2005 to December 2005, a cross-sectional seroprevalence study was carried out among healthy subjects in Rafsanjan (a city that located in Kerman province, in South-East of Iran). In total, 586 subjects were studied, including 386 children (187 males; 199 females aged 1-15 years with a mean of 9.5 ± 3.9 years) and 200 adults (114 males; 86 females aged 20-60 years with a mean of 48.1 ± 15.9 years). All subjects were basically health, with no acute or chronic illnesses. The criteria for enrolment included no history of peptic ulcer disease, no abdominal surgery, no history of therapy for *H pylori* infection, and no symptoms of upper gastrointestinal disease such as indigestion, nausea, vomiting and epigastric burning pain.

The adults were recruited among blood donors of Rafsanjan Blood Transfusion Center. They were randomly selected according to registration number. Children were recruited from randomly selected schools and health centers. School students were randomly selected for blood samplings by their registration number and similar procedures were performed in health centers. Informed consents were obtained from parents of all the children before blood samplings. Children were recruited if their parents agreed with the study and signed the informed consents. Moreover, this study was evaluated and approved by the Ethical Committee of Rafsanjan University of Medical Sciences.

Two to three mL of peripheral blood was collected from each participant at the time of interviewing. The blood samples were centrifuged and the sera were separated and frozen at -20℃ until analysis.

Determination of H pylori-specific antibodies in serum

The serum levels of anti-*H pylori* immunoglobulin G were measured by using the commercial enzyme-linked immunosorbent assay (Trinity Biotec, Ireland); previously the sensitivity of this method was estimated > 98% in Iranian subjects[8]. According to manufacturer guideline the results were obtained as Immune Status Ratio (ISR) and the values of ⩾ 1.1 were considered as positive. Serum anti-CagA IgG antibody levels were also assayed by ELISA method using commercial kits (Diagnostic Bioprobes, Italy). The serum concentration of anti-CagA antibodies were expressed in arbitrary units per milliliter (Uarb/mL) as no International Standard is available. According to the manufacturer's guidelines the value of 5 Uarb/mL used to discriminate the negative from positive samples. Moreover, in each group the serum concentrations of anti-CagA antibody expressed as mean ± SD.

Statistical analysis

Differences in variables were analyzed using Kruskal-Wallis, Mann-Whitney *U*-test, Chi-square and Fisher exact tests as appropriate and *P*-values of less than 0.05 were considered significant. All the available data were analyzed by a computer program (SPSS, Chicago, IL, USA).

Table 1 Seroprevalance of *H pylori*-specific antibodies in children and adults

Group	Sex	Anti-*H pylori* seropositivity	Anti-CagA seropositivity	Mean titer of anti-CagA (Uarb/mL)
Children	Male	97/187 (51.9%)	76/97 (78.4%)	69.24 ± 70.83
	Female	83/199 (41.7%)	55/83 (66.3%)	58.08 ± 63.58
	Total	180/386 (46.6%)	131/180 (72.8%)	64.1 ± 67.63
Adults	Male	82/114 (71.9%)	62/82 (75.6%)	31.6 ± 31.6
	Female	53/86 (61.6%)	29/53 (54.71%)	29.2 ± 34.1
	Total	135/200(67.5%)	91/135 (67.4%)	30.7 ± 32.5

Table 2 Seroprevalance of *H pylori*-specific antibodies in children and adults according to their age

Group	Age subgroup (yr)	Anti-*H pylori* seropositivity	Anti-CagA seropositivity	Mean titer of anti-CagA (Uarb/mL)
Children	1-5	47/125 (37.6%)	28/47 (59.57%)	75.94 ± 93.23
	6-10	60/128 (46.9%)	45/60 (75%)	63.32 ± 59.63
	11-15	73/133 (54.9%)	58/73 (79.45%)	57.11 ± 52.56
Adults	20-30	36/61 (59.01%)	30/36 (83.33%)	52.06 ± 40.43
	31-40	38/57 (66.6%)	23/38 (60.52%)	23.62 ± 23.02
	41-50	36/49 (73.46%)	23/36 (63.88%)	21.52 ± 24.43
	51-60	25/33 (75.75%)	15/25 (60%)	21.80 ± 27.26
Total	1-60	315/586 (53.75%)	222/315 (70.47%)	50.40 ± 53.22

RESULTS

Anti-H pylori IgG seropositivity

The overall seroprevalence of anti-*H pylori* IgG was 53.75%. The seroprevalence of *H pylori* in adults was significantly higher than that observed in children (67.5% *vs* 46.6%; $P < 0.000003$). In children, the seropositivity rate in males (51.9%) was significantly ($P < 0.05$) higher than that observed in females (41.7%). Similarly, in adults, the prevalence of anti-*H pylori* IgG was higher in males compare to females but the difference did not reach statistically, significant (Table 1).

In children, the age-specific seropositive rate of anti-*H pylori* IgG was 37.6% at age 1-5 years, 46.9% at age 6-10 years and 54.9% at age 11-15 years. Furthermore, in adults the age-specific seropositive rate of anti-*H pylori* IgG was also 59.01% at age 20-30 years, 66.6% at age 31-40 years, 73.46% at age 41-50 years and 75.75% at age 51-60 years (Table 2).

Anti-CagA seropositivity

The overall seroprevalence of anti-CagA IgG was 70.47% in asymptomatic subjects. The prevalence of serum anti-CagA antibody was 72.8% and 67.4% in infected children and adults with mean titer of 64.1 ± 67.63 Uarb/mL and 30.7 ± 32.5 Uarb/mL, respectively (Table 1). There was no significant difference between children and adults regarding the prevalence of serum anti-CagA antibodies, although this parameter was higher in children than that in adults. However, the mean titer of serum IgG anti-CagA antibodies was significantly higher among children in

comparison to adults ($P < 0.03$).

In infected children, the prevalence of serum anti-CagA IgG antibodies was markedly higher in males compared to females (78.4% *vs* 66.3%; $P = 0.07$). Moreover, in infected adults, statistical analyses showed that the prevalence of anti-CagA IgG antibodies was significantly higher in males in comparison to females (75.6% *vs* 54.71%; $P < 0.04$). No significant differences were observed between males and females regarding titer of serum anti-CagA antibodies.

In infected children, the age-specific seropositive rate of anti-CagA IgG was 59.57% at age 1-5 years, 75% at age 6-10 years and 79.45% at age 11-15 years, with mean titer of 75.94, 63.32 and 57.11 Uarb/mL. Furthermore, in infected adults the age-specific seropositive rate of anti-CagA IgG was also 83.33% at age 20-30 years, 60.52% at age 31-40 years, 63.88% at age 41-50 years and 60% at age 51-60 years, with mean titer of 52.06, 23.62, 21.52 and 21.80 Uarb/mL (Table 2). Accordingly, the seroprevalence of anti-CagA antibody increased with age, up to 30 years and then decreased. Collectively, the seroprevalence of anti-CagA antibody in those with age of < 30 years was significantly higher than that observed in those with age > 30 years (74.53% *vs* 61.61; $P < 0.02$). However, the mean titer of serum anti-CagA antibodies declined with age and statistical analysis showed that there was significant differences between mean serum anti-CagA antibodies in age subgroups ($P < 0.001$).

DISCUSSION

H pylori infection is thought to play an etiologic role in several gastroduodenal diseases. In epidemiological studies, serum assaying of anti-*H pylori* IgG or IgA antibodies could offer high sensitivity and specificity and could be used to determine prevalence of infection[9,10]. The results of the present study showed that the overall seroprevalence of *H pylori* infection was 46.6% in children at age 1-15 years and was 67.5% in adults at age 20-60 years. In different studies, the prevalence of *H pylori* is variable in children such that it has been reported to be 60% at age 4 years in Ethiopia[11], 7.5% at age 2-18 years in Czech[12], 44% at age 6 mo to 17 years in Turkey[13], 8% at age 1-3 years in USA[14], 50% at age 1-9 years and 80% at age 10-19 years in Libya[15], 56% at age 1-14 years in Brazil[16], 96% at age 1-14 years in Saudi Arbia[17] and 80% at age 1.5-5 years in Bangladesh[18]. Moreover, It has been reported that children in Gambia and Nigeria are almost all infected by *H pylori* at age of 5 years[19,20]. On the other hand, Heuberger *et al*[21] reported the prevalence of *H pylori* infection among adolescents between 15-16 years of age, living in Switzerland. They found one of the lowest prevalence of H pylori infection among adolescents in Europe (7.3%). In developing countries, more than 80% of adults are colonized with *H pylori* compared to 30% of the adults in developed countries[2]. This discrepancy may be attributed largely to differences including race and ethnic background and socioeconomic status such as family income, size of the family, type of housing, location of housing, water supply, health and education level, and keeping pets[22,23].

In the present study, there was an increasing in prevalence of *H pylori* infection with age, reached to a maximum of 75.75% at 51-60 years of age, suggesting a steady colonization rate through the different age groups. This overall infection rate curve shared common patterns with other reports, although considerable differences exist between developing and developed countries[23].

The results of the present study showed that prevalence of anti-CagA antibody was 72.8% and 67.4% among asymptomatic infected children and adults, respectively. The cagA has been identified as a possible marker of virulence of *H pylori*[24]. Since the cytotoxin-associated gene product (CagA, 120 to 140 kDa) encoded by cagA is immunodominant, therefore, serum IgG antibodies to the CagA antigen may be a reliable marker of carriage of a $CagA^+$ *H pylori* strain[25]. In other studies the seroprevalence of anti-CagA antibody in *H pylori*-infected asymptomatic subjects was evaluated, so that it has been reported to be 56.7% at age 1-14 years in Saudia Arbia[17], 82% at age 1.5-5 years in Bangladesh[18], 46.9% at age 1-15 years in Mexico[26], 88.5% and 81.3% at age 3-12 years in two counties of China[27], 83% at age 20-65 years in Turkey[28], 95.3% at age 20-60 years in Nigeria[29]. Accordingly, our observation confirms that CagA seroprevalence varies geographically.

Another interesting result observed here was that the seroprevalence of anti-cagA was correlated with age, increasing with age up to 30 years and then decreasing. Moreover, the prevalence of anti-CagA was higher in children compared to adults, although the difference was not significant. Thus, susceptibility to colonization by a CagA-positive strain seems to be linked to age. This differential prevalence may be related to differential expressions of gastric mucosa adherence molecules, which may be modified by age or may be dependent on differential expression of bacterial adhesin molecules. It has been reported that the fucosylated blood group antigens Lewis b (Leb) and H-1 (the precursor for Leb) involve in adherence of *H pylori* to human gastric epithelial cells *in situ*[30]. Ilver *et al*[31] showed that 66% of clinical isolates of *H pylori* bound the Leb antigen. These authors demonstrated that bacterial adhesin to the Leb antigen is coded by the babA2 gene and is associated with the presence of the cagA gene. An interesting and surprising finding, demonstrated by Çelik *et al*[32], is that children probably have few Leb receptors on surface mucous cells, which may explain the fact that susceptibility to colonization with a CagA-positive strain is linked to age.

An interesting finding was also that the mean titer of the IgG response to CagA were significantly ($P < 0.05$) higher in children compared to adults. Another result observed here was that the decrease of antibody response to CagA through the different groups of ages. Accordingly, there is a reverse association between levels of anti-CagA antibody with advanced age and it seems that the age of the subjects may also influence the antibody response to CagA. These observations are difficult to interpret and may attributed to differential CagA expression and/or changes in the host immune response with aging. Moreover, one possibility would be that at older ages (> 30 years) the bacterial colonization may gradually shift

from cagA-positive strains to cagA-negative strains and accordingly, in some adult subjects cagA-positive strains may disappear. Based on these observation it seems that colonization of some younger subjects (< 30 years) with CagA-positive strains is a transient phenomenon. The accumulating evidence regarding CagA-positive strains are more susceptible than CagA-negative strains to eradication treatment[33] is consistent with our observation.

Our result showed that the prevalence of anti-*H pylori* antibody was significantly higher in males compared to females. Although, similar results reported from other countries[34,35]. Our results were inconsistent with other finding recently, reported in Iranian children from Tehran city, so that higher prevalence of *H pylori* were found in females compared to males[8]. However, in some studies no significant statistical differences observed between both sexes[25]. Our results for the first time showed that the prevalence of anti-CagA was markedly higher in males compared to females. Accordingly, it seems that the male gender are more susceptible to infection and colonization by CagA-positive strains of *H pylori*. This differential susceptibility may be directly related to the long-term clinical outcome. It has been reported that the males are at a greater risk of *H pylori* clinical manifestations[36,37]. These observation may account for higher prevalence of duodenal ulcer and gastric cancer in males. More studies should be conducted to document that this differential susceptibility in males and females can cause the male preponderance to peptic ulcer disease and gastric cancer.

In conclusion the results of present study showed that anti-*H pylori* and anti-CagA antibodies were common in children and adults. In both groups the prevalence of *H pylori*-specific antibodies were higher in males compared to females. It seems that the males are more susceptible to infection with $CagA^+$ strains compared to females. It has been shown that the prevalence of *H pylori* infection was increased progressively with advanced age. However, the seroprevalence of anti-CagA antibody was increased with age, up to 30. It was also found that the magnitude of the IgG response to CagA decreased with advanced age.

COMMENTS

Background

H pylori infection play an etiologic role in several gastroduodenal diseases. Both bacterial and host factors may influence the clinical outcomes of the *H pylori* infection. The CagA is thought to be the major virulence factor is produced by only a subset of *H pylori* isolates, defined as *H pylori* type Ⅰ. This study conducted to evaluate the IgG response to *H pylori* and CagA protein, across age groups and in healthy children and adults.

Research frontiers

Because universal eradication therapy is not feasible and *H pylori* vaccine is not available at this point, it is of the utmost to acquire better understanding of the role of both bacterial and host factors in the infection by this pathogen to improve diagnostic and therapeutic modalities.

Innovations and breakthroughs

The prevalence of *H pylori* have been reported in a large numbers of epidemiologic studies from developing and developed countries. However, this study provided new insights regarding the humoral immune response to *H pylori* and CagA antigen across age groups and in healthy children and adults and with respect to their sex.

Applications

Because the distribution of *H pylori* strains are not similar in age groups and both gender, it is recommended that physicians consider *H pylori* strain characteristics (such as cagA status), and host factors (such as age and gender) in attempting to optimize care for their patients.

Terminology

The cytotoxin-associated gene A (CagA) has been identified as a virulence marker of *H pylori*. Cytotoxin-associated gene product (CagA) encoded by a chromosomal pathogenicity island, which also contains genes coding for a secretion system that is able to translocate CagA to the cytoplasm of the host cell which interferes with intracellular signaling leading to morphological as well as functional changes. It has been reported that testing of serum IgG to the CagA antigen may be a reliable marker of carriage of a $CagA^+$ *H pylori* strains.

Peer review

This paper described the seroprevalence of anti-*H pylori* IgG antibody and anti-CagA antibody in healthy children and adults at ages from 1 to 60 years old in a city of Iran. There are two new findings, one is a sex difference in the seroprevalence of anti-*H pylori* IgG and anti-CagA antibodies, and the other is a peak seroprevalence of anti-CagA antibody at age of 20-30 years old and titers of this antibody declined with age.

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RAPID COMMUNICATION

Clinical significance of telomerase activity in peritoneal lavage fluid from patients with gastric cancer and its relationship with cellular proliferation

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Abstract

AIM: To evaluate the efficacy of telomerase activity assay and peritoneal lavage cytology (PLC) examination in peritoneal lavage fluid for the prediction of peritoneal metastasis in gastric cancer patients, and to explore the relationship between telomerase activity and proliferating cell nuclear antigen expression.

METHODS: Telomeric repeated amplification protocol (TRAP)-enzyme-linked immunosorbent assay (ELISA) was performed to measure the telomerase activity in 60 patients with gastric cancer and 50 with peptic ulcer. PLC analysis of the 60 patients with gastric cancer was used for comparison. The proliferating cell nuclear antigen (PCNA) in gastric carcinoma was immunohistochemically examined.

RESULTS: The telomerase activity and PLC positive rate in peritoneal lavage fluid from patients with gastric cancer was 41.7% (25/60), and 25.0% (15/60), respectively. The positive rate of telomerase activity was significantly higher than that of PLC in the group of pT_4 (15/16 *vs* 9/16, $P < 0.05$), P_{1-3} (13/13 *vs* 9/13, $P < 0.05$) and diffuse type (22/42 *vs* 13/42, $P < 0.05$). The patients with positive telomerase activity, peritoneal metastasis, and serosal invasion had significantly higher levels of average PCNA proliferation index (PI), (55.00 ± 6.59 *vs* 27.43 ± 7.72, 57.26 ± 10.18 *vs* 29.15 ± 8.31, and 49.82 ± 6.74 *vs* 24.65 ± 7.33, respectively, $P < 0.05$).

CONCLUSION: The TRAP assay for telomerase activity is a useful adjunct for cytologic method in the diagnosis of peritoneal micrometastasis and well related to higher proliferating activity of gastric cancer. The results of this study also suggest a promising future therapeutic strategy for treating peritoneal dissemination based on telomerase inhibition.

Key words: Gastric cancer; Telomerase activity; Peritoneal lavage cytology; Peritoneal metastasis; Proliferating cell nuclear antigen

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INTRODUCTION

In spite of improvement in postoperative survival with advances in surgical techniques and multimodal adjuvant therapy, gastric cancer nevertheless, remains a top killer among cancers of the gastrointestinal tract[1]. The postoperative survival rate of patients with advanced gastric cancer is very low[2-4]. Peritoneal dissemination is the most frequent pattern of recurrence in patients with gastric carcinoma[5]. About 50%-60% of gastric cancer patients with serosal invasion after curative resection eventually developed peritoneal metastases[6,7]. The incidence of peritoneal recurrence after curative resection was 13.46% and the survival after peritoneal recurrence was 4.9 mo in average[8]. The 5-year survival rate of patients with positive peritoneal lavage cytology was only 2%[9]. However, the mechanism of peritoneal dissemination has not yet been clearly defined so far. Free cancer cells exfoliated from cancer lesions have been postulated as the main cause of metastasis[5,8-10].

Recent advances on the molecular mechanisms of peritoneal dissemination have elucidated some of the target molecules and the development of new multimodal therapies in an attempt to eliminate peritoneal exfoliated

free cancer cells has also improved survival[11]. Of course, it is very important to develop reliable methods for the accurate selection of patients for whom adjuvant intraperitoneal therapy will be required. PLC examination has been generally considered as the gold standard for predicting peritoneal recurrences and is seen as one of the most accurate prognostic tools[10]. In the absence of macroscopic peritoneal metastases, positive cytological results have been shown to correlate with the survival of patients with advanced gastric cancer[12]. However, the sensitivity of this assay is relatively low[13-15], ranging from 21%-35%[9]. Some patients with negative cytological findings have been diagnosed later with peritoneal metastasis[15]. Hence, further studies are required to eliminate the above limitations to improve the accuracy of this technique.

Telomerase is a ribonucleoprotein polymerase that adds TTAGGG repeats to telomeric ends[16]. Telomerase activity is closely linked to attainment of cellular immortality, while lack of such activity contributes to cellular senescence. Telomerase is inactive in most normal somatic tissues except for germ and some stem cells, but has been found to be reactivated in approximately 85% of human malignancies[17,18]. Telomerase activity can be detected in trace amount of tissues, such as that collected in fine-needle aspiration cytology or even in the body fluid of cancer patients[19]. Recently, telomerase expression also was found in peritoneal fluid of patients with gastric cancer[20-22]. The presence of telomerase was associated with advanced disease or peritoneal dissemination.

In the present study, we reported the telomerase activity of peritoneal lavage fluid and expression of PCNA in gastric tissues. We aimed to compare the efficacy of telomerase activity assay and conventional cytological examination of peritoneal lavage fluid for prediction of peritoneal metastasis in gastric cancer, and to explore the relationship between telomerase activity and proliferating cell nuclear antigen expression.

MATERIALS AND METHODS

Patients

Between January 2004 and January 2005, we observed 60 patients with gastric cancer who underwent surgical resection at West China Hospital, including 40 men and 20 women, aged from 30-78 years (mean of 58 years). Another 50 patients with peptic ulcer who underwent surgical resection at same hospital were also studied as control, including 27 men and 23 women, ranged in age from 26 to 60 years (mean of 43 years). No patient had received preoperative radiation therapy or chemotherapy. Whole body bone scan and sonography were performed for all of the patients to rule out distant metastases. Histological type was established on haematoxylin/eosin stained sections according to Lauren's classification. Well and moderately differentiated tubular adenocarcinomas were referred to as the intestinal type, while poorly differentiated, signet ring cells and mucinous adenocarcinomas were referred to as the diffuse type. The histological type included diffuse type in 42 and intestinal type in 18. According to the criteria of the International Union Against Cancer (UICC) TNM Classification 5th edition, the depth of invasion was classified as follows: T_1 in 3, T_2 in 17, T_3 in 24, and T_4 in 16. Macroscopically evident peritoneal metastasis (P) and microscopically evident serosal invasion were also confirmed by histopathology.

Peritoneal lavage and samples

Specimens of lesion tissue and peritoneal lavage fluid were collected at the time of operation from patients undergoing surgical resection. Immediately after the laparotomy, before the manipulation of the process, 50 mL of isotonic sodium chloride was instilled into the left subphrenic or Douglas cavities with 16F catheter and recovered after being gently stirred. If ascites was identified within the peritoneum, the fluids were sucked directly. The lavage fluids were collected in heparinized tubes and centrifuged at 3000 r/min for 10 min at 4℃ (centrifugate radius: 90 mm). The supernatant was discarded. The superficial "white" layer of the sediment was picked up with a Pasteur pipette and was smeared on three glass slides and fixed for 1 min in Delaunay's solution (1 L Delaunay's contains 500 mL of absolute alcohol, 500 mL of pure acetone, and 1 mL of 3 mol/L trichloracetic acid) for Papanicolaou staining. At the end of this examination, the rest of the material was suspended in phosphate buffered saline (PBS) and the centrifugation step was repeated. To avoid contamination with red blood cells that could potentially interfere with polymerase chain reaction (PCR), cells were washed 2-4 times with ice-cold hypotonic solution (50 mmol/L KCl) and then kept frozen at -80℃ until telomerase assay. Tissue specimens were obtained from the mucosal surface of the resected lesions. Lesion material (10-20 g) was dissected from cancers without interfering with resection margins or the macroscopic appearance of the lesion. Necrotic areas from the centre of the lesions were avoided. After surgical removal, all the samples were snap frozen immediately and stored at -80℃ until further use. Telomerase assay and cytological examination were performed independently in a blinded manner.

Immunohistochemical assay

The streptavidin peroxidase method was used to quantify PCNA protein expression. All reagents were purchased from Beijing Zhongshan Biotechnology Co., Ltd. Resected gastric cancer samples were fixed in 10% buffered formalin and embedded in paraffin, and 4-μm sections were cut, dewaxed and rehydrated by sequential immersion in xylene, graded ethanol and water. Mouse anti-PCNA monoclonal antibody (Santa Cruz, USA) and biotinylated goat anti-mouse IgG antibody (Santa Cruz, USA) were used as the first and second antibodies, respectively. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 15 min in methanol. Dewaxed tissue sections were heated in a steamer for 2 min to retrieve the antigens and cooled to room temperature. Tissue sections after the enzyme digestion were then treated with the primary antibodies against PCNA. After washing in PBS and exposure to 10% normal goat serum for 10 min to reduce

the nonspecific antibody binding, the slides were incubated at 37℃ with mouse monoclonal antibody against PCNA (Santa Cruz, USA) at 1:100 dilution, in humid chambers for 1 h. After further PBS washing, slides were incubated at 37℃ with biotinylated goat anti-mouse IgG antibody in humid chambers for 20 min. Primary antibodies were visualized with an Envision System (DAKO, Denmark). After further PBS washing, slides were incubated with substrate diaminobenzidine and hydrogen peroxide for 10 min. Finally, sections were counterstained with hematoxylin. Negative control experiments were carried out as above by normal rabbit serum for the primary antibodies. Sections from previously studied cases of gastric cancer known to express PCNA were used as positive controls.

The assessment of all the samples was conducted blindly by calculating the average ratio of positive cells in 10 vision fields (the plasma staining brown yellow) under a × 400 microscope. The sections were initially scanned at low power to determine the areas that were evenly labeled. The cases were evaluated independently by two pathologists, and discrepancies in estimation were reconciled by concurrent review using a multiheaded microscope. At least 10 high-power fields were chosen randomly, and 2000 cells were counted. Tumor sections were considered negative if staining was absent or present in 10% of the tumor cells. In each specimen, PI was expressed as a percentage of positive nuclei per total nuclei counted in the section.

Telomerase activity assay

Telomerase activity was measured using a telomerase polymerase chain reaction (PCR) ELISA kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. The frozen samples were suspended in 200 mL lysis reagent, pre-cooled on ice by retropipetting at least 3 times and incubated on ice for 30 min. The lysate was centrifuged at 16000 × *g* for 20 min at 4℃. The supernatant was removed carefully and transferred to a fresh tube for the TRAP assay. For each sample to be tested and the controls, 25 μL reaction mixture was transferred into a tube suitable for PCR amplification. The extended products were amplified by PCR using Taq polymerase, the P1-TS, P2 primers and nucleotides. After a 30 min incubation at 25℃ to allow the telomerase mediated extension of the TS primer and followed by 94℃ for 10 min to inactivate the telomerase, the reaction mixture was subjected to 30 PCR cycles at 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 90 s on a DNA thermocycler (GeneAmp PCR System 9700, ABI, USA). Using the ELISA method, the amplified products were immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction, and then detected by anti-digoxigenin antibody conjugated to peroxidase. After the addition of the peroxidase substrate (3, 3', 5, 5'-tetramethyl benzidine), the amount of TRAP products were determined by measurement of their absorbance at 450 nm (with a reference wave length of 690 nm). The maximum value of absorbance for the negative control should be 0.25 $A_{450\ nm}$-$A_{690\ nm}$ units. The absorbance readings obtained with the positive control (supplied with the kit) should be higher than 1.5 $A_{450\ nm}$-$A_{690\ nm}$ units after 20 min substrate reaction. Samples are regarded as telomerase-positive if the difference in absorbance (ΔA) is higher than 0.2 $A_{450\ nm}$-$A_{690\ nm}$ units.

Statistical analysis

Data were analyzed by *t* test and χ^2 test. $P < 0.05$ was considered statistically significant. The SPSS software version 12.0 was used for statistical analysis.

RESULTS

Expression of telomerase activity in gastric cancer tissue and peritoneal lavage fluid

In the tissue specimens, telomerase activity was positive in 31 (51.7%) of 60 patients with gastric cancer, and 2 (4%) of 50 patients with peptic ulcer ($P < 0.005$). In the peritoneal lavage fluid, telomerase activity was found positive in 25 (41.7%) of 60 patients with gastric cancer, and none in peptic ulcer subjects ($P < 0.005$). Although the telomerase activity positive rate in tissues tended to be higher than that in the peritoneal lavage fluid, there was no significant difference statistically ($P > 0.05$). The sensitivity, specificity, positive predictive value, and negative predictive value of telomerase activity in peritoneal lavage fluid from patients with gastric cancer were 84%, 91%, 91%, and 83%, respectively.

Comparison between telomerase activity assay and conventional cytological method

Conventional cytological examinations were performed routinely for gastric cancer samples tested by the telomerase activity assay. All 50 control specimens were cytologically negative. The positive rate of telomerase activity in peritoneal lavage fluid collected from patients with gastric cancer was 41.7% (25/60), which was well related to serosal invasion, Lauren's classification, depth of infiltration and peritoneal metastasis of cancer, and rose with the increased depth of infiltration and serosa-involved areas ($P < 0.05$). The positive rate of PLC was 25.0% (15/60), which was obviously high in the group with macroscopic peritoneal metastasis (the group of $P_{1\text{-}3}$), and also increased with the increased depth of infiltration and serosa-involved areas ($P < 0.05$). All PLC positive cases were telomerase test positive in their peritoneal lavage fluids. Of the 45 PLC negative cases, 4 cases with macroscopic peritoneal metastasis ($P_{1\text{-}3}$) and 6 cases with serosal invasion were telomerase test positive. Although the positive rate of telomerase activity in peritoneal lavage fluids from patients with gastric cancer was not significantly higher than that of PLC in general, it was significantly higher than that of PLC in the group of pT_4, $P_{1\text{-}3}$ and diffuse type (Table 1).

Relationship between telomerase activity and proliferating cell nuclear antigen expression

As shown in Figure 1, the PCNA protein was expressed intensely, mainly in the nucleus of gastric cancer cells. PCNA expression was positive in 51 (85%) of 60 patients

Table 1 Comparison of telomerase activity and cytological test in peritoneal lavage fluid from patients with gastric cancer

Clinicopathological features	*n*	Telomerase activity		PLC	
		Positive cases *n* (%)	*P*	Positive cases *n* (%)	*P*
Lauren's classification			0.01		0.104
Intestinal	18	3 (16.7)		2 (11.1)	
Diffuse	42	22 (52.4)[e]		13 (31.0)	
Invasion depth			0		0.001
PT_{1-2}	20	0 (0)		0 (0)	
PT_3	24	10 (41.7)		6 (25.0)	
PT_4	16	15 (93.8)[a,e]		9 (56.3)[a]	
Peritoneal metastasis			0.035		0
P_0	47	12 (25.5)		6 (12.8)	
P_{1-3}	13	13 (100.0)[e]		9 (69.2)	
Serosal invasion			0.014		0.092
Absent	23	5 (21.7)		3 (13.0)	
Present	37	20 (54.1)		12 (32.4)	
Serosa-involved areas (cm^2)			0		0
0	23	1 (4.3)		0 (0)	
< 10	7	1 (14.3)		0 (0)	
10-20	17	12 (70.6)		7 (41.2)	
> 20	13	11 (84.6)[c]		8 (61.5)[c]	

[a]$P < 0.05$ *vs* pT_{1-2} and pT_3; [c]$P < 0.05$ *vs* 0, < 10 and 10-20; [e]$P < 0.05$ *vs* PLC.

with gastric carcinoma. In 25 patients with positive telomerase activity, the PI was significantly higher than those with negative telomerase activity (55.00 ± 6.59 *vs* 27.43 ± 7.72, $P < 0.05$). The 13 patients with macroscopic peritoneal metastasis had significantly higher levels of PI than negative ones (57.26 ± 10.18 *vs* 29.15 ± 8.31, $P < 0.05$). Furthermore, the PI in 37 patients with serosal invasion was significantly higher than in those without serosal invasion (49.82 ± 6.74 *vs* 24.65 ± 7.33, $P < 0.05$).

DISCUSSION

To date, there has been no effective therapy for peritoneal carcinomatosis. Therefore, attention has been paid to detecting peritoneal free cancer cells in patients with advanced gastric carcinoma without overt peritoneal metastasis to prevent peritoneal metastasis[23]. Several studies have shown that cytological examination of peritoneal lavage fluid is still the gold standard for assessing the presence of free cancer cells in the peritoneal cavity[10,12]. However, cytological examination lacks sensitivity for detecting free cancer cells[9,15]. Telomerase activity in body fluids, as measured by the TRAP assay, is a sensitive potential tumor marker that might help increase the cancer detection rate and the cancer treatment success rate when combined with conventional cytopathological methods[24,25].

High expression of telomerase was demonstrated in gastric cancer. Hiyama *et al*[26] first reported telomerase activity in advanced gastric cancer tissue and its metastases. The survival time of patients with detectable telomerase activity in their tumors was significantly shorter than those without telomerase activity. In another study by Tahara *et al*[27], reactivation of telomerase was found to be involved at the early stage of gastric carcinogenesis and its expression correlated well with malignant progression of the cancer. In the present study, telomerase activity was detected in 51.7% (31/60) of gastric cancer tissue specimens and 41.7% (25/60) of these cases in the peritoneal lavage fluid. These results were comparable to the findings of other studies on gastric cancer[20,21].

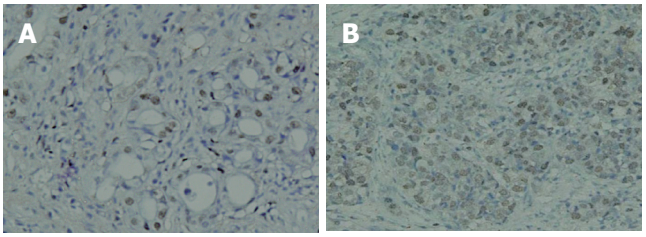

Figure 1 PCNA was expressed in cell nuclear of gastric cancer (SP, × 200). **A**: tubular adenocarcinoma; **B**: mucinous adenocarcinoma.

This study demonstrated significant correlations between positive rate of telomerase activity in peritoneal lavage fluid from patients with gastric cancer and invasion depth of cancer, serosal invasion, histological types, serosa-involved areas, presence and extent of peritoneal metastasis, which are important biological features of gastric cancer and reflect the invasiveness of tumor cells. In particular, our data demonstrated that 100% (13/13) of gastric cancers with peritoneal metastasis had detectable telomerase activity in peritoneal lavage fluid, although the sample size was relatively small. Thus, telomerase activity expression in peritoneal lavage fluid can be used as an indication of the invasive potential and poor prognosis of the gastric cancer.

With regard to clinically evident peritoneal metastasis, 100% (13/13) of these cases showed detectable telomerase activity, while only 69.2% (9/13) of these cases produced positive cytological results, the positive rate of telomerase activity was also significantly higher than that of PLC in the group of pT_4 and diffuse type, thus confirming that telomerase assay is more sensitive than PLC methods. The positive rate of tolemerase activity of the 37 specimens with serosal invasion was 54.1% (20/37), while PLC positive rate of those was 32.4% (12/37). As we know, serosal invasion appears to be mainly responsible for the exfoliation of free cancer cells into the peritoneal cavity[11]. It has been reported that approximately half of patients with serosal invasion develop peritoneal recurrence even if curative resection is performed[6,7,28]. In general, although using the presence of telomerase activity did not show results that surpass PLC methods, the test might offer the possibility of detecting cancer cells in cytology-negative specimens. Our data suggest that the telomerase test in peritoneal fluids can be used as an adjuvant to PLC test in the diagnosis of peritoneal micrometastasis, particularly in cases of negative cytology. In these cases, a review of peritoneal histocytology is advised. Statistically, the sensitivity and specificity of telomerase activity in peritoneal lavage fluid for gastric cancer was 84% and 91%, respectively. Our study also indicates that telomerase activity is a candidate molecular marker for the presence of free cancer cells in peritoneal cavity.

Telomerase activity was not detected in peritoneal lavage fluids in some cases of gastric cancer, which might be attributable to several reasons. One possible explanation may be that the concentration of free cancer cells in peritoneal lavage fluids was insufficient. We can not completely rule out the possibility of technical error or that perhaps some peritoneal lavage fluids did not contain cancer cells. However, the peritoneal lavage fluid can also be contaminated with lymphocytes or other blood cells in the presence of inflammation or ulceration[29]. The presence of these cells may give rise to false-positive results in TRAP assay[30]. Further studies are required to eliminate the above limitations to improve the accuracy of this technique.

The rate of PCNA expression in 60 gastric carcinoma specimens was 85% (51/60).The patients with positive telomerase activity, peritoneal metastasis, and serosal invasion showed significantly higher levels of PI. It was suggested that telomerase activity was well related to higher proliferating activity of gastric cancer, which was the very important reason of peritoneal carcinomatosis and serosal invasion.

In conclusion, to detection of telomerase activity in peritoneal lavage fluid and tumor cells by cytology could be useful to predict subclinical metastasis to the peritoneum in patients with gastric cancer. Telomerase activity assay could be a useful adjunct for cytologic method in the diagnosis of peritoneal micrometastasis and well related to higher proliferating activity of gastric cancer. Our results also suggest a promising future therapeutic strategy for peritoneal dissemination of gastric cancer based on telomerase inhibition.

COMMENTS

Background

Peritoneal dissemination is the most common pattern of metastasis in advanced gastric carcinoma. There has been no standard treatment for peritoneal carcinomatosis and the results are poor. The mechanism of peritoneal dissemination has not yet been clearly defined so far. Free cancer cells exfoliated from cancer lesions have been postulated as the main cause of metastasis.

Research frontiers

A close association has been demonstrated between positive results for peritoneal free cancer cells and low survival rate. Cytological examination has been the gold standard for detecting free cancer cells in the peritoneal lavage fluid. However, the sensitivity is relatively low, and the reliability of morphologic diagnosis is limited. Telomerase activity in body fluids is a sensitive potential tumor marker that might help increase the cancer detection rate. A PCR-based assay called telomeric repeat amplification protocol (TRAP), is quite sensitive and can detect as few as 10 telomerase positive cells.

Innovations and breakthroughs

The TRAP assay for telomerase activity in the peritoneal lavage fluid could be a useful potential marker in the diagnosis of peritoneal micrometastasis and well related to higher cellular proliferative activity of gastric cancer. It suggested a promising future therapeutic strategy for treating peritoneal dissemination based on telomerase inhibition.

Applications

Measurement of telomerase activity could be a useful adjunct of cytologic method for detecting free cancer cells in the peritoneal lavage fluid during operation.

Terminology

Telomerase is a critical enzyme responsible for cellular immortality. Telomerase activity is closely linked to attainment of cellular immortality, while lack of such activity contributes to cellular senescence. With the exception of germ and some stem cells, normal somatic cells do not have telomerase activity. Conversely, telomerase activity has been observed in 80%-90% of malignancies.

Peer review

The aim of this study was to evaluate the efficacy of telomerase activity assay and conventional cytological examination in peritoneal lavage fluid for the prediction of peritoneal metastasis in gastric cancer. The authors compared the positive rate of telomerase activity with that of cytology and found that telomerase activity has stronger correlations with clinicopathological factors than cytology. The authors concluded that telomerase activity could be a useful adjunct for cytologic method in the diagnosis of peritoneal micrometastasis and malignant progression.

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RAPID COMMUNICATION

Role of interventional therapy in hepatic artery stenosis and non-anastomosis bile duct stricture after orthotopic liver transplantation

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Abstract

AIM: To analyze the clinical manifestations and the effectiveness of therapy in patients with orthotopic liver transplantation (OLT)-associated hepatic artery stenosis (HAS) and non-anastomosis bile duct stricture.

METHODS: Nine cases were diagnosed as HAS and non-anastomosis bile duct stricture. Percutaneous transluminal angioplasty (PTA) was performed in four HAS cases, and expectant treatment in other five HAS cases; percutaneous transhepatic bile drainage, balloon dilation, stent placement were performed in all nine cases.

RESULTS: Diffuse intra- and extra-bile duct stricture was observed in nine cases, which was associated with bile mud siltation and biliary infection. Obstruction of the bile duct was improved obviously or removed. Life span/follow-up period was 13-30 mo after PTA of four HAS cases, 6-23 mo without PTA of other five cases.

CONCLUSION: Progressive, non-anastomosis, and diffuse bile duct stricture are the characteristic manifestations of HAS and non-anastomosis bile duct stricture after OLT. These are often associated with bile mud siltation, biliary infection, and ultimate liver failure. Interventional therapy is significantly beneficial.

Key words: Liver transplantation; Bile duct; Postoperative complication; Stricture; Interventional therapy

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INTRODUCTION

The pathogenesis of bile duct obstruction following orthotopic liver transplantation (OLT) is multifactorial, and is associated with significant morbidity and mortality[1]. With the improvement of pipeline anastomosis methods and techniques, the rate of bile duct anastomosis stricture and bile duct leak caused by these techniques has declined, and non-anastomosis bile duct stricture has become the major bile duct complication after OLT. Hepatic artery stricture (HAS) is an important cause of non-anastomosis bile duct stricture following OLT. The use of interventional therapy for resolving blood vessel and bile duct complications associated with liver transplantation has been indicated. However, the application of such techniques for both the hepatic artery and bile duct within an individual patient is rare. Here, we retrospectively analyzed the treatment (including dual intervention) and outcome of nine patients diagnosed with both HAS and non-stoma bile duct stricture following OLT.

MATERIALS AND METHODS

Patients

From September 2003 to October 2006, 82 of 643 OLT patients had bile duct complications, 9 of which were diagnosed both HAS and non-stoma bile duct stricture (Table 1). Eight of these patients were male and 1 female. The average age was 45.1 (range, 32-65) years. The indications for liver transplantation were hepatic cirrhosis in four patients, hepatocellular cancer in two patients, recurrent hepatitis in two patients, and hepatic giant

Table 1 General data pertaining to patients with HAS and non-stoma bile duct stricture

Case	Sex	Age	Primary disease	Hepatic artery			Bile duct		
				Anastomosis	Manifestation	Time after OLT (mo)	Anastomosis	Manifestation	Time after OLT (mo)
1	M	41	Hepatic cirrhosis	End-to-end	Stoma stricture	6	End-to-end	Diffuse stricture	7
2	M	51	HCC	End-to-end	Stoma stricture	3	End-to-end	Diffuse stricture	4
3	M	42	Hemangiomas	End-to-end	Stoma stricture	4 d	End-to-end	Diffuse stricture	10
4	M	32	Hepatic cirrhosis	End-to-end	Stoma stricture	2	End-to-end	Diffuse stricture	21
5	M	65	HCC	End-to-end	HA occlusion	15	End-to-end	Diffuse stricture	17
6	M	37	Hepatic cirrhosis	End-to-end	Stoma stricture	12	End-to-end	Diffuse stricture	15
7	M	40	Re-hepatitis	Bypass	Stoma stricture	3	R-Y	Diffuse stricture	4
8	F	59	Re-hepatitis	Bypass	Stoma stricture	1	R-Y	Diffuse stricture	3
9	M	39	Hepatic cirrhosis	End-to-end	Stoma stricture	9	End-to-end	Diffuse stricture	11

HCC: Hepatocellular carcinoma; Re-hepatitis: Recurrent hepatitis; R-Y: Roux-en-Y anastomosis.

Table 2 Results of intervention therapy in patients with HAS and ITBL

Case	Hepatic artery				Bile duct				Survival/Follow-up period (mo)
	Time after OLT (mo)	Treatmen	Frequency	Outcome	Time after OLT (mo)	Treatment	Frequency	Outcome	
1	6	Stent	1	Disengaged (stent occlusion 3 mo later)	7	PTBD	1	Smooth drainage	21
2	8	Stent	1	Disengaged	8	PTBD/balloon	4/2	Smooth drainage	17
3	4 d	Stent	1	Disengaged	10	Stent/PTBD	1/4	Removal 5 mo later	18
4	2	Balloon	1	Disengaged	21	PTBD	3	Smooth drainage	30
5	15	Expectant	0	Occlusion	17	PTBD/balloon	3/2	Smooth drainage	23/died of liver failure
6	12	Expectant	0	Stricture	15	PTBD/balloon	1/1	Smooth drainage	18
7	3	Expectant	0	Stricture	4	PTBD	2	Smooth drainage	6
8	1	Expectant	0	Stricture	3	PTBD/balloon /bile mud removal	8/1	Smooth drainage	9/third OLT
9	10	Expectant	0	Stricture	11	PTBD/balloon	2/1	Smooth drainage	13

hemangioma in one patient. The OLT modus operandi was improved piggyback liver transplantation. End-to-end HA anastomosis was performed in 7, and blood vessel bypass in 2 patients. Seven patients received choledochocholedochostomy, and 2 choledochojejunostomy.

Diagnosis and treatment of HAS

HAS was diagnosed by color Doppler flow image (CDFI), computed tomography (CT), magnetic resonance imaging (MRI) and digital subtraction angiography (DSA). DSA is the gold standard. The criteria for CDFI were an intrahepatic arterial blood flow resistance index $\leqslant 0.5$ and an acceleration time of $\geqslant 0.08$ s. HAS was defined by CT and MR angiography when a stricture ratio $\geqslant 50\%$ was evident. Diagnosis by DSA was indicated when HAS was $\geqslant 40\%$, and when the pressure difference between pre- and post-stricture was $\geqslant 5$ cmH_2O.

Percutaneous transluminal angioplasty (PTA) or expectant treatment was used to treat stenotic HA.

Diagnosis and treatment of non-stoma bile duct stricture

Non-stoma bile duct stricture was diagnosed by physical examination, liver function tests and image analysis. Direct cholangiography is the gold standard. If diffuse intra- and extra-hepatic bile duct stricture, especially intra-hepatic and hepatic hilum bile duct stricture, but few bile duct expansions were shown by direct cholangiography, a non-stoma bile duct stricture could be diagnosed.

Interventional therapy consisted of percutaneous transhepatic bile drainage (PTBD), percutaneous transhepatic bile duct balloon dilation and/or stent placement, and removal of bile duct mud *via* basket extraction, and administration of antibiotics.

Evaluation of therapeutic effect

The following parameters were considered to indicate effective outcome: HAS ratio < 50%; pressure difference between pre- and post-stricture $\leqslant 3$ cm H_2O; decreased fever, jaundice, abdominal pain; and alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), total bilirubin (TB) and direct bilirubin (DB) levels decreased by > 50%.

RESULTS

HAS was found in eight patients, and one hepatic artery occlusion in one patient. Non-anastomosis and diffuse bile duct stricture was seen in nine patients, associated with bile mud siltation, biliary infection, and two patients aggravated to ultimate liver failure (Table 2).

HA and bile duct intervention therapy

PTA was successfully performed in 4 HAS patients

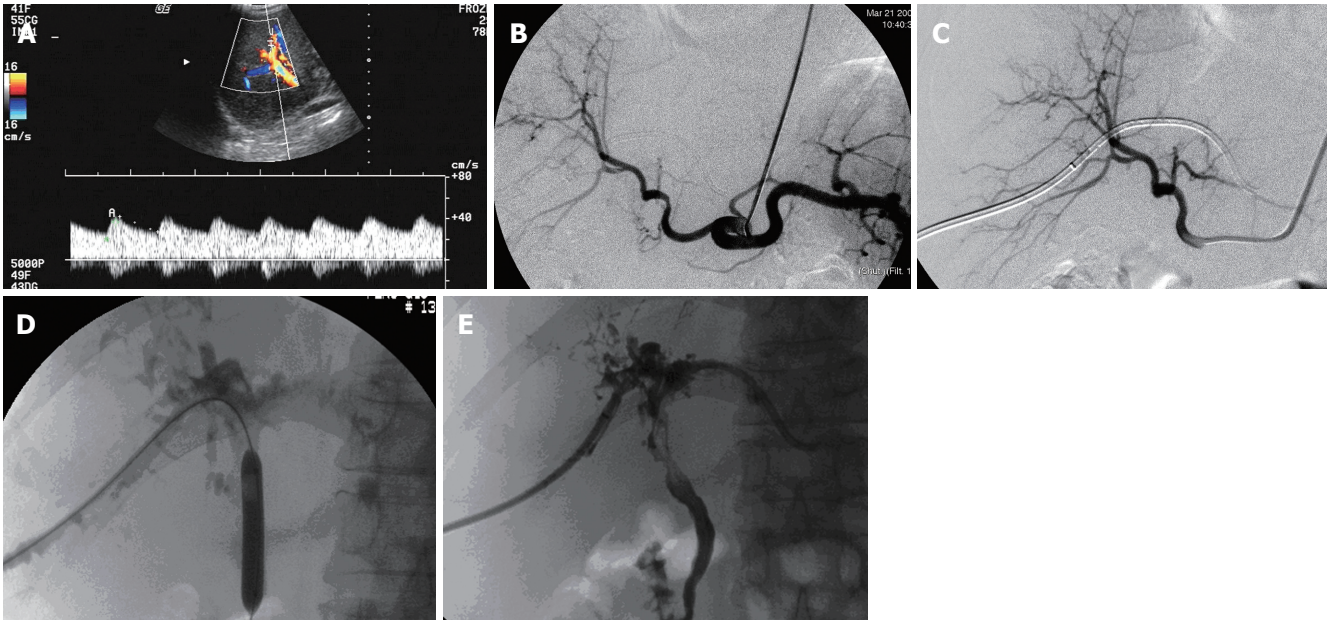

Figure 1 Images pertaining to case 2. HA stoma stricture was diagnosed 3 mo after OLT, no treatment was performed; coronary artery stent placement was performed after 5 mo. Diffuse intra- and extra- hepatic bile duct stricture was apparent 8 mo after OLT. PTBD and balloon dilation therapy were performed. **A**: Tardus parvus frequency spectrum shown by color Doppler ultrasonography indicating HA stoma stricture; **B**: HA stoma stricture as indicated by celiac axis angiography; **C**: Celiac axis angiography revealing HAS disappearance following HA stent placement; **D**: Treatment of bile duct stricture by percutaneous transhepatic balloon dilation; **E**: Cholangiography revealed bile duct stiffness, rare branching, and filling defect in the bile duct lumina.

(Figures 1A-C and 2A). Stents were inserted in 3 of these 4 individuals while balloon dilation was performed in the other. Stricture was disengaged after PTA. Stent re-occlusion was detected by CDFI in one patient 3 mo after intervention, and a bypass circuit was established around the stent. Blood flow was normal in the other three patients.

Diffuse stricture of the hepatic hilum and intra-hepatic bile duct was evident in 3 patients (Figures 1D, E and 2B). In an another case, common bile duct stoma stricture was initially diagnosed. This evolved to diffuse stricture of the hepatic hilum and intra-hepatic bile duct 3 mo later. PTBD was initiated in all 4 patients. Simultaneous balloon dilation of the left and right hepatic and common bile duct, and further stent placement were performed in one patient. Bile duct clysis with heparinate and physiological saline was performed regularly. Chronically implanted bile duct drainage tubes were replaced every 1 to 2 mo. Bile duct drainage tube was retained for a long time.

Following intervention, symptoms related to fever, jaundice, abdominal pain improved markedly in all 4 patients. GGT, ALP, TB and DB levels decreased by more than 50% in all patients, and in two patients, fell to the normal reference range. Post-intervention survival/follow-up period ranged from 17 to 30 mo.

Intervention therapy for bile duct only

Four of the remaining 5 patients were diagnosed with HAS; one with HA occlusion. PTA was not performed in any of these patients. Expectant treatment was performed in each case. Hepatic artery stenosis or occlusion was apparent in all of these individuals.

Common bile duct stoma, which evolved to diffuse intra- and extra-hepatic stricture, was confirmed in all 5 patients. PTBD was performed in all cases. Simultaneous balloon dilation of the bile duct was undertaken in 4 patients, while bile mud was removed *via* net-basket extraction in the other. Bile duct clysis with heparinate and physiological saline was performed regularly. Chronically implanted bile duct drainage tubes were replaced every 1 to 2 mo.

Following intervention, symptoms related to fever, jaundice, abdominal pain improved markedly in all 5 patients. GGT, ALP, TB and DB levels decreased by more than 50% in all patients. Post-intervention survival/follow-up period ranged from 6, 9, 13 and 18 to 23 mo. Liver failure occurred in two patients, one of whom received another OLT, while the other died.

Specific case example

Case 2 (Figure 1) was a 51-year-old male. OLT was performed due to liver cirrhosis and hepatic cell carcinoma.

HAS was confirmed by CDFI (Figure 1A) and CT scanning 3 mo after OLT. However, no interventional therapy was initiated due to the lack of signs and symptoms. Hepatic arteriography revealed hepatic arterial stoma stricture 8 mo after OLT (degree 70%, length 5 mm) (Figure 1B). Balloon dilation and stent placement were performed, and stricture was subsequently eliminated (Figure 1C). Hepatic artery blood flow was smooth, and remained so for 12 mo follow-up period as determined by CDFI.

Common bile duct stoma stricture was diagnosed by CDFI 4 mo after OLT. Expectant therapy was chosen. However, this evolved to diffuse stricture of the hepatic

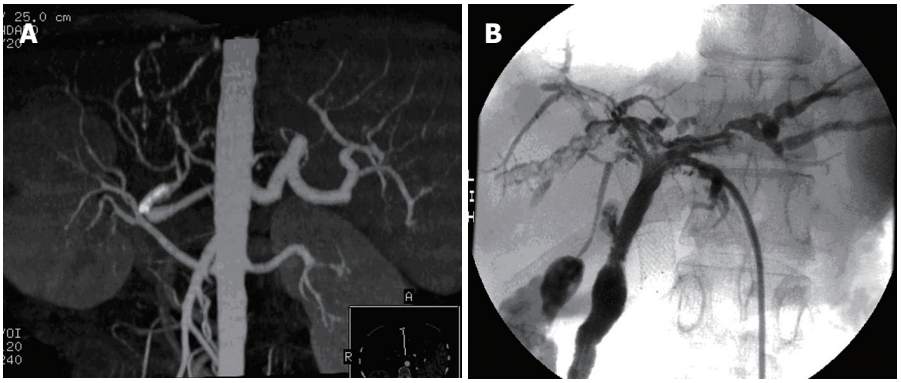

Figure 2 Images pertaining to case 3. HAS was diagnosed 4 d after OLT, and coronary artery stent placement was performed. Diffuse intra- and extra-hepatic bile duct stricture was apparent 10 mo after OLT. PTBD, balloon dilation and bile duct stent placement therapy were performed. **A**: Computed tomography angiography revealing HAS disappearance following HA stent placement; **B**: Cholangiography showing bile duct stiffness, rare branching, and filling defect in the bile duct lumina.

hilum and intra-hepatic bile duct 4 mo later. PTBD and bile duct balloon dilation were performed. The drainage tube was retained and replaced every month.

GGT, ALP, TB, and DB levels proceeded to decrease after intervention, with TB value stabilizing at 100 ng/mL. The patient was discharged with drainage *in situ*, and remained symptom-free at 17 mo follow-up.

DISCUSSION

Ischemia-reperfusion injury to bile duct at OLT

During the process of liver transplantation, there are periods of unequal cold and warm ischemia, which may lead to ischemia-reperfusion injury. When the bile duct becomes ischemic, epithelial cells produce less ATP, leading to calcium overload and subsequent membrane disorganization. This may cause endothelial xerochase, ablation, full-thickness mucosal necrosis/perforation, and fiber formation. These changes can promote bile duct stricture[2]. Ostroff *et al*[3] have suggested that if the time interval between HA and portal vein blood flow recovery is excessive, the bile system may be exposed to long-term warm ischemic conditions. Partial blood flow restoration at this point may further result in HA contraflow, and induce the formation of microthrombi in the HA, leading to consequent bile duct stricture following reconstruction. This theory implies that if portal vein and HA blood flow is restored simultaneously, the extent of ischemic injury to the bile duct should be lessened. In our cohort of patients, the time interval between HA and portal vein reopening was 5 to 16 min. This cannot indicate that the time of HA anastomosis definitively relates to non-stoma bile duct stricture following OLT.

Non-stoma bile duct stricture following OLT

The pathogenesis of non-stoma bile duct stricture following OLT is not clear. According to the causes of non-stoma bile duct stricture, it can be divided into two types: ischemic and immunologic. The possible causes of ischemic type non-stoma bile duct stricture include HAS, hepatic artery thrombosis, long donor liver cold/warm ischemia time[4]. Hintze *et al*[4] thought that the breakdown of bile duct blood supply and damage of the hepatic artery could increase the rate of non-stoma bile duct stricture following OLT. Zheng *et al*[5] reported that 3 of 5 hepatic artery thromboses and 8 HAS patients had hepatic hilum and intra-hepatic bile duct stricture, accounting for 45.5%, which exceeded 23.5% of which without earlier period poor blood supply in the hepatic artery among 32 cases of bile duct complication. Stange *et al*[6] reported that more than 50% of patients with HAS following OLT also had cholangitis, non-stoma bile duct necrosis, or liver abscess. Close to half of these patients required further transplantation. All 9 cases presented in the current report had both HAS or hepatic artery thrombosis and non-stoma bile duct stricture, suggesting that HAS is an important mediator of non-stoma bile duct stricture.

Relationship between non-stoma bile duct stricture and treatment of HAS following OLT

Through prompt PTA operation, HAS could be eliminated, normal blood flow in hepatic artery could be recovered, bile duct blood supply could be improved, therefore, the non-stoma bile duct stricture could be delayed or avoided following OLT[7]. When HAS was not eliminated in a timely manner, the probability of non-stoma bile duct stricture occurrence was increased. In our study, through PTA in four HAS patients following OLT, the time of bile duct stricture occurrence was delayed, symptoms of fever and abdominal pain improved significantly, the values of hemobilirubin decreased obviously and survival or follow-up period was prolonged, thereby emphasizing the importance of timely and correct interventional therapy for HAS.

Value of interventional therapy in non-stoma bile duct stricture

The treatment of bile duct stricture following OLT has been transitioned from pure operation to non-operation[8]. Indeed 90% of bile duct obstruction can be improved through balloon expansion, endoprosthesis placement and bile duct drainage[9].

Interventional bile duct therapy was associated with minimal trauma, intact bypass circuitry, and bile duct blood supply. Repeated interventional bile duct therapy can prolong graft survival time, delay or avoid re-transplantation. Symptomatic improvements were noted in all 9 patients with regard to fever, jaundice, abdominal pain and biliary infection. Overall quality of life was also improved. Hence, it is apparent that interventional therapy for non-stoma bile duct stricture has a great clinical value, and could be considered a first line treatment option.

However, patients with HAS and non-stoma bile duct stricture had a relatively poorer prognosis. Presumably this

was due to HAS-associated bile duct capillary embolism, leading to diffuse bile duct stricture and bile mud siltation, biliary infection and liver failure. Safdar *et al*[10] studied 57 patients with bile leakage after liver transplantation and discovered an incidence of 28% HAS necessitating re-transplantation. Dong *et al*[11] reported that two patients underwent re-transplantation for treatment of non-stoma bile duct stricture. The fact that two of our patients progressed to liver failure demonstrates that sometimes intervention therapy cannot cure non-stoma bile duct stricture radically. Accordingly, intervention therapy for non-stoma bile duct stricture has its own limitation.

Single bile duct stoma stricture was presumably caused by the anastomosis technique, stoma hydroncus or scarring, and was not associated with HAS. Balloon dilation and stent placement were found to be effective methods of treatment.

In conclusion, progressive, diffuse, intra- and extra-hepatic bile duct strictures were the characteristic manifestations of HAS with non-stoma bile duct stricture following OLT, while HAS could be the cause of non-stoma bile duct stricture. These were associated with biliary mud siltation, biliary infection, and ultimately liver function failure. Our data demonstrate that intervention therapy for HAS and bile duct stricture is of significant value in ameliorating these pathologies and symptoms, and should be applied first.

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RAPID COMMUNICATION

Isolation of Kupffer cells and their suppressive effects on T lymphocyte growth in rat orthotopic liver transplantation

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Abstract

AIM: To develop a practical method for isolation, purification and culture of hepatic Kupffer cells (KCs) and to observe their suppressive effects on the proliferation of alloreactive T cells.

METHODS: Perfusion *in situ in vivo* combined with density gradient centrifugation was applied in isolation, purification and culture of hepatic KC. The suppression by KCs on the T cell proliferation in mixed lymphocyte reaction (MLR) was observed.

RESULTS: This method resulted in a satisfactorily high yield of $(1.1 \pm 0.2) \times 10^7$ KCs per liver, (93.5% ± 1.8%) viable cells, over 90% purity and positive for ED-2. After the first 24 h in culture, a great number of KCs which exhibited typical characteristics were observed. Using ^{3}H-TdR incorporation assay, non-irradiated KCs significantly suppressed allo-MLR. The KCs recovered from accepted liver allografts in groups D and E were more effective in suppressing allo-MLR.

CONCLUSION: A standardized procedure for isolation of highly purified rat KCs is proposed and KCs have suppressive effects on the proliferation of alloreactive T cells, especially those derived from accepted liver allografts.

Key words: Liver transplantation; Kupffer cell; T cell

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INTRODUCTION

Kupffer cells (KCs), the resident macrophage population in the liver, which comprise one of the major population (20%) of the hepatic nonparenchymal cell fraction, may play an important role in immunomodulation and the induction of tolerance after liver transplantation. The inherent tolerogenicity of the liver poses important questions about how immune reactivity in the liver is regulated. The interaction between KC and lymphocytes in the liver sinusoids was described first by Gassel[1], who showed that KCs within the hepatic sinusoidal space are in physical contact with two lymphocytes at a time. Therefore, increasing attention has focused on the key role of hepatic allograft-derived KCs in regulating immune responses and facilitating tolerance induction. The primary aim of this study was to develop a practical method for isolation, purification and culture of hepatic KCs and to observe their suppressive effects on the proliferation of alloreactive T cells.

MATERIALS AND METHODS

Animals

Inbred male Sprague-Dawley rats weighing 230-250 g and Wistar rats weighing 250-270 g served as donors and recipients, respectively. All animals were purchased from the Animal Resource Center of Science Academy of China in Shanghai. Animals were cared for under a protocol approved by the Jiao Tong University Animal Ethics Committee.

Reagents

Mouse anti-rat ED2 antibody was purchased from Serotec Inc. (UK) and goat anti-mouse ED2 antibody was provided by Jing Mei Biological Inc. (Beijing, China). The other reagents included RPMI-1640 (Gibco Inc., USA), Ⅳ-type collagenase (Sigma Inc., USA), Percoll's solution (Pharmacia Inc., Switzerland), *etc.*

Experimental design

Orthotopic rat liver transplantation was performed by the cuff technique without hepatic artery reconstruction as described by Kamada[2]. Recipients were divided into five groups: group A (control group, SD→SD); group B (SD →Wistar without any immunosuppression); group C (SD →Wistar, CsA from d 1 to d 5); group D (SD→Wistar, CsA from d 1 to d 5 and anti-CD40L mAb on d 0 and

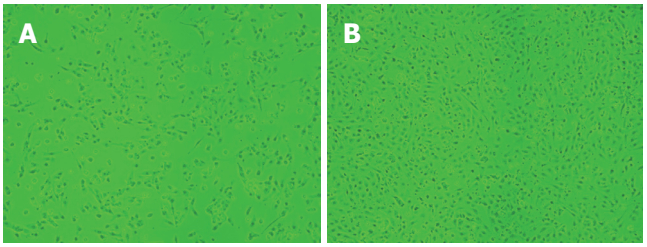

Figure 1 **A**: Single-layer KCs formed with irregular shape and kidney-like nucleus after 24 h (× 100); **B**: KCs increased and became prominently larger after 48 h, showing typical macrophage morphologic features with irregular shape, transparent cytoplasm and kidney-like nucleus (× 100).

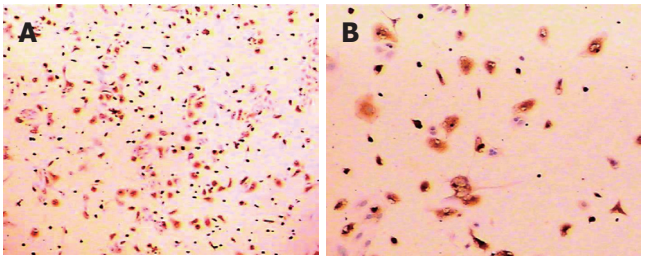

Figure 2 **A**: The cells were stained positively for ED2 and the purity was ⩾ 90% consistently (× 100); **B**: The same as Figure 3 (× 400).

d 2); and group E (SD→Wistar, the same as group D in combination with donor specific blood transfusion). CsA was diluted in normal saline to 1 g/L and injected into the recipients subcutaneously once a day (1.5 mg/kg per day). Anti-CD40L mAb was administered intraperitoneally once a day (1 mg/kg per day).

Isolation and culture of KC

Non-parenchymal cell (NPC) suspensions were acquired by collagenase *in situ* collagenase perfusion of the liver and then KCs were isolated by sedimentation in a two-step Percoll gradient with selective adherence of cells to plastic flasks. The morphologic features of KCs were observed under light microscopy and the viability was determined by trypan blue exclusion. In addition, purity of the KC fraction was determined by ED-2 staining.

Spleen T cell preparation

We prepared suspensions of spleen cells by squeezing mechanically dissociated spleen through a 50-μm stainless steel screen followed by erythrocyte lysis with Tris-ammonium chloride and washings in PPMI 1640. T cells were purified further by passage over nylon wool columns.

Suppression on T cell proliferation of KCs in MLR

Mixed lymphocyte reaction (MLR) was performed: 2.5 × 10^5 SD T cells and 2.5 × 10^5 Wistar T cells from normal rats were cocultured in 96-well plate in the presence of 3 × 10^4 KCs or γ-ray irradiated KCs as control. All cultures were incubated for 6 d at 37℃ in a 5% CO_2-humidified air atmosphere and 18 h before harvesting, One μCi of ^{3}H-thymidine was added into each well. Incorporation of ^{3}H-TdR into DNA was assayed by liquid scintillation

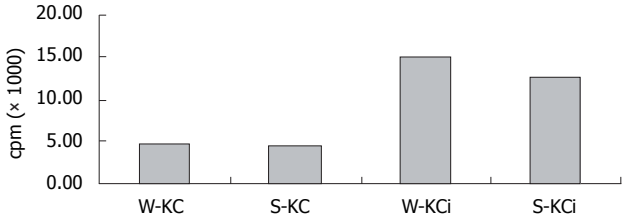

Figure 3 Effects of irradiated or non-irradiated KCs on T lymphocyte proliferation. Non-irradiated KCs (W-KC and S-KC) showed significant suppressive effects on T cell proliferation in MLR while irradiated KCs (W-KCi and S-KCi) showed decreased inhibitory effects in MLR.

counting system. Based on the above experiment, 3 × 10^4 KCs derived from the different groups on the 6th day after operation were added into the above-mentioned MLR system and their suppressive effects on T cell proliferation *in vitro* were observed.

Statistical analysis

The data of liquid scintillation counting were expressed in mean ± SD and ANOVA was applied for comparison in different groups. A P value less than 0.05 was considered significant.

RESULTS

Identification of KCs

Viability and purity: The technique of cell isolation in this study yielded about (1.1 ± 0.2) × 10^7 KCs per liver with (93.5 ± 1.8)% viability determined by trypan blue exclusion. The purity of KC fraction was consistently ⩾ 90% determined by morphology in combination with ED2 staining.

Morphology and immunohistobiochemical staining: The freshly isolated cells had a ball-like shape, 20 μm in diameter when viewed under light microscopy. About 3-4 h later, most of them adhered to the wall of plastic flasks and 48 h later they became larger, prominent and showed typical macrophage morphologic features with irregular shape, transparent cytoplasm and kidney-like nucleus (Figure 1A and B). By immunohistobiochemical technique, the cells were stained positively for ED2 (Figure 2A and B).

Suppressive effects of KCs on T lymphocyte proliferation in vitro

Effects of irradiated and non-irradiated KCs on T lymphocyte proliferation: To observe the effect of KCs on T cell response, we examined T cell proliferation in two-way MLR. By ^{3}H-TdR incorporation assay, non-irradiated KCs showed significantly suppressive effects on T cell proliferation in MLR. However, when irradiated KCs were used, there was a marked decrease in the inhibitory effect upon the MLR (Figure 3).

Effects of KCs derived from liver allograft on T lymphocyte proliferation: The KCs derived from liver allograft of different groups were added into the MLR system in order to determine the effects of those cells. The results showed that there was a marked decrease of cpm value in MLR when KCs from groups D and E were

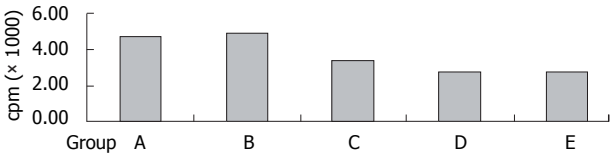

Figure 4 Suppressive effects of KCs derived from liver allograft on T lymphocyte proliferation. There was a marked decrease of cpm value in MLR when KCs were present in groups D and E, i.e., KCs derived from those two groups had more significantly suppressive effect on T cell proliferation.

present, i.e., KCs derived from those two groups had more significantly suppressive effects on T cell proliferation. The cpm of different groups was 4810 ± 132, 5036 ± 315, 3480 ± 201, 2825 ± 118 and 2786 ± 122, respectively (Figure 4).

DISCUSSION

Kupffer cells (KCs), the most important resident macrophages of the liver, comprise one of the major populations (about 20%) of the hepatic nonparenchymal cell fraction, and the tolerogenic properties of the liver are generally accepted[3]. Therefore increased attention has focused on the possible role and mechanism of KCs from liver allograft in tolerance induction[4,5]. In recent years, some authors have put forward a novel KC-dependent immunomodulatory mechanism which may be strengthened markedly after liver transplantation, especially those donor-derived KCs expressing Fas ligand which exhibit immune privilege *in vivo*[6]. But precise mechanisms accounting for this phenomenon have not been illustrated. It is of great importance to establish a kind of stable, economical and reliable technique of KC isolation and culture for the study of tolerance induction and immune regulation. Various methods have been applied and basic steps include collagenase in situ perfusion of the liver, sedimentation in two-step Percoll gradient and selective adherence. The isolated KCs should be kept with biological and immunological characteristics *in vivo* such as phagocytosis, lysosomal enzymes and expression of specific antigens[7-9].

By collagenase in situ perfusion in combination with gradient centrifugation, we successfully established a method of isolating, purifying and culturing KCs. And the technique in this study yielded about $(1.1 \pm 0.2) \times 10^7$ KCs per liver with a 93.5% ± 1.8% viability and a consistently ⩾ 90% purity. Twenty-four hours later, single layer of KCs formed with an irregular shape and kidney-like nucleus. Forty-eight h later KCs increased and became larger, more prominent and showed typical macrophage morphologic features with irregular shape, transparent cytoplasm and kidney-like nucleus. With immunohistobiochemical techniques, the cells were stained positively for ED2. The isolated KCs could survive as long as about 4 weeks while keeping biological and immunological characteristics *in vitro*.

In comparison with the literature, we made some modification in isolation and culture of KCs. Firstly, we blocked the suprahepatic inferior vena cava (IVC) while perfusing collagenase *in situ*. Secondly, we increased perfusion velocity in order to wash off thoroughly erythrocytes in sinusoid. And thirdly, digestion of liver cells in vitro was applied. Collagenase and Pronase E were used at the same time when isolating KCs both at home and abroad[10-12]. Pronase E will destroy CD14 molecules on the surface of KCs which are independent in the activation of macrophages by LPS so that the isolated KCs may not keep physiological functions[13,14]. Therefore, only collagenase was used in perfusion of liver. The results showed that the technique in our study was able to yield sufficient number of KCs with high viability and purity.

On the basis of the successful isolation and culture of KCs, we observed suppressive effects of KCs on T lymphocyte proliferation *in vitro*. Non-irradiated KCs showed significant suppressive effects on T cell proliferation in MLR while there was a marked decrease in the inhibitory effect upon the MLR when irradiated KCs pretreated by 100Gy γ-ray were used. When KCs derived from liver allograft of groups D and E were added into the MLR system, there was a marked decrease of cpm value, i.e., KCs derived from those two groups had more significantly suppressive effect on T cell proliferation. Administration of KCs derived from chronically accepted liver allografts might be able to significantly prolong the survival of hepatic allografts in an acute rejection model in an alloantigen-specific manner[15-17]. There are different explanations for this phenomenon. FasL expression of KCs is increased after liver transplantation which is associated with T lymphocyte apoptosis through Fas-FasL pathway[18]. At the same time, KCs can increase the secretion of IL-10 and TGF-β by up-regulating the expression of Th2/Th3 cytokines mRNA so that KCs may regulate the differentiation of Th2/Th3 cells[19,20]. And KC itself secretes an array of cytokines such as IL-4, IL-10, IL-12 and TGF-β which may drive the differentiation of T cell subsets towards Th2/Th3[21]. Because Th2 cells are not sensitive to FasL-induced apoptosis, selective Th2 survival coupled with rapid death of Th1 cells may be a mechanism for differential regulation of the two T cell subsets by KCs[22,23]. Due to the restriction of instruments in our lab, mechanisms proposed have not yet been validated convincingly.

In conclusion, KCs had significantly suppressive effects on allo-reactive T lymphocyte proliferation, especially those recovered from chronically accepted liver allografts. The hepatic professional and non-professional APC including KCs, liver sinusoidal endothelial cells(LSEC), DC, even hepatocytes may play key roles in regulating immune responses and facilitating tolerance induction after liver transplantation[24]. The precise mechanism needs to be further investigated in the future.

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CASE REPORT

Intraocular complications of IFN-α and ribavirin therapy in patients with chronic viral hepatitis C

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Abstract

We report a panel of severe inflammatory and vascular intraocular disorders occurring during interferon-alpha (IFN-α) treatment in eight hepatitis C virus (HCV)-infected patients. These events include three cases of Vogt-Koyanagi-Harada like (VKH) disease (an association of panuveitis, retinal detachment, ear and meningeal detachment and skin and hair changes), two cases of central retinal vein occlusion, one case of central retinal artery occlusion, one case of severe hypertensive retinopathy and one case of bilateral ischemic optic neuropathy with severe visual impairment. Rare as they are, such severe ophthalmological complications require a close follow-up of HCV-infected patients under IFN-α treatment with ophthalmological monitoring if any ocular manifestation occurs.

Key words: Hepatitis C virus; Interferon-alpha; Intraocular complications; Central retinal vein occlusion; Central retinal artery occlusion; Acute anterior ischemic optic neuritis; Vogt-Koyanagi-Harada like disease

Sène D, Touitou V, Bodaghi B, Saadoun D, Perlemuter G, Cassoux N, Piette JC, Hoang PL, Cacoub P. Intraocular complications of interferon-α and ribavirin therapy in patients with chronic viral hepatitis C. *World J Gastroenterol* 2007; 13(22): 3137-3140

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INTRODUCTION

Treatment of chronic hepatitis C virus (HCV) infection included initially standard interferon-α (IFN-α) given three times a week. Since 2000, it has been proposed to administer pegylated IFN-a once a week[1]. Most frequent side effects include flu-like syndrome, asthenia, and weight loss. Ophthalmological complications are rare. The most typical ocular adverse effect is the IFN-α related retinopathy, which is characterized by cotton wool spots and retinal haemorrhages especially around the optic nerve. Visual loss is usually absent or limited and reversible after interruption of the therapy[2,3]. Involvement of the posterior segment of the eye is rare but may lead to permanent visual loss in the absence of appropriate therapy[4]. In the present report, we prospectively recorded and analyzed eight patients who presented with severe ophthalmological complications during the treatment with IFN-α and ribavirin for chronic hepatitis C. Clinical and angiographic findings were monitored. IFN-α was discontinued in all cases.

CASE REPORT

All patients were chronically HCV-infected (HCV RNA positive). Epidemiological, clinical and biological features were prospectively recorded. Patients were referred to the ophthalmologist only in case of ocular symptoms. They were managed and followed in a single ophthalmological department. Ophthalmological examination included visual acuity, slit lamp examination, fundoscopy, and fluorescein angiography if necessary. Final diagnosis and therapeutic management were collegially assumed. Severe ophthalmological complications during IFN-α therapy included inflammatory ocular diseases [3 cases of Vogt-Koyanagi-Harada (VKH) like disease] and vascular disorders (5 cases), including central retinal vein occlusion (CRVO) in 2, central retinal artery occlusion (CRAO) in 2, severe hypertensive retinopathy in 1 and ischemic optical neuritis in 1. The three cases of VKH-like disease have been already reported elsewhere[5].

Intraocular inflammatory disorders

The intraocular inflammatory disorders reported herein are three cases of VKH disease. The VKH disease is a rare autoimmune disorder with an ocular involvement,

Table 1 Main characteristics and course of HCV-infected patients with VKH-like disease

	Case 1	Case 2	Case 3
Age (yr)	43	51	42
Sex	F	F	M
HCV genotype	3	1	1
liver biopsy (Metavir)[1]	A1F2	A1F2	A3F1
Anti-HCV therapy	PEG-IFN α-2b + Ribavirin	PEG-IFN α-2b + Ribavirin	PEG-IFN α-2b + Ribavirin
Interval before first ocular manifestations[2]	4 mo	3 mo	4 mo
Ocular manifestations	-Visual acuity 20/200 OS -Macular edema and a bilateral serous retinal detachment.	-Bilateral vision loss -Bilateral uveitis, major papillar and retinal edema	-Bilateral vision loss -Episcleritis and bilateral uveitis
Retinal fluorescein angiography	Pin-points and bilateral serous retinal and pigmented epithelium detachments, suggestive of a Vogt-Koyanagi-Harada like [VKH] disease		
Therapeutic management	-PEG-IFN and ribavirin disruption	-PEG-IFN and ribavirin disruption	-PEG-IFN and ribavirin disruption
	-Methylprednisolone IV and per os	- Methylprednisolone IV and per os	- Methylprednisolone IV and per os
Course	-Complete recovery under low dose steroids (< 10 mg/d)	-Low improvement of ocular lesions	-Partial improvement of ocular lesions
	-Steroids were stopped after one year of treatment without ocular relapse.	-Cortico-dependency > 25 mg/d	-Cortico-dependency > 25 mg/d
		-Failure of cyclosporine course	-Re-introduction of PEG-IFN and ribavirin 5 mo later[3]
		-Introduction of azathioprine	-Full recovery of ocular manifestations 10 mo after IFN was reintroduced

[1]Metavir scoring system for the appraisal of HCV-related liver disease. [2]Time or interval between introduction of the anti-HCV therapy and the first ocular manifestations. [3]Forcase 3, PEG-IFN and ribavirin was re-introduced because of the high level of cortico-dependency and based upon the reported efficacy of IFN in some cases of severe and refractory uveitis[14].

mainly granulomatous panuveitis associated with exudative retinal detachments, with skin and hair (vitiligo, poliosis and alopecia) changes and ear and meningeal involvement (meningitis, cranial nerve palsy, focal signs, dysacusis, hearing loss). The diagnosis is confirmed by the retinal fluorescein angiography that shows typical pin-points and bilateral serous retinal and pigmented epithelial detachments.

VKH-like disease after 4 mo of pegylated (PEG)-IFNα-2b therapy: A 43-year-old woman, HCV-infected (genotype 3) since 1982 (after blood transfusion), had a non-symptomatic mixed cryoglobulinemia and significant liver histological damages at liver biopsy (Metavir A1F2), which required pegylated IFN-α (PEG-IFN-α) (1.5 μg/kg per week) and ribavirin (10.5 mg/kg per day). Four months later, she was admitted for decreased vision of the left eye. Visual acuity was 20/20 OD and 20/200 OS. Fundus examination disclosed a left macular edema and bilateral serous retinal detachment. Fluorescein angiography revealed several pin-points, bilateral serous retinal and pigmented epithelial detachments. VKH-like disease was diagnosed. PEG-IFN and ribavirin were both discontinued. Intravenous pulses of methylprednisolone (1 g/d, 3 d) were performed, followed by oral prednisone (1 mg/kg per day). One month later, visual acuity remained 20/20 P2 for the right eye and improved to 20/40 P2 for the left eye. Retinal detachments disappeared. Seven months later, visual acuity was 20/20 P2 OD, 20/25 P2 OS and prednisone was slowly tapered. After one year of treatment, steroids were stopped without any ocular relapse.

Clinical features of the patients with VKH disease occurring during IFN therapy are reported in Table 1.

Intraocular vascular complications

Five patients presented with severe intraocular complications under PEG-IFN + Ribavirin treatment, including 2 cases of central retinal vein occlusion, one case of central retinal artery occlusion, one case of acute anterior ischemic optic neuritis and one case of severe exudative hypertensive retinopathy. The common hallmark of all these cases is the complete or severe and definitive vision loss despite the treatment withdrawal and an adequate therapeutic management. This reflects the severity of such complications. Clinical features of the patients with intraocular vascular side effects during IFN therapy are reported in Table 2.

DISCUSSION

Most of ophthalmological side effects occurring during IFN-α treatment are benign, transient, and are mainly represented by the classical IFN-related retinopathy. In the present work, we describe two mechanisms of severe and sight-threatening ocular complications during IFN therapy: the first group of complications includes inflammatory disorders and the second group vascular intraocular diseases. Concerning the cases of inflammatory intraocular disorders, we report three cases of VKH-like disease-defined by the association of a panuveitis with exudative retinal detachment, skin and hair changes and ear and meningeal involvement- occurring during IFN-α

Table 2 Main features of HCV-infected patients with intraocular vascular complications under PEG-IFN and ribavirin treatment

Main features	Case 4	Case 5	Case 6	Case 7	Case 8
Age (yr)	51	70	55	40	40
Sex	M	M	F	M	M
HCV genotype		1	2		
Liver biopsy (Metavir)	ND	F4	A2F1	A2F2	F4 (clinical cirrhosis)
Antecedents	Sarcoidosis	Arterial hypertension Smoking	-	Splenic lymphoma with villous lymphocytes Mixed cryoglobulin-associated glomerulonephritis Severe arterial hypertension Dyslipidemia, smoking	Hypertension with past hypertensive retinopathy
Anti-HCV therapy	PEG-IFN α-2b + Ribavirin	PEG-IFN α-2b + Ribavirin	PEG-IFN α-2b + Ribavirin	Standard IFN α-2b + ribavirin	Standard IFN α-2b + Ribavirin
Interval before first ocular manifestations	7 mo	5 mo	6 mo	18 mo	6 mo
Ocular manifestations	-Initial visual acuity: OD (< 20/200), OS (20/20) -Papillar edema, macular edema and retinal hemorrhages -Cotton wool spots	-OD vision loss (20/200 OD; 20/20 OS)	-Bilateral vision loss (20/400 OD, 20/80 OS)	-Visual acuity OS: 10/10; OD: < 20/200	-Bilateral vision loss (20/64 P2 OD, 20/200 OS) -Bilateral macular edema and retinal hemorrhages -Cotton-wool spots (IFN-α-induced retinopathy) (Figure 1A and B)
Diagnosis	Central retinal vein occlusion OD	Central retinal vein occlusion OD	Acute anterior ischemic optic neuritis	Central retinal artery occlusion OD	Exsudative hypertensive and IFN -induced retinopathy
Treatment	-Withdrawal of PEG-IFN and ribavirin -Steroids and IV heparin	-Withdrawal of PEG-IFN and ribavirin -Steroids, IV heparin and aspirin	-Withdrawal of PEG-IFN and ribavirin -Steroids	-Withdrawal of standard IFN and ribavirin -Steroids and IV heparin	-Withdrawal of standard IFN and ribavirin -Better control of hypertension (nadolol, benazepril)
Course	-6 mo later, radiary neurotomy -At the end of follow-up, definitive loss of vision OD (< 20/200)	-2 mo later, radiary neurotomy -At the end of follow-up, definitive vision loss OD	-At the end of follow-up, severe visual impairment (< 20/400 OD; 20/80 OS)	-4 mo later, slow improvement (20/64 OD) -Died of severe sepsis 5 mo later	-2 mo later, significant improvement of the visual acuity (20/40 OD; 20/40 OS) -Introduction of PEG-IFN α-2b and Ribavirin without recurrence after more than 1 yr follow-up

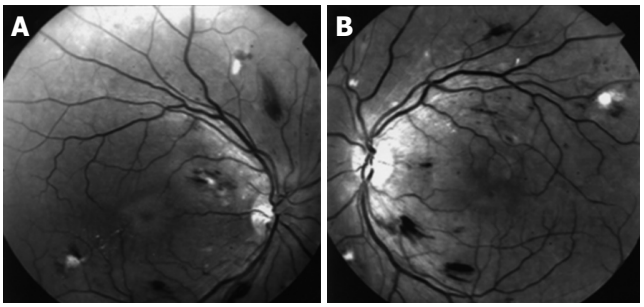

Figure 1 Interferon-induced retinopathy with cotton-wool spots and retinal hemorrhages. **A**: OD; **B**: OS.

treatment for HCV infection. In all cases, high doses of steroids were required and sometimes associated with an immunosuppressive drug (azathioprine, cyclophosphamide, and cyclosporine). These complications mostly resulted in a definitive and severe visual impairment.

VKH-like disease during interferon therapy is rare, and to our knowledge, apart from our three patients[5], only three cases of VKH under an IFN-α course have been reported[6,7]. This evidence and the third patient of our report who benefited from a second course of PEG-IFN for both HCV-infection and VKH disease, show the complex relationship between IFN-α course and VKH disease. However, considering the severity of the visual impairment which can be induced by such a syndrome, ophthalmological examination should be systematically proposed during IFN treatment. In case of intraocular inflammation, the diagnosis of VKH-like disease must be considered and interferon therapy can be disrupted.

Retinal vascular disorders associated with IFN treatment include central retinal venous and central retinal arterial occlusion, and severe hypertensive retinopathy. Only few cases have been reported[8,9]. Arterial and venous occlusions were associated with a severe visual defect that did not improve despite the combination of heparin, steroids and the withdrawal of the IFN therapy. Most of arterial occlusive events occurred in presence of ill-

controlled vascular risk factors, such as hypertension, dyslipidemia and smoking.

Finally, cases of ischemic optic neuritis have been reported in HCV-infected patients under IFN-α[10] and may impair dramatically visual functions. Predisposing factors are not clearly identified, except for classical vascular risk factors. These data point out the necessary control of known vascular risk factors before and during IFN-α treatment.

Other ophthalmological complications of IFN-α therapy include transient blurred vision, increased intraocular pression, neovascular glaucoma, and "specific IFN-α"-related retinopathy characterized by cotton wool spots, retinal hemorrhages, and microaneurysms[11-13]. Functional abnormalities seemed also to be frequent under IFN-α but without clinical expression[12].

Besides previous case reports of ocular side effects of IFN-α therapy, our study raises the possibility of severe ophthalmological manifestations (occlusion of retinal, choroidal or optic nerve vessels, or VKH-like diseases). These results confirm the potential severity of intraocular complications during IFN-α therapy in HCV-infected patients. A close ophthalmological monitoring and efficient control of systemic and ocular vascular risk factors (hypertension, diabetes, and dyslipidemia) seem mandatory before further IFN reintroduction.

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CASE REPORT

Distant skeletal muscle metastasis from intrahepatic cholangiocarcinoma presenting as Budd-Chiari syndrome

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Abstract

Intrahepatic cholangiocarcinoma is a malignant neoplasm arising from the biliary epithelium, which frequently invades adjacent organs or metastasizes to other visceral organs such as the lungs, bones, adrenals, and brain. However, distant skeletal muscle metastasis of cholangiocarcinoma has never been described before to the best of our knowledge and, furthermore, Budd-Chiari syndrome secondary to intrahepatic cholangiocarcinoma is also extremely rare. Here we present the first case overall of distant muscle metastasis from intrahepatic cholangiocarcinoma presenting as Budd-Chiari syndrome. A 44-year-old man admitted to the hospital with complaints of abdominal distension, edema of both legs, back pain and anorexia of 30 d' duration. Computed tomography and ultrasonography-guided percutaneous muscle biopsy established intrahepatic cholangiocarcinoma with disseminated thrombosis from inferior vena cava to bilateral iliac and femoral veins, and multiple skeletal muscle metastases in bilateral buttock and erector spinal muscle.

Key words: Intrahepatic Cholangiocarcinoma; Metastasis; Skeletal muscle; Budd-Chiari syndrome

Kwon OS, Jun DW, Kim SH, Chung MY, Kim NI, Song MH, Lee HH, Kim SH, Jo YJ, Park YS, Joo JE. Distant skeletal muscle metastasis from intrahepatic cholangiocarcinoma presenting as Budd-Chiari syndrome. *World J Gastroenterol* 2007; 13(22): 3141-3143

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INTRODUCTION

Intrahepatic cholangiocarcinoma is a malignant neoplasm arising from the biliary epithelium and a devastating malignancy that presents late, is notoriously difficult to diagnose, and often invades adjacent organs or metastasizes to other visceral organs such as lungs, bones, adrenals, and brain. Skeletal muscle is one of the most uncommon sites of metastasis from any malignancy. Although direct muscle invasion by primary malignancy is well recognized, few cases of metastasis to skeletal muscle distant from the primary carcinoma have been published[1]. Primary carcinoma sites to distant skeletal muscle metastasis included the stomach, esophagus, lung, colon, and pancreas[2]. However intrahepatic cholangiocarcinoma has never been mentioned as the primary carcinoma site for skeletal muscle metastases to the best of our knowledge. Budd-Chiari syndrome which is defined as any pathophysiologic process that results in interruption of the normal flow of blood out of the liver, and is commonly associated with a hypercoagulable state which is often secondary to malignancy. But the Budd-Chiari syndrome secondary to intrahepatic cholangiocarcinoma is so rare that only three cases have been reported in the literature so far.

We report the first case of distant skeletal muscle metastasis of intrahepatic cholangiocarcinoma presenting as Budd-Chiari syndrome and acute thrombus extended down into the bilateral iliac veins and femoral veins.

CASE REPORT

A 44-year-old man visited the gastroenterology department with complaints of abdominal distension, dyspnea, low extremity edema, back pain and anorexia of one month's duration. He was previously healthy and his past medical and family histories were not remarkable. He consumed alcohol (3 bottles of Soju distilled liquor per week) until one month before admission.

Physical examination on admission revealed a height of 174 cm, body weight of 78 kg, temperature of 36.4℃, blood pressure of 110/60 mmHg, and pulse rate of 78/min. Upon examination, the abdomen was not tender, distended with a shifting dullness. The liver was not palpable below the costal margin. Ascites was severe and both low limb edema was moderate. Patient laboratory tests included a red blood cell count (RBC) of 3.93×10^{12}/L, hemoglobin concentration of 121 g/L,

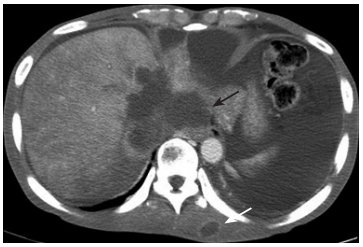

Figure 1 Computed tomography shows a large mass with rim enhancement around intrahepatic portion IVC of liver which encircles IVC (black arrow). Metastatic lesions seen in the left spinal erector muscle (white arrow) and right psoas muscle (not shown).

hematocrit of 35.9%, white blood cell count of 5.8×10^9/L, platelet count of 1.18×10^{11}/L, prothrombin activity of 75.9%, activated partial thromboplastin time of 30.1 s, aspartate aminotransferase (AST) level of 50 IU/L, alanine aminotransferase (ALT) level of 26 IU/L, alkaline phosphatase level of 813 IU/L, γ-glutamyl transferase level of 144 IU/L, lactate dehydrogenase level of 494 IU/L, total bilirubin level of 0.8 mg/dL, and albumin level of 37 g/L. Renal function tests showed a blood urea nitrogen level of 39.5 mg/dL and a creatinine level of 1.6 mg/dL. HBsAg and anti-HBeAb were positive but anti-HBs antibodies, anti-HBc antibodies, HBeAg, HBV DNA, and anti-hepatitis C virus antibodies were all negative. Peritoneal fluid analysis revealed WBC of 6.90×10^8/L, RBC of 1.05×10^9/L, polymorphonuclear cell of 19%, lymphocytes of 81%, albumin level of 13 g/L, and the serum ascites albumin gradient (SAAG) was 24 g/L. Levels of carcinoembryonic antigen and alpha-fetoprotein were normal. The levels of thyroxin stimulating hormone and thyroid hormones were within normal limits. An abdominal ultrasonography showed a large amount of ascites, splenomegaly and hydronephrosis of the left kidney. The ascites was intractable and the edema and pain of both lower extremities were aggravated. Abdominal computed tomography (CT) without contrast enhancement was performed and it showed a large lobulated area of low density in the left lobe of liver (7 cm × 4 cm), thrombosis in inferior vena cava (IVC), two round, low density areas in the right psoas muscle (2cm) and an oval low density area at the left paravertebral area between left psoas muscle and quadratus lumbrom muscle (3.5 cm × 2 cm), mild left hydronephrosis and a large amount of ascites. For further evaluation, CT scan with contrast enhancement from chest to lower extremity was performed. The constrasted CT scan revealed a large liver mass (8 cm × 8 cm) with peripheral rim enhancement around intrahepatic portion of IVC, extensive thrombosis in IVC to bilateral iliac and femoral veins, multiple muscle metastasis in bilateral buttock, left erector spinae, right psoas muscles, multiple bony metastasis in the 10th and 11th thorasic spine and the 1st lumbar spine, and multiple patches or nodules of increased density in the lungs suggesting metastasis (Figure 1). Tests for coagulation activity showed D-dimer level of 6200 ng/mL (normal less than 300 ng/mL), antithrombin Ⅲ level of 20 mg/dL (normal 21.0-34.0 mg/dL), protein-C activity level of 55% (normal 70%-130%), and protein-S activity level of 61% (normal 77%-143%).

Ultrasonography-guided percutaneous needle biopsy was performed at the erector spinae muscle which was suspected to have metastatic lesions. Microscopic examinations showed that the skeletal muscle was infiltrated by

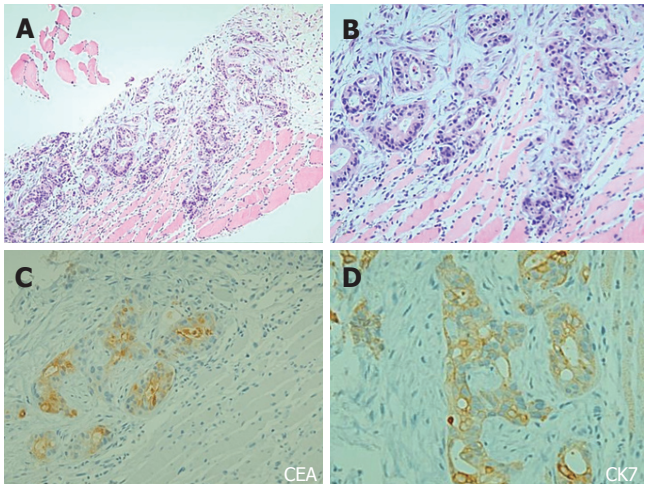

Figure 2 **A**, **B**: Neoplastic glandular infiltration in the skeletal muscle, suggesting metastatic adenocarcinoma. (HE, x 100 and x 200); **C**: Neoplastic glands are positive for immunohistochemical staining on CEA (CEA immunostain, x 200); **D**: Neoplastic glands are positive for immunohistochemcal staining on cytokeratin 7 (Cytokeratin 7 immunostain, x 200).

neoplastic glands accompanying moderate desmoplasia (Figure 2). Immunohistochemical staining showed that the neoplastic cells were strongly positive for cytokeratin 7 and CEA, weak and focally positive for cytokeratin 20, and negative for TTF-1. The findings of CT scan and the histopathologic feature would be compatible with intraductal cholangiocarcinoma and its distant skeletal muscle metastasis.

DISCUSSION

The incidence of skeletal muscle metastases is reported to be less than 1% of metastases of hematogeneous origin, despite of the fact that skeletal muscle accounts for nearly 50% of the total body weight and is characterized by rich blood supply[3]. The cause for the low incidence of skeletal muscular metastases of primary cancer is still unclear, but may be related to various factors as follows: tumor suppressors in skeletal muscles[4], the constant movement of skeletal muscles which may represent a difficult condition for the implantation and growth of metastatic cells under the high tissue pressure related to the exercise-associated increase of blood flow, the local production of lactic acid which would create an unfavorable environment for metastatic cell growth, the inhibition of cell invasion by protease inhibitors located in the basement membrane, and the antitumor activity of lymphocytes or natural killer cells within the skeletal muscle[3].

The reason why distant skeletal muscle metastasis from intrahepatic cholangiocarcinoma has been not been reported may be because of the relative rarity and poor prognosis of intrahepatic cholangiocarcinoma, and another possibility is that physicians and patients tend to overlook the distant skeletal muscle metastasis, which is frequentely asymptomatic, nonspecific, and in hidden locations[5]. Under-diagnosis of skeletal muscle metastases may contribute to their apparent low incidence. In 194 autopsies involving tumor metastasis to skeletal muscles,

which were performed at the Marque de Valdecilla National Medical Center from 1980 to 1982, metastases to skeletal muscle were noted in 11%, and 20% of the patients with carcinoma had muscle metastasis[6].

Budd-Chiari syndrome secondary to intrahepatic cholangiocarcinoma is very rare, only three cases have been reported to our knowledge. The first was from a case series that attempted to etiopathophysiologically classify Budd-Chiari syndrome[7]. The second was in a woman who had presented with recurrent venous thrombosis during the third episode endoscopic cholangiopancrea tography, and guided biopsy established a diagnosis of cholangiocarcinoma at the mid portion of common bile duct[8]. Law *et al*[9] reported the third case of metastatic intrahepatic cholangiocarcinoma presenting as acute Budd-Chiari syndrome with a large thrombus in the inferior vena cava and plain thrombus extending from the right atrium down into the iliac veins by postmortem examination.

In summary, intrahepatic cholangiocarcinoma presenting Budd-Chiari syndrome is extremely rare, and distant skeletal muscle metastasis from intrahepatic cholangiocarcinoma presenting with Budd-Chiari syndrome has not been reported previously to our knowledge. In the present report, we described the first case of metastasis to the distant erector spinae muscle from intrahepatic cholangiocarcinoma presenting as Budd-Chiari syndrome. Intrahepatic cholangiocarcinoma tends to have a poor prognosis because of its typically late presentation. In our case, the presentation of distant skeletal muscle metastasis and Budd-Chiari syndrome is a reflection of the severe malignant potential of this carcinoma and our limited options in the management of this disease.

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CASE REPORT

Gallbladder villous adenoma in a patient with acromegaly: A case report

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Abstract

Villous adenomas are benign epithelial lesions with malignant potential that can occur in any part of the gastrointestinal tract. We present a case of a middle age woman with acromegaly who was investigated for nonspecific gastrointestinal complaints. Ultrasonography and subsequent endosonography diagnosed a large (4.5 cm), hyperechoic, sessile polyp with numerous pedicles. An open cholecystectomy was performed and revealed a villous adenoma with several foci of carcinoma *in situ*. Detailed investigations showed no other tumors of the gastrointestinal tract. After five years of follow-up, the patient reports no complaints, and the results of laboratory testing and imaging studies are within the normal range.

Key words: Villous adenoma; Gallbladder; Acromegaly; Endosonography

Krstic M, Alempijevic T, Stimec B, Micev M, Milicevic M, Micic D, Jankovic G. Gallbladder villous adenoma in patient with acromegaly: A case report. *World J Gastroenterol* 2007; 13(22): 3144-3146

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INTRODUCTION

A polypoidal lesion may be defined as an elevation on the mucosa. Such polypoid lesions in the gallbladder are present in about 5% of the adult population[1]. As ultrasound technology improves, an increasing number of polypoid lesions of the gallbladder are being detected[2]. Although some patients are symptomatic, others are found incidentally. The potential range of pathological lesions is large, and the differentiation between benign, malignant and potentially malignant lesions is a major diagnostic dilemma. Christensen and Ishak[3] categorized polypoid lesions of the gallbladder into: true tumors (adenoma, leiomyoma, lipoma and others), pseudotumors (cholesterol polyp, adenomyomatosis, heterotopias and inflammatory lesions), and malignant lesions.

Adenomas of the biliary tract are uncommon. Gallbladder adenomas are found in 0.5% of cholecystectomy specimens[4]. The vast majority of gallbladder adenomas have a tubular structure while tubulovillous adenomas are exceedingly rare. The incidence of pure villous adenomas of the gallbladder is only 0.08% in autopsy series[5].

CASE REPORT

A middle aged female with acromegaly diagnosed 5 years earlier, was admitted to the hospital because of nonspecific gastrointestinal complaints. Conventional ultrasonography (US) revealed a large heterogeneous polypoid lesion in the gallbladder. Endoscopic ultrasonography (EUS) of the upper GI tract and biliary system was performed, using an Olympus radial 7.5/12 MHz switchable probe. The lesion was described as a large sessile polyp, measuring 4.5 cm, and consisting of numerous pedicles which were moving independently during the examination (Figure 1). The tumor was hyperechoic and hetrogenous, with an irregular surface, and did not show any penetration of the muscularis propria layer. There were several small polyps surrounding the large lesion. No polyps were identified in the gastrointestinal tract on colonoscopy, push enteroscopy and small bowel follow through examination. The patient underwent an open cholecystectomy which confirmed the findings of EUS (Figure 2). Histopathology revealed a villous adenoma with regular arborization of the villo-papillar adenomatous epithelium and peripheral stromal edema of the villi (Figure 3). Most of the microscopic fields showed low grade epithelial dysplasia, with a few small foci of prominent atypia consistent with intraepithelial carcinoma. Numerous small polyps located in the periphery of the larger one were tubular adenomas, with only low grade epithelial atypia. The muscularis

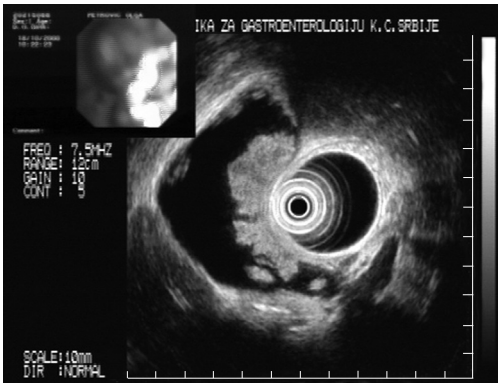

Figure 1 Endosonography picture of the gallbladder polyp.

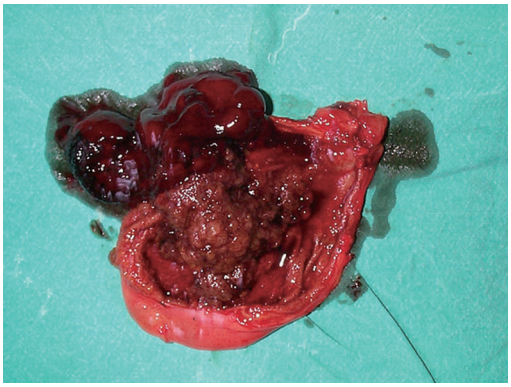

Figure 2 Resected specimen of the gallbladder.

propria layer of the gallbladder was clearly preserved and the local lymph nodes were unremarkable. After five years, the patient reports no complaints and repeated clinical examinations, laboratory testing, and imaging studies have been within the normal range.

DISCUSSION

Villous adenomas are benign epithelial lesions that can occur anywhere in the gastrointestinal tract. These tumors are usually found in the rectum and colon, less frequently in the small intestine, and rarely in the biliary tree[6,7]. This case report describes a patient with acromegaly and a villous adenoma of the gallbladder, the first such presentation seen in our region.

Seven hereditary syndromes associated with hamartomatous polyps have been described. These include familial juvenile polyposis syndrome, Cowden's syndrome, Bannayan-Ruvalcaba-Riley syndrome, Peutz-Jeghers syndrome, basal cell nevus syndrome, neurofibromatosis 1, and multiple endocrine neoplastic syndrome 2B. Hereditary mixed polyposis syndrome is a variant of juvenile polyposis characterized by both hamartomatous and adenomatous polyps. The hamartomatous syndromes occur at approximately 1/10th of the frequency of the adenomatous syndromes and account for < 1% of colorectal cancers in North America[8]. In these inherited diseases, there is a higher incidence of gallbladder polyps, but there is also

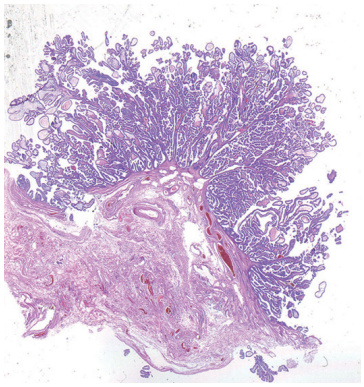

Figure 3 Low power light microscopy (HE) of the villous adenoma.

a higher risk of extraintestinal malignancy[4]. While the diagnosis of these inherited syndromes is primarily clinical, genetic testing is now available for the six syndromes noted above. However, a significant number of spontaneous mutations have been described in each of these syndromes.

Patients with acromegaly are at an increased risk of developing neoplastic lesions, especially of the large bowel secondary to an increased incidence of colonic polyps and carcinomas. Histological examination of the polyps shows both adenomas as well as hyperplastic polyps. The pathogenesis is multifactorial. The major mechanism is believed to be IGF- I hypersecretion, as well as mutation of the tumor suppressor genes, such as adenomatous polyposis coli and *p*53, DDC (deleted in colon cancer), and DNA mismatch repair genes[9,10]. Nevertheless, despite several analyses the findings remain controversial[11-13]. To our knowledge, there is no link between gallbladder polyps and acromegaly. In our patient, the gallbladder polyp was the only neoplasm identified, and polyps were not detected in the gastrointestinal tract, and no other neoplastic diseases were observed.

A review of the literature revealed ten cases of bile duct villous adenomas[6]. The most common location of these tumors was at the ampulla of Vater[14]. Most patients presented with obstructive jaundice and were treated surgically, supporting the importance of meticulous preoperative assessment and judicious use of surgical exploration.

In our patient, cholecystectomy was the ideal procedure. Laparoscopic cholecystectomy is generally the preferred operative choice. However, if the chances of malignancy are considered high, for example in polyps larger than 2 cm, open surgery is preferable in order to reduce the risk of tumor seeding associated with laparoscopic surgery[15]. This is why an open cholecystectomy was performed in our patient. Besides the clinical features, certain US and EUS findings are highly indicative of malignancy. Choi[16] presented a scoring system based on five variables: layer structure, echo pattern, margin of the polyp stalk, and number of polyps. Based on this scoring system, the appearance of the polyp in our patient (heterogeneous echo pattern, lobulated margin, sessile, multiple), despite preserved layer structure was highly suspicious for a malignant lesion. Thus, the scoring system is more accurate if the polypoid lesion measures between 5-15 mm.

Based on an analysis of surgical specimens of gallbladder

polyps, it has been suggested that in symptomatic patients there should be no reservation about the need for surgery, regardless of the size of the lesion. Factors associated with an increased risk for malignancy are: age of the patient (> 60 years), size of the polyp (> 10 mm) and coexistence of gallstones. Other authors have suggested the following features as suspicious for malignancy and therefore an indication for surgical treatment: wide base lesion, lesion tending to enlarge in a short period, long pedicle, polyps at the neck preventing emptying of the gallbladder, and polyps associated with irregular thickening of the gallbladder wall[2,17,18].

A causal relation between the acromegaly and villous adenoma of the gallbladder cannot be established on the basis of a single case. Although the vast majority of gallbladder polyps are benign, these patients need careful evaluation and appropriate follow-up. In the present case, the polypoid lesion in the gallbladder was a villous adenoma, which has a significant potential for malignant transformation.

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Treatment of duodenal ulceration with Furazolidone in China preceded the discovery of its association with *H pylori*

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TO THE EDITOR

It is not generally known that patients with duodenal ulceration were being treated with an antibiotic, Furazolidone, in China five or more years before Marshall and Warren[1] published their seminal paper in 1984 about the association between duodenal ulceration and Campylobacter like organisms in the stomach, later named *H pylori*. Marshall and Warren won the 2005 Nobel Prize in physiology or medicine for their work on how a bacterium can relate to gastric inflammation or peptic ulceration.

In 1981 I was invited by the Bureau of Health to a lecture/research tour of rice-growing areas of China in connection with research into the geographical prevalence of duodenal ulceration in relationship to staple diets. During that visit I met Professor Zhi-Tian Zheng at the Third Teaching Hospital in Beijing, and he told me about a series of duodenal ulcer patients, 80% of whose ulcers had healed, and had remained healed for 3 years, following a 2 wk course of treatment with Furazolidone. At the time I was very sceptical about this.

I was invited back to China in 1984, this time to make a tour of the wheat and millet-growing areas, and once again I visited Professor Zhi-Tian Zheng in Beijing. By then he had gathered a much larger number of patients whose duodenal ulcers had healed following treatment with Furazolidone, and who were remaining in remission. I persuaded him to publish this, and a letter from him and his colleagues appeared in *The Lancet* in 1985[2].

Later in this tour I found that Professor Huai-Yu Zhao in Lanzhou had similar findings which he and his colleagues also reported later in the same year in a letter to *The Lancet*[3].

It seems only right that Professors Zhi-Tian Zheng and Huai-Yu Zhao and their colleagues in China should have some of the credit for having linked persistence and recurrence of duodenal ulceration with a bacterial infection.

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symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
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cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

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11-15 April 2007
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easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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