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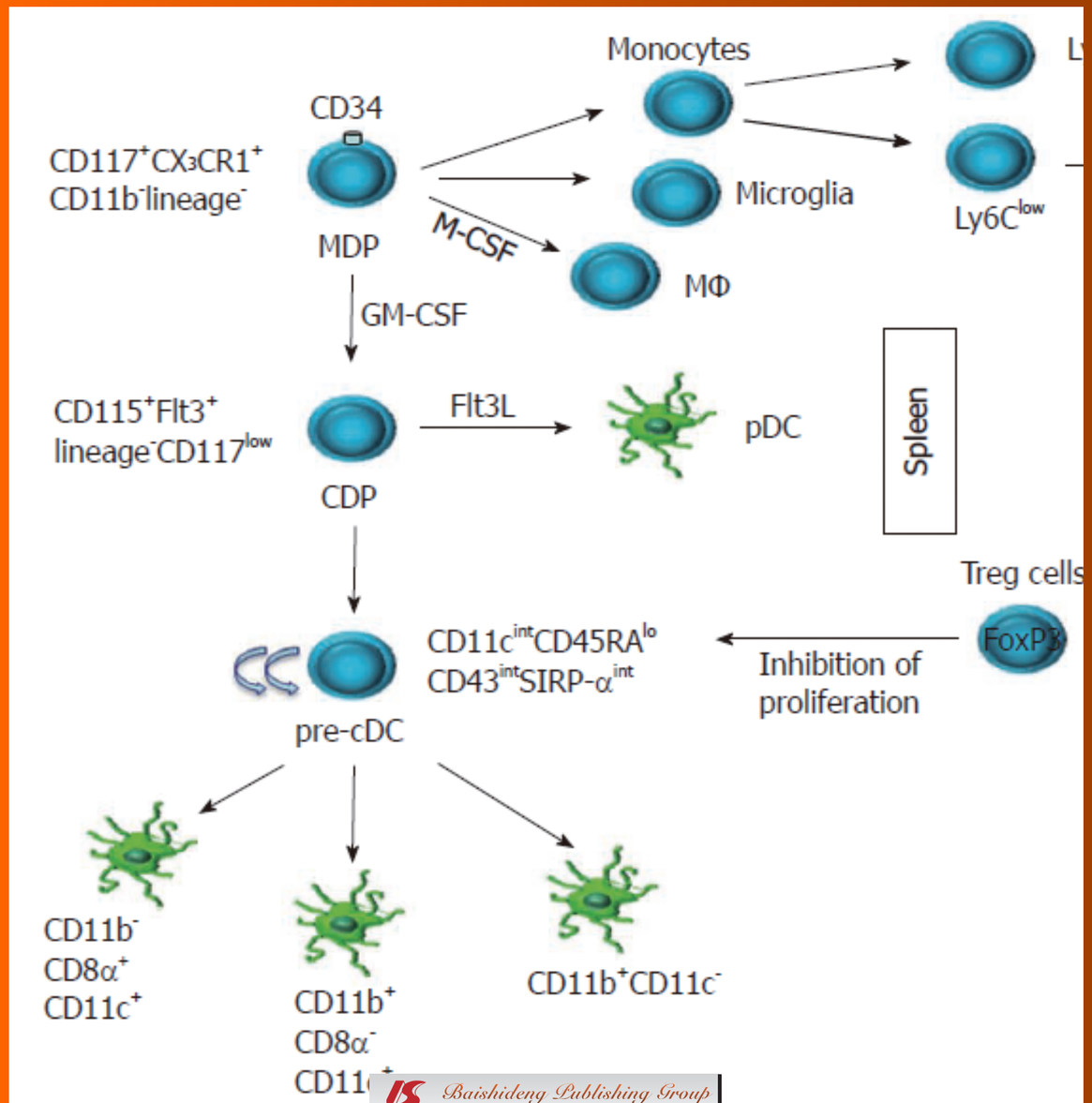

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EDITORIAL

Intestinal dendritic cells in the pathogenesis of inflammatory bowel disease

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Abstract

The gastrointestinal tract harbors a large number and diverse array of commensal bacteria and is an important entry site for pathogens. For these reasons, the intestinal immune system is uniquely dedicated to protect against infections, while avoiding the development of destructive inflammatory responses to the microbiota. Several models have been proposed to explain how the immune system discriminates between, and appropriately responds to, commensal and pathogenic microorganisms. Dendritic cells (DCs) and regulatory T cells (Treg) are instrumental in maintaining immune homeostasis and tolerance in the gut. DCs are virtually omnipresent and are remarkably plastic, having the ability to adapt to the influences of the microenvironment. Different DC populations with partially overlapping phenotypic and functional properties have been described in different anatomical locations. DCs in the draining mesenteric lymph nodes, in the intestinal lamina propria and in Peyer's patches partake both in the control of intestinal inflammation and in the maintenance of gut tolerance. In this respect, gut-resident DCs and macrophages exert tolerogenic functions as they regularly encounter and sense commensal bacteria. In contrast, migrating DC subsets that are recruited to the gut as a result of pathogenic insults initiate immune responses. Importantly, tolerogenic DCs act by promoting the differentiation and expansion of Treg cells that efficiently modulate gut inflammation, as shown both in pre-clinical models of colitis and in patients with inflammatory bowel disease (IBD). This article reviews the phenotypic and functional features of gut DC subsets and discusses the current evidence underpinning the DC contribution to the pathogenesis of the major clinical subtypes of human IBD. It also addresses the potential clinical benefit derived from DC targeting either *in vivo* or *in vitro*.

Key words: Dendritic cell; Tolerance; Gut; Inflammatory bowel disease; Cytokine; Regulatory T cells

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INTRODUCTION

The digestive tract has a surface area nearly 200 times greater than that of the skin. Being an important port of entry for microorganisms, the gut must be protected by effective immune responses. However, immune reactivity must be prevented from damaging gut tissues in response

to benign foreign material to which the gut is continuously exposed. T cell immunity relies on the recognition of antigenic peptides processed and presented to T cells by dendritic cells (DCs), which act as initiators, stimulators and regulators of antigen-specific T cell responses, but also play a pivotal role in the maintenance of tolerance towards the commensal microflora[1,2].

DCs are specialized accessory cells distinguishable from other mononuclear phagocytes (MPs) such as monocytes and macrophages by their unique morphology and ability to capture and process antigens for presentation to effector T cells. Upon encounter with pathogens and activation, DCs undergo rapid maturation characterized by the upregulation of major histocompatibility complex (MHC) and costimulatory molecules and migrate to the draining lymph nodes. The remarkable flexibility of DC functions likely results from their ability to sense the local environment and to shape the ensuing immune response[3,4]. Intestinal MPs are distributed in organized lymphoid organs, such as Peyer's patches (PP) and mesenteric lymph nodes (MLN), and are highly abundant in the loose connective tissue underlying the epithelium, the lamina propria (LP)[5].

It is now established that DCs play a crucial role in both immunity and tolerance[1,6]. In a tolerogenic setting, DC can induce anergy in antigen-specific T cells or generate protective FoxP3$^+$ regulatory T cells (Treg) in the lymph nodes. Under steady-state conditions, DCs continuously migrate from peripheral organs *via* the lymph to secondary lymphoid organs, where they present self-antigens or innocuous environmental antigens to maintain peripheral tolerance. The chemokine receptor, CCR7, is a key regulator of the homeostatic and inflammation-induced trafficking of DCs from skin, lung and gut to their respective draining lymph nodes[7].

Human inflammatory bowel disease (IBD) consists of 2 dominant disease subtypes, Crohn's disease (CD), largely arising from a Th1 response, and ulcerative colitis (UC), largely mediated by interleukin (IL)-5- and IL-13-producing T cells or natural killer T cells[8]. The immunopathology of human IBD relates to an inappropriate and exaggerated immune response to constituents of the gut flora in a genetically predisposed individual. Amongst other cell types[9], DCs play a role in IBD pathogenesis, as suggested by mouse models of colitis and by observations in humans. The local microenvironment regulates the function of mucosal DCs through the presence of immune cells, non-immune cells and luminal bacteria[10]. In principle, DC dysfunction may promote the development of gut inflammation by priming T-cell responses against bacteria, by sustaining T cell reactivity within the inflamed mucosa and by functioning as effector cells releasing pro-inflammatory cytokines[11].

DC LINEAGE AND SUBSETS IN MICE AND HUMANS

DC origin and precursor-progeny relationships have remained a matter of controversy and debate for decades[12]. Recent landmark studies have led to a better definition of DC ontogeny in mice (Figure 1), unraveling that a macrophage and DC precursor (MDP) serves as a common bone marrow progenitor for classical or conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocytes (Table 1). Specifically, Fogg *et al*[13] have identified a clonogenic MDP with a CD117$^+$CX$_3$CR1$^+$CD11b$^-$lineage$^-$ phenotype, representing ~0.5% of total bone marrow cells and giving rise to monocytes, to several macrophage subsets and, ultimately, to steady-state CD11c$^+$CD8α^+ and CD11c$^+$CD8α^- DC. Lymphoid organ DCs in the steady state originate from a bone marrow precursor with a Lin$^-$CD115$^+$Flt3$^+$CD117^{lo} phenotype, termed common DC progenitor (CDP)[14]. Migratory DC precursors (pre-DCs) also exist in the peripheral blood and are in equilibrium with DCs in lymphoid organs and in non-lymphoid tissues, such as skin, lung, kidney and intestine. DCs actively divide *in vivo* and their lifespan varies from 5 to 7 d in the spleen, lymph node, liver and kidney and can be as long as 25 d in the lung[15].

DCs lack a unique surface marker, but rather express a distinct set of cell surface antigens. The number of DC subsets that have been phenotypically characterized and functionally designated is increasing steadily. In addition to the classical integrin marker CD11c distinguishing DCs, the integrin αE (CD103) recently gained attention and has been used to sub-classify DC subsets based on specific functional activities and anatomic location (see below for a thorough discussion)[16]. CD103 mediates T cell adhesion to epithelial cells through its binding to E-cadherin, which is expressed on the basolateral side of epithelial cells but not on endothelial cells. Mice with a targeted disruption of *cd103* show a mild reduction in T cell numbers in the intraepithelial and LP compartments, coupled with the inability to reject islet allografts[17].

MECHANISMS UNDERLYING DC-MEDIATED TOLERANCE IN THE GUT

One of the major functions of tolerogenic DCs may be the differentiation of Treg cells from naïve T cells. Two major subtypes of Treg cells have been described to date, namely, naturally occurring CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (nTreg) and inducible type 1 Treg cells (Tr1).

DCs as inducers of nTreg cells

Naturally occurring Treg cells, a functionally specialized subset of CD4$^+$ T cells, have been involved in preventing T cell-mediated and innate immune pathology in a number of disease models[18]. The transcription factor FoxP3 is expressed by CD4$^+$CD25$^+$ Treg cells and is fundamental for Treg development and function. nTreg cells mainly suppress effector T cells through a cell contact-dependent and largely contact-independent mechanism. Membrane-bound transforming growth factor (TGF)-β has been implicated in nTreg-mediated inhibition of T cell responses. Moreover, TGF-β1 acts as a co-stimulatory factor for FoxP3 expression, leading to Treg differen-

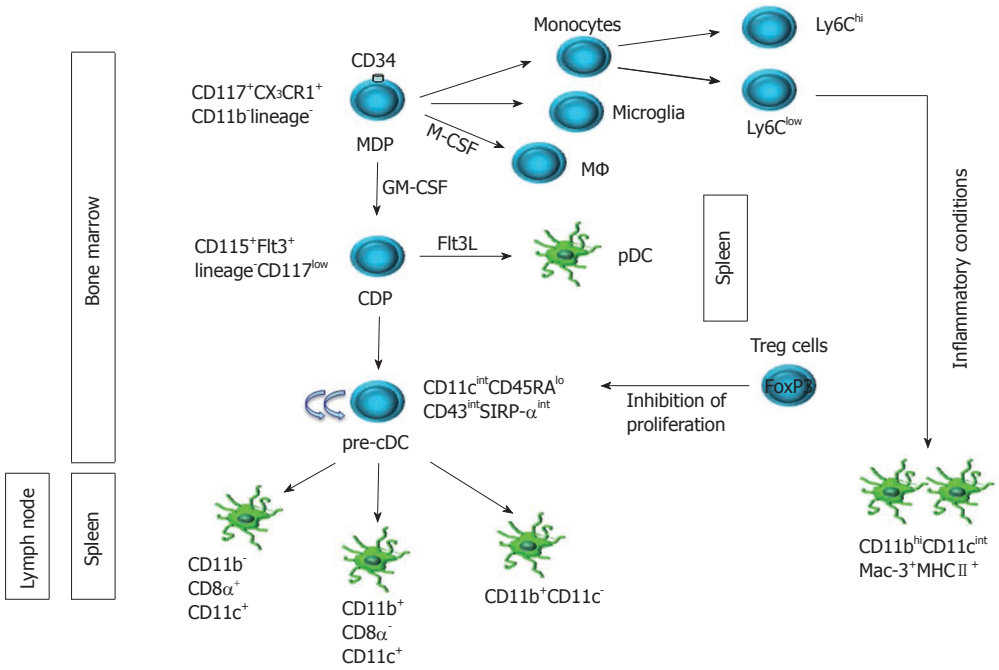

Figure 1 Ontogeny of dendritic cell subsets in mice. The most recent evidence elucidating dendritic cell (DC) ontogeny and unraveling the complexity of the DC compartment in mice is summarized. The curved arrows in cyan denote proliferation potential. Regulatory T (Treg) cells may contribute to DC development and homeostasis in mice, as suggested by studies where Treg depletion has been associated with a 2- and 12-fold increase in precursor conventional DC (pre-cDC) and cDC in spleen and lymph node, respectively[14]. MDP: Macrophage and dendritic cell progenitor; CDP: Common dendritic cell progenitor; pDC: Plasmacytoid DC.

tiation from $CD4^+CD25^-$ T cells. Interestingly, TGF-β1 production by Treg cells is not required for inhibition of colitis, suggesting that Treg cells may induce TGF-β release by other hematopoietic or stromal cells[19]. Support for this hypothesis is provided by the observation that suppression of colitis by TGF-$β1^{-/-}$ Treg cells was inhibited by anti-TGF-β antibodies, indicating that TGF-β is central to the function of Treg cells even when they do not synthesize it themselves[19]. In this respect, DCs remain a key and intriguing candidate for TGF-β production *in vivo*. It is conceivable that Treg cells be required to express TGF-β1 on the cell surface and to present it to pathogenic T cells, as previously shown[19].

Treg cells are believed to play a crucial role in inhibiting intestinal inflammation and IBD. Notably, Treg cells may contribute differentially to the modulation of experimental autoimmune gastritis and colitis. Protection from colitis, but not from gastric inflammation, has been reported to depend on IL-10 expression by $CD4^+CD25^+$ nTreg cells[20]. The T cell transfer model of colitis allows an understanding of Treg-mediated mechanisms controlling intestinal inflammation. During cure of experimental colitis, Treg cells proliferate and accumulate in MLN and colonic LP, in contact with $CD11c^+$ DCs and effector T cells[21]. Interestingly, IL-10-producing Treg cells selectively enrich within the colonic LP, whereas FoxP3-expressing Treg cells are present in similar frequencies in both the secondary lymphoid organs and LP of colitic animals[22]. Transfer of $CD4^+CD45RB^+$ T cells into $RAG^{-/-}$ mice causes colitis. Disease development requires β7-integrin-dependent intestinal localization. Importantly, β7-deficient Treg cells prevent colitis, suggesting that Treg accumulation in the intestine is dispensable for disease suppression[23]. The presence of Treg cells impacts on $CD4^+CD45RB^+$ T cell accumulation in the intestine, indicating that one major function of Treg cells may involve the inhibition of tissue localization of Th1 effector cells.

Peripheral blood $CD4^+CD25^{high}$ T cells may be decreased in active human IBD compared with inactive disease[24]. Notably, Treg cells are increased in mucosal IBD lesions, coincident with an increase in transcripts for IL-8, a hallmark of inflammation in the gut, and for FoxP3[24]. The higher degree of Treg infiltration in the gut LP of patients with diverticulitis compared with IBD suggests that an insufficient increase of Treg cells in IBD accounts for inflammation and intestinal pathology[24]. In the LP of human colon, Treg accumulation has been detected in a variety of inflammatory conditions, such as diverticulitis, pseudo-membranous colitis and cytomegalovirus-induced colitis, and may not be a specific feature of CD or UC[22]. The presence of $FoxP3^+$ T cells in the LP of patients with IBD suggests that defects in Treg numbers may not account for the pathology, and that ineffective Treg activity may rather contribute to sustained gut inflammation.

DCs as inducers of Tr1 cells

Tr1 have been described as a $CD4^+$ T-cell subset releasing high levels of IL-10, in the absence of measurable IL-2 and IL-4 production, and exerting suppressive functions in an IL-10/TGF-β-dependent but cell contact-independent manner[25]. The production and release of interferon (IFN)-γ and TGF-β by Tr1 cells are comparable with those of Th0 and Th1 clones, respectively[25].

Table 1 Intestinal dendritic cell subsets and other mononuclear phagocytes described to date

Subset	Anatomic location	Function, if known	Ref.
$CD11c^{hi}CD11b^{+}CD8\alpha^{-}$	Peyer's patches	Localized in the subepithelial dome	[57]
$CD11c^{hi}CD11b^{+}CD8\alpha^{+}$		Localized in the interfollicular regions	[57]
$CD11c^{+}CD11b^{-}CD8\alpha^{-}$		Localized in both subepithelial dome and interfollicular regions; secretion of IL-10 in response to CD40 cross-linking; induction of T-cell release of IL-4/IL-10; promotion of T-cell proliferation	[58]
$CD11c^{mid}$ plasmacytoid DC			
$CD11c^{+}CD11b^{+}CD8\alpha^{+}$ DC	Small-intestinal and colonic lamina propria	Low T-cell responses *in vitro*; $IL\text{-}12p40^{low}IL\text{-}10^{+}$; tolerance to OVA (m); secretion of IFN-α; differentiation of Tr1-like cells that secrete IL-10, IL-4 and IFN-γ	[30,55]
CX_3CR1^{+} DC		Pro-inflammatory activity dependent upon TNF-α production	[101]
$CD11c^{hi}$class $II^{hi}CD103^{+}$ DC		Generation of $CCR9^{+}\alpha_4\beta_7^{+}$ T cells with gut tropism	[51]
$CD11b^{+}CD8\alpha^{-}CD103^{+/-}$ DC		Conversion of $Foxp3^{-}$ T cells to $Foxp3^{+}$ T cells; retinoic acid and TGF-β required	[54]
$CD11c^{lo}B220^{+}CD8\alpha^{+}$	Mesenteric lymph node	Promotion of $CD4^{+}CD25^{+}$ Treg function; differentiation of Tr1-like cells ($IL\text{-}10^{+}IL\text{-}4^{+}IFN\text{-}\gamma^{+}$) from naïve T cells after repeated stimulations	
$CD11c^{hi}CD11b^{+}CD8\alpha^{+}$		See above	
$CD11c^{hi}CD11b^{-}CD8\alpha^{-}$		See above	
$CD103^{+}$ DC		~50% of $CD11c^{+}$ DC in the MLN; conversion of naïve T cells into Treg cells; retinoic acid and TGF-β required; induction of CCR9 on gut-tropic T cells	[16]
$CD103^{-}$ DC		Expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-23p19) and genes such as *Tbet*, *tlr2* and *tbx21*	[16]
$CD11c^{hi}$class $II^{hi}CD103^{+}$ DC		Induction of CCR9 gut-homing receptor on $CD4^{+}$ T cells	[53]
$CD11b^{+}CD11c^{-}$ macrophages	Small and large intestine	Induction of Treg cells, secretion of IL-10 but not IL-12p40 or IL-12p70	[105,106]

DC: Dendritic cell; OVA: Ovalbumin; IFN: Interferon; MLN: Mesenteric lymph node; Tr1: Type 1 Treg cells; TGF: Transforming growth factor; IL: Interleukin; M: Mouse studies.

Colitis in the severe combined immunodeficient (SCID) mouse model involves the development of Th1 cells responding primarily to the intestinal flora. The transfer of ovalbumin (OVA)-specific Tr1 cells in SCID mice with $CD4^{+}CD45RB^{hi}$ T cell-induced colitis prevents disease manifestations, an effect that is dependent upon the *in vivo* activation of Tr1 cells by feeding mice with OVA[25]. This observation indicates that Tr1 cells can inhibit immune responses to unknown antigens by a bystander suppression mechanism. Another report has shown that $IL\text{-}10^{-/-}$ mice lack $CD4^{+}CD45RB^{lo}$ Treg cells capable of controlling intestinal inflammatory responses, pointing to IL-10 as a crucial mediator of tolerance in the gut[26]. Similarly, TGF-β is required to suppress Th1-mediated colitis induced by $CD4^{+}CD45RB^{hi}$ T cells[27], indicating that IL-10 and TGF-β play non-redundant roles in the functioning of intestinal Treg cells.

The source of IL-10 which regulates colitis remains to be unequivocally identified. Treg-derived IL-10 was recently shown to be dispensable for suppression of colitis in $Rag1^{-/-}$ mice, but host IL-10 was required to inhibit disease development[28]. Specifically, IL-10 production by myeloid $CD11b^{+}F4/80^{+}$ cells, mostly macrophages, was important for the maintenance of Foxp3 expression by Treg cells[28]. IL-10 acted directly on Treg cells, because Treg cells lacking IL-10Rβ chain failed to suppress colitis when transferred together with $CD4^{+}CD45RB^{hi}$ T cells. In addition, this study demonstrates that IL-10 is not required to maintain FoxP3 expression in non-inflammatory conditions, because Treg development and function are unaffected in $Il10rb^{-/-}$ mice[28]. It is conceivable that the differential requirement for IL-10 for FoxP3 expression and maintenance in inflammatory *vs* non-inflammatory conditions may reflect the need for an additional signal to counter inflammatory mediators such as IL-6 or tumor necrosis factor (TNF)-α[29]. It remains to be determined whether IL-10-mediated mechanisms are unique to the gut microenvironment or whether IL-10 may be required to maintain FoxP3 expression in other organs.

Other studies pointed to Treg-derived IL-10 as a major contributor to Treg-mediated suppression[25]. These discrepancies may be attributed to differences in the endogenous flora and/or in the model systems studied. Mucosal $CD8\alpha^{+}$ DCs with a $CD11c^{lo}B220^{+}$ phenotype can be isolated from mouse MLN and have been reported to promote the suppressive function of $CD4^{+}CD25^{+}$ Treg cells and to promote the conversion of naïve T cells into Tr1-like cells[30]. At variance with classical Tr1 cells, the Tr1-like cells described in this study released IL-10, IL-4 and IFN-γ and suppressed T helper proliferation[30]. The $CD8\alpha^{+}$ DC were capable of supporting Tr1-like cell differentiation also in the presence of a maturational stimulus, such as CpG, as reported for other tolerogenic, semimature DC preparations[6,31].

DC expression of indoleamine 2,3-dioxygenase 1 and gut tolerance

Indoleamine 2,3-dioxygenase 1 (IDO1) is a tryptophan-catabolizing enzyme implicated in maternal allograft acceptance and in immune tolerance to tumors[32-36]. IDO1 converts tryptophan into immune suppressive kynurenines that profoundly affect T-cell functions, promoting T-cell unresponsiveness, T-cell apoptosis and differentiation of Treg cells. IDO expression has been associated with $CD103^{+}$ DCs in the gut LP and MLN of mice[37]. Similarly, human intestinal $CD11c^{+}CD103^{+}$ DCs express higher levels of IDO mRNA compared with $CD11c^{+}CD103^{-}$ DCs. IDO inhibition of mouse $CD103^{+}$ DCs with the D

isomer of 1-methyl-tryptophan (1MT) reduced the ability of IDO$^+$ DCs to convert Treg cells and augmented the generation of IL-17-producing T cells. Mice treated with 1MT concomitant with adoptive transfer of OVA transgenic T cells and oral immunization with OVA led to a reduction in the frequency of Treg cells in the LP, PP and MLN. *Ido1*$^{-/-}$ mice displayed a decreased percentage of Foxp3$^+$ Treg cells in the LP and an almost double the proportion of IL-17$^+$CD4$^+$ and IFN-γ$^+$CD4$^+$ T cells in the intestine compared with wild-type animals. Finally, *Rag1*$^{-/-}$ mice injected with colitogenic T cells from C57BL/6 mice experienced more extensive gut inflammation and aggressive disease if treated with 1MT. Similar effects were demonstrated in mice with dextran sodium sulfate (DSS)-colitis, where 1MT administration worsened the mortality rate and colon shortening. Collectively, these experiments indicate that IDO may play a previously unappreciated and fundamental role in regulating gut inflammation through the control of Th1/Th17/Treg balance.

The expression of IDO in the murine gut may increase with age via an IFN-γ-dependent mechanism that involves commensal microorganisms[38]. IDO-deficient mice have abnormally high levels of both IgG and IgA, a phenomenon driven by the commensal flora. IDO may then physiologically restrict B-cell responses to intestinal commensal bacteria. The elevated levels of IgG and IgA in IDO-deficient mice might in principle confer resistance to enteric pathogens such as *Citrobacter rodentium*, a gram-negative bacillus similar to human enteropathogenic *Escherichia coli*. When infected orally with *Citrobacter*, IDO-deficient mice appeared well throughout the course of the experiment, at variance with wild-type animals that had decreased activity, ruffled fur and hunched posture, and had attenuated gut colonization by the pathogen[39]. IDO-deficient mice had reduced edema, inflammatory cell infiltration and epithelial damage in colonic tissue sections, associated with lower levels of TNF-α compared with wild-type mice. These observations point to IDO as a novel target to manipulate intestinal inflammation and to control diseases caused by enteric pathogens.

Crosstalk between DCs and intestinal epithelial cells

Intestinal epithelial cells (IECs) are a central component of the immune system of the gut. They express receptors for microbial-associated molecular patterns that activate signaling cascades leading to the production of antimicrobial products and chemokines[40]. IECs can also recruit leukocytes to complement their barrier function or to participate in the activation of gut adaptive immune responses, including the production of IgA and the differentiation of effector Th1, Th2 and Th17 cells.

IECs are in close contact with LP DCs and have been shown to release molecules that influence DC functions. Thymic stromal lymphopoietin (TSLP) is a cytokine secreted by IECs under steady-state conditions and imparts a Th2-polarizing phenotype to DCs[41]. IEC-derived factors also stimulate the expression of both chains of TSLP receptor on DCs, namely the common IL-7 receptor α chain and the TSLP receptor, thus conferring the ability to respond to TSLP and to drive Th2 responses. Importantly, TSLP expression by primary IECs may be deregulated in a proportion of patients with IBD. The same study also showed that mRNA signals for TSLP are readily detected in IECs from healthy controls, although the protein is consistently below the detection limit by immunoprecipitation, unless IECs are challenged with bacteria such as *S. typhimurium*. TSLP has been detected in epithelial cells of the Hassall's corpuscles and activates myeloid CD11c$^+$ DCs in the thymic medulla[42]. These apparently mature DCs promote the development of Treg cells through a mechanism that requires peptide-MHC class Ⅱ interactions, and the presence of CD80, CD86 and IL-2. Plasmacytoid DCs can be also activated by TSLP and become efficient generators of Treg cells from thymocytes through an IL-10-dependent mechanism[43]. CD4$^+$ T cells triggered through the T cell receptor, but not resting CD4$^+$ T cells, respond to TSLP with robust proliferation and acquire sensitivity to low doses of IL-2[44].

INTESTINAL DCs UNDER STEADY-STATE CONDITIONS AND IN EXPERIMENTAL COLITIS

DCs in the non-inflamed gut

Cells with antigen-presenting function within the intestine and associated lymphoid tissue include macrophages, conventional CD11c-expressing DCs and plasmacytoid DCs. Macrophages belong to a family of tissue cells that includes Kupffer cells in the liver and glial cells in the brain and have predominantly innate immune functions, such as capturing and killing of microbes, scavenging of apoptotic and dead cells, and production of regulatory cytokines[45]. Macrophages are the most abundant population of phagocytic cells in the intestine. Distinctive characteristics have also been assigned to intestinal macrophages as compared with splenic macrophages or blood monocyte-derived macrophages. Early studies identified macrophages in the small and large intestine in the mouse, based on the expression of the F4/80 glycoprotein in association with CD11b[46]. LP macrophages are detected in juxtaposition to CD4$^+$ T cells and in close contact with the epithelium[23,47]. CD11b$^+$CD11c$^-$ macrophages are scattered throughout the villus-tip axis of small and large intestine, express immune regulatory molecules such as programmed death ligand 1 (PD-L1) and PD-L2, and secrete IL-10 but not IL-12p40 or IL-12p70. They are hyporesponsive to Toll-like receptor (TLR) stimulation, suppress the differentiation of Th1 and Th17 cells, and promote the differentiation of Treg cells[23]. Local macrophages may contribute to colitis development in IL-10$^{-/-}$ mice[48]. The pharmacological depletion of macrophages in this model of colonic inflammation ameliorated colitis, suggesting that IL-10 deficiency impedes the conditioning of macrophages, leading to macrophage-mediated destructive inflammatory responses.

Under steady-state conditions, the functional properties of the DC subpopulations vary according to their anatomical location. For instance, functional differences among DCs from PP, from MLN and from small intestinal and colonic LP have been reported. Within a single anatomical site, DCs can de distinguished and further subdivided according to their surface membrane phenotype. Under inflammatory conditions, DC recruitment to the intestine occurs, although it is presently unclear whether these DC populations are separate from DCs present in the steady-state or whether DCs arriving in the inflamed intestinal microenvironment acquire the ability to foster pro-inflammatory responses as a result of their exposure to pathogens and local inflammatory mediators.

LP DCs can be isolated in the absence of overt inflammatory stimuli and perform a tolerogenic function by constitutively migrating to the draining MLN, where they present antigen to T cells. The carriage of antigens from commensal bacterial strains to the MLN might be triggered by low-level production of pro-inflammatory cytokines. In this respect, the chemokine receptor CCR9 is crucial for the positioning of plasma cells[49] and plasmacytoid DCs[50] to the small intestine, suggesting that the chemokine CCL25/TECK may regulate DC homing during inflammatory processes. After their migration to the MLN, DCs interact with T and B cells and initiate immune responses aimed at maintaining a non-inflammatory state in the intestine. Intestinal DCs have been reported to promote the peripheral induction of FoxP3-expressing Treg cells from naïve T cells. Such Treg cells with specificity for commensal bacteria and dietary antigens may prevent naïve T cells from inducing pathological responses, thus complementing the pool of thymus-derived Treg cells. In this respect, gut-associated lymphoid tissue DCs may synthesize the vitamin A metabolite retinoic acid, that selectively induces CCR9 and $\alpha_4\beta_7$ integrin on $CD8^+$ T cells with gut tropism[51]. This phenomenon occurs more efficiently after oral as compared with intraperitoneal antigen administration, indicating differential DC targeting by the 2 immunization routes[51].

CD103 is the α chain of the $\alpha E\beta_7$ integrin expressed by most mouse and human intestinal lymphocytes and mediating lymphocyte adhesion to E-cadherin-expressing intestinal epithelial cells. CD103-expressing DCs may also be required to induce gut-tropic effector T cells in the MLN[51]. Interestingly, TGF-β plays a dominant role in CD103 induction on gut-tropic $CD8^+$ T cells, as shown in a mouse model of post-transplantation graft-*versus*-host disease (GVHD) with T-cell infiltration of the intestinal epithelium[52]. T cells from 2C T cell receptor-Tg mice that express a dominant negative TGF-β type Ⅱ receptor were incapable of upregulating CD103 upon migration into the intestinal epithelium[52]. In addition, CD103 expression on host-reactive $CD8^+$ T cells was causally related to the development of GVHD pathology and mortality. Although TGF-β activity is present locally within the intestinal milieu, this study did not exclude the possibility that CD8 effectors encounter TGF-β and upregulate CD103 expression before their entry into the intestinal epithelium. $CD103^{-/-}$ T cells migrate into the host intestine but are retained much less efficiently than wild-type T cells, indicating that CD103 expression may also contribute to T-cell accumulation in the gut[52]. In a T-cell transfer model of colitis, disease-inducing $CD4^+CD45RB^{high}$ T cells were shown to promote colitis development irrespective of their expression of CD103[53]. However, anti-CD103 antibodies abrogated the suppression of colitis mediated by Treg cells. Further experiments suggested that CD103 expression by Treg cells was not essential for their function, indicating the requirement for CD103 on non-T host cells for protection from colitis[53]. Of interest, ~50% of $CD11c^+$ DCs in the MLN co-expressed CD103 at high density, at variance with ~30% of splenic DCs. Sorted $CD103^+$ DCs activated the proliferation of allogeneic $CD4^+$ T cells to a similar extent compared with the $CD103^-$ counterpart but were potent inducers of CCR9 co-expression by day 4 of culture, suggesting their ability to impart gut tropism on T cells. In addition, $CD103^+$ DCs were inefficient at inducing IL-10 and IFN-γ production by T cells. Collectively, this study suggested that DC subsets that are primed in the immunosuppressive environment of the gut may be unable to drive the release of pro-inflammatory cytokines such as IFN-γ, thus preventing the development of unwanted effector responses to ingested antigens. Another report by the same investigators has shown that $CD103^+$ DCs isolated from the MLN may both induce *de novo* expression of Foxp3 in naïve T cells and maintain pre-existing $Foxp3^+$ cells[16]. The conversion of naïve T cells into Treg cells by CD103-expressing DCs was completely inhibited by anti-TGF-β antibodies, but further enhanced by exogenous TGF-β, so that provision of 1 ng/mL TGF-β to the T-cell/DC co-cultures translated into the expression of Foxp3 by ~50% of T cells[16]. Even the provision of high concentrations of TGF-β to $CD103^-$ DCs did not allow the generation of similar percentages of Treg cells to $CD103^+$ DCs, suggesting that $CD103^-$ DCs may lack an essential cofactor. Further experiments led the authors to identify retinoic acid as the cofactor for the TGF-β-driven conversion of Treg cells from naïve T cells. Compared with the $CD103^+$ DCs, $CD103^-$ DCs released higher amounts of pro-inflammatory cytokines (TNF-α, IL-6), and expressed higher levels of IL-23p19 and Tbet[16]. Collectively, this study showed the existence of functionally distinct DC populations in the MLN of normal mice, with apparent diverging functions. A companion paper by Sun *et al*[54] has shown, both in a lymphopenic mouse transfer model and in an immunologically complete setting, that retinoic acid released by LP DCs promotes Treg conversion in the presence of TGF-β. The LP DCs expressed a $CD8\alpha^-CD11c^+$ phenotype and displayed the morphologic features of conventional DCs, consisting of a stellar shape comparable to freshly isolated splenic DCs[54].

LP DCs

The extensive phenotypic and functional characterization of mouse LP DCs so far pursued has revealed a greater complexity than previously appreciated. The majority of

LP DCs express a $CD11b^+CD8\alpha^-$ phenotype, although $CD11b^-CD8\alpha^+$ and $CD11b^-CD8\alpha^-$ DCs have also been identified[55]. Treatment of mice with Flt3 ligand increases the proportion of LP DCs without significantly altering the relative proportion of DC subsets, thus allowing the purification of a higher DC number for detailed functional analyses. Using this approach, some authors have shown that LP DCs are not fully mature *in situ* but they can be induced to differentiate in response to appropriate stimuli[55]. LP DCs were also less efficient at stimulating OVA-specific T-cell proliferation *in vitro* when compared with splenic DCs, and mediated the development of tolerance when transferred to mice fed with OVA[55]. LP DCs exhibited a unique cytokine profile, consisting of low levels of IL-12p40 mRNA associated with constitutive IL-10 and type I IFN production[55]. A specialized subset of LP DCs with a $CD8\alpha^+$ phenotype has been identified in mice[30]. Gut-derived $CD8\alpha^+$ DCs secrete IFN-α and support antigen-specific suppression mediated by $CD4^+CD25^+$ Treg cells. Furthermore, $CD8\alpha^+$ DCs favor the differentiation of Tr1-like cells that release high quantities of IL-10, IL-4 and IFN-γ upon activation with plate-bound anti-CD3 antibodies[30]. The ability of $CD8\alpha^+$ DCs to induce Tr1-like cells was not affected by their exposure to maturation stimuli, as reported for other populations of maturation-resistant, tolerogenic DCs[31].

Other LP DC subsets identified in mice include $CD11b^-CD103^{hi}$ and $CD11b^+CD103^{-/low}$ DCs. LP DCs can be further subdivided into $CD11b^-CD103^{hi}CX_3CR1^-$ DCs and $CD11b^+$ DCs with different CX_3CR1 (fractalkine receptor) expression levels[56]. The $CD103^{hi}CX_3CR1^-CD11b^-$ LP DCs originate through a DC-committed non-monocytic intermediate from MDP, a differentiation pathway that is driven by Flt3L. Conversely, $CD103^-CX_3CR1^+CD11b^+$ LP DCs derive from $Ly6C^{hi}$ monocytes and their derivation involves an extensive, granulocyte-macrophage colony-stimulating factor (GM-CSF)-driven local expansion in the mucosa. Importantly, mice that were persistently or transiently depleted of LP DCs neither developed spontaneous intestinal inflammation nor were susceptible to colitis development. In contrast, mice that harbored predominantly $CD103^-CX_3CR1^+CD11b^+$ LP DCs developed severe colitis in response to a DSS challenge, as evaluated by colonoscopy and histological examination. This pro-inflammatory activity was dependent on TNF-α secretion with ensuing epithelial damage, and might also be regulated through IL-10/TGF-β production by the $CD103^{hi}CX_3CR1^-CD11b^-$ LP DC subset[23]. This study highlighted the importance of a critical balance between LP DC subsets for tissue repair and gut homeostasis.

PP DCs

PP are the primary sites for the induction of immune responses in the intestinal mucosa and are representative of lymphoid follicles present in diffuse mucosal tissues. DCs from PP possess a unique capacity to induce T-cell responses that regulate systemic immunity through the release of IL-4 and IL-10 and that provide help for IgA B-cell differentiation. It has been shown that PP DCs reside in different anatomical sites, with $CD11b^+CD8\alpha^-$ DCs being localized in the subepithelial dome, $CD11b^-CD8\alpha^+$ in the interfollicular regions and $CD11b^-CD8\alpha^-$ [double-negative (DN) DCs] in both compartments[57]. The DN DCs constitute approximately 30% of PP DCs, are interspersed within the follicle-associated epithelium, with processes extending to the luminal surface, and occasionally associated with M cells within the M-cell pocket[58]. DN DCs express intracellular MHC molecules, indicating their immaturity, and secrete IL-12p70, suggesting functional similarity to the lymphoid DC subset[58]. Collectively, these studies indicate that DN DCs should be able to induce Th1 differentiation, at variance with myeloid DC subsets that have been implicated in IL-10 release and in skewing the immune response towards a Th2 profile. It has been proposed that orally delivered antigens may initially encounter the myeloid and DN DCs located underneath or within the follicle-associated epithelium. As a result of feeding with low-dose antigen, antigen uptake by the DCs would not result in DC activation and migration but rather in the differentiation of Th2 or Th3 cells with regulatory properties. The T cells interacting with antigen would then secrete IFN-γ in the absence of activation signals by the DN DCs, thus becoming anergized. If soluble protein antigen is given at high dose, T-cell activation would occur in the PP and LP as a result of DC stimulation and migration to the interfollicular regions or the MLN. In this scenario, IL-10 produced by the myeloid DCs may serve to control detrimental inflammation induced by microbial antigens, whereas DN DCs and lymphoid DCs may be acting as the primary source of IL-12 for the induction of Th1 responses[58].

DCs in experimental colitis

The availability of mice expressing the diphtheria toxin receptor under the control of the *Cd11c* promoter has allowed the selective depletion of DCs and the study of DC role in the development of intestinal inflammation. DC ablation has been correlated with the amelioration of DSS-induced colitis[59]. DSS-stimulated bone marrow-derived DCs release high quantities of proinflammatory cytokines and chemoattractants *in vitro*. Furthermore, DC adoptive transfer exacerbated disease manifestations, whereas DC ablation attenuated disease severity as shown by histological examination of tissue sections. However, DC activation with TLR9 ligands before colitis induction with DSS exacerbated disease manifestations. Since DSS injures the colonic epithelium, it is conceivable that, at least in this model, DCs exerted protective effects through stimulating repair of colonic epithelial cell layers rather than modulating the immune response.

There is evidence that DCs may play both protective and detrimental roles in intestinal pathology. In DSS colitis, an experimental model resembling acute colitis, DC ablation during DSS administration ameliorated disease manifestations[60]. Conversely, colitis was exacerbated if DCs were ablated before DSS treatment, suggesting that DCs are protective in initial phases of colitis but play a pathogenic role during the disease course[60].

In a T-cell transfer model of colitis induced by CD45RB^{hi}CD4$^+$ T cells, transplanted T cells formed aggregates with sub-epithelial CD11c$^+$ DCs in the MLN[61]. Blocking OX40-OX40L interactions prevented the development of colitis. DC activation *via* CD40 has been reported to cause colitis in the absence of T and B cells and through a cytokine-dependent mechanism[62].

Intestinal inflammation is correlated with significant changes in the cellular composition of the colonic LP. Gut inflammation in mice is accompanied by a marked infiltration of CD11c$^+$ DCs within the LP[63]. From a phenotypic standpoint, these DCs express high levels of CD80 and resemble mature activated DCs, while secreting low levels of IL-10 and IFN-α. Of interest, CD103$^+$ DCs were dramatically reduced in the LP of colitic mice but were detectable in the spleen, suggesting that intestinal CD103$^+$ DCs may migrate to lymphoid organs during inflammation.

The observation that IL-10-deficient and TGF-β-deficient mice develop spontaneous colitis point to IL-10 and TGF-β as important determinants of DC function in the gut. In a T-cell transfer model of colitis, Treg production of IL-10 was dispensable for disease suppression but IL-10 secreted by LP CD11b$^+$ macrophages was crucial to maintain FoxP3 expression in Treg cells[28]. It is conceivable that intestinal bacteria are a fundamental trigger of IL-10 production by LP macrophages through the activation of TLR signaling. It is presently unknown whether IL-10 signals are also required to maintain FoxP3 expression in Treg cells from other organs during inflammation[29]. Serum IL-10 is reportedly normal in patients with IBD[64]. However, LP mononuclear cells are impaired in the ability to release and respond to IL-10[64], suggesting that IL-10 provision might be beneficial in human IBD through effects on the DC compartment[65].

DCs IN HUMAN IBD

DCs accumulate at sites of inflammation in patients with IBD, whereas both myeloid DC and pDC populations are depleted in the peripheral blood of patients with active disease. DC recruitment to the gut may be the result of an increased expression of chemokines such as CCL20 or of addressins, such as mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1). CCL20 is a nuclear factor-κB-regulated molecule that mediates the CCR6-dependent recruitment of DCs and T cells to mucosal surfaces. CCL20 has been detected at increased levels in the mucosal epithelium covering lymphoid follicles in patients with both types of IBD, in proximity to CCR6-expressing cell types, such as langerin$^+$ DC, B cells and memory T cells[66]. Phenotypically heterogeneous populations of DCs have been identified in colonic tissues and MLN from patients with IBD[67]. One population consists of immature DCs expressing DC-specific intercellular adhesion molecule-grabbing non-integrin (SIGN) and mainly located at antigen-capturing sites in the mucosa and medullary cords. A second population expresses CD141 and has a similar localization as that of DC-SIGN$^+$ DCs. The third DC subset consists of mature DCs expressing S-100 and CD83 and is located in the T-cell areas in both the colonic lymphoid follicles and the MLN. In contrast, pDCs were hardly detected in the colon and MLN. The fraction of circulating DC precursors has been found to correlate with established IBD activity indices[68]. Specifically, higher percentages of pDC and myeloid DCs were measured during disease remission compared with acute flares, suggesting DC migration to secondary lymphoid organs. In line with this hypothesis, DC precursors from patients with IBD expressed $\alpha_4\beta_7$, a gut-homing integrin marker and receptor for MAdCAM-1 also detected on LP T cells[69]. Importantly, immature DCs are significantly reduced in active IBD, indicating that potentially tolerogenic DC subsets may be defective during disease reactivation.

M-DC8$^+$ DCs have been detected in the subepithelial dome of ileal PP from 3 patients with untreated CD[70]. In one of these patients, an ileal biopsy performed 6 mo after glucocorticoid-induced clinical remission documented the complete disappearance of M-DC8$^+$ DCs from the ileal mucosa. The observation that M-DC8$^+$ DCs secrete large amounts of TNF-α but not IL-10 upon stimulation with lipopolysaccharide (LPS) suggests that these cell types might contribute to the pathogenesis of IBD[70].

Colonic CD11c$^+$ DCs from patients with either CD or UC express higher levels of TLR2 (interacting with peptidoglycan and bacterial lipoproteins), TLR4 (a receptor for LPS) and CD40 compared with non-inflamed CD tissues and tissues from healthy controls[71]. This may lead to enhanced recognition of bacterial products and an increased response to them. Importantly, treatment with TNF-α blocking antibodies translated into the downregulation of CD40 expression on DCs, irrespective of resolution of inflammation at the tissue level. Also, production of IL-6 and IL-12 at the single-cell level was increased in DCs from patients with CD but not with UC compared with healthy controls.

Although the DC abnormalities documented in UC generally resemble those evidenced in CD, differences may exist when comparing these 2 major forms of IBD. Epstein-Barr virus-induced gene 3 (EBI3) encodes a secreted protein that shares 27% amino acid sequence identity with IL-12p40. EBI3 can substitute for p40 to form a heterodimer with IL-12p35, and is an IL-27 subunit[72]. EBI3 expression is upregulated by macrophage/DC-like cells within the LP of patients with active UC but not CD[73]. These data are consistent with a scenario in which EBI3 opposes the IL-12p40/p35 heterodimer and downregulates the cytotoxicity promoted by IL-12. In addition, this study reinforced the view that macrophages and DCs serve more than one role in the pathogenesis of IBD, being either protective or detrimental.

DCs AS TOOLS AND TARGETS FOR THERAPY IN IBD

Different approaches have been proposed to restore and/or enhance the tolerogenic properties of DCs, including *in vitro* treatment with growth factors and use of drugs that target DC number and/or function (Figure 2)[74,75].

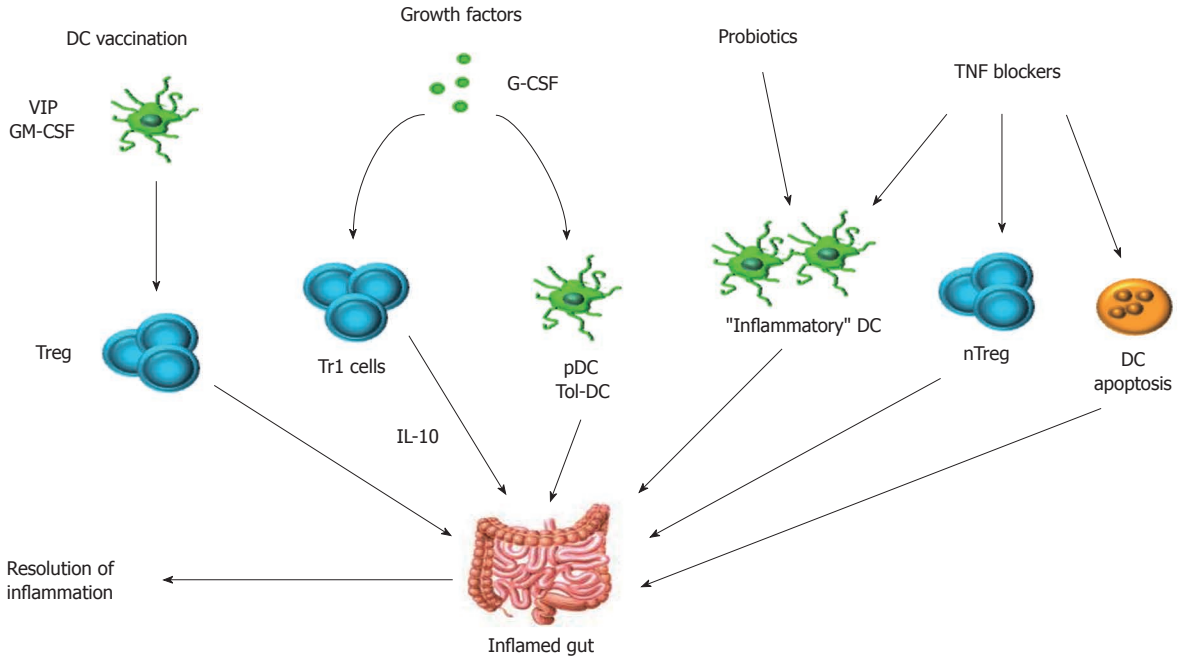

Figure 2 Potential strategies to modulate dendritic cell functionality in human inflammatory bowel disease. *In vitro* differentiated tolerogenic dendritic cell (DC) have been administered to mice with inflammatory/autoimmune disorders. Vasoactive intestinal peptide (VIP) has a unique ability to skew DC function towards a tolerogenic profile and has been used to vaccinate animals with colitis, rheumatoid arthritis and post-transplantation graft-*versus*-host disease[76,77]. Selected growth factors have shown to modulate immune reactivity *in vivo*. For instance, granulocyte colony-stimulating factor (G-CSF) has been successfully given to patients with Crohn's disease, leading to accumulation of pDC in the lamina propria and increase in IL-10 production, with favorable repercussions on disease manifestations[89]. GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL: Interleukin; TNF: Tumor necrosis factor.

Adoptive transfer of cytokine-modulated DCs

DCs have been targeted in animal models of intestinal inflammation. Regulatory DCs differentiated with vasoactive intestinal peptide (VIP) and GM-CSF (DC-VIP) have been transferred to BALB/c mice with colitis induced by trinitrobenzene sulfonic acid (TNBS), a Th1-mediated disease requiring T-cell activation with subsequent macrophage recruitment and activation[76]. Mice received the DC preparations either 8 h after TNBS instillation or 6 d after colitis induction in order to assess their therapeutic effects both on colitis induction and on established disease. DC infusion ameliorated disease severity and histopathology, being associated with inhibited Th1 responses and with the *in vivo* differentiation of IL-10-producing Treg cells. DC-VIP decreased the production of proinflammatory cytokines both systemically and locally, and deactivated spleen macrophages, blunting the *in vitro* production of TNF-α and IL-12 in response to LPS challenge. Importantly, DC-VIP augmented the number of TGF-β/IL-10-secreting $CD4^+$ T cells within LP mononuclear cells cultured in the presence of colonic proteins extracted from colitic mice. Also, $CD4^+$ T cells obtained by the MLN of DC-treated mice suppressed proliferation and IL-2 production by autoreactive $CD4^+$ T cells in response to colonic proteins. These $CD4^+$ T cells were also capable of reversing the body weight loss which is characteristic of TNBS-induced colitis when transferred to colitic mice, suggesting the acquisition of a potent regulatory activity after their *in vivo* encounter with DC-VIP. Finally, the therapeutic effect of $CD4^+$ T cells was dependent on TGF-β and IL-10 production, being reversed by *in vivo* blocking of these cytokines. This elegant study suggests that *in vitro* conditioning of DC preparations with VIP and self-antigens might be pursued as therapeutic strategy in colitis and possibly other inflammatory disorders[77,78], also to minimize patients' dependence on non-specific immune suppressive drugs currently in use for human IBD.

TGF-β1 gene-modified immature DCs with enhanced tolerogenicity undergo efficient transport to inflamed colonic tissues and delay the progression of murine IBD induced by DSS[79]. DC injections in mice with established colitis alleviated weight loss and reduced intestinal bleeding, translating into a lower disease activity index compared with control DC or untreated mice. DC vaccination was associated with increased Treg numbers in the MLN and with increased TGF-β1 levels in mouse colon tissues[79].

Probiotic bacteria

Probiotics, mainly belonging to the lactic acid bacteria (LAB) family, exert beneficial effects in human or animal health and are presently considered as peace-keepers in the gut[40,80]. The regular intake of probiotic bacteria may contribute to immune homeostasis by altering microbial balance or by interacting with intestinal immune cells. Dysbiosis, namely, an imbalance between pro-inflammatory and anti-inflammatory bacteria in favor of the former, may have a causative role in patients with IBD. Probiotics have been tested both in animal models of colitis and in patients with IBD. The potential mechanisms of action of probiotic bacteria include their interaction with

TLR and DCs in the gut. The demonstration of anti-inflammatory effects after systemic administration of probiotics suggests that regulatory cell populations may be induced distant from the site of inflammation[81].

Importantly, probiotics may present strain-specific *in vitro* immune modulating actions, that are strictly correlated with their *in vivo* anti-inflammatory effects. For instance, *L. salivarius* Ls33 and *L. rhamnosus* Lr32 possess high immunoregulatory capacities and efficiently protect from murine TNBS-induced colitis, at variance with other strains such as *L. acidophilus* NCFM and *L. lactis* MG1363 that exhibit an opposite immunological profile[82]. The protective effect of probiotic-treated DCs was attributed to a downregulation of proinflammatory mediators such as IL-12 and IL-17, paralleled with an acute overexpression of IFN-γ and IDO[82]. Of interest, pre-formed naturally occurring Treg cells were required for the protective effect of probiotic-treated DCs, as shown by experiments with an anti-CD25 rat monoclonal antibody.

The probiotic mixture designated IRT5 contains 5 different probiotic strains. IRT5 has been shown to induce T-cell and B-cell hyporesponsiveness when administered for 20 d to mice by the oral route[83]. Even more intriguingly, IRT5 increased FoxP3 expression in MLN as a result of the enhanced conversion of naïve T cells into Treg cells and the augmentation of the suppression function of pre-existing natural Treg cells. These effects were mediated through the promotion of DC tolerogenic activity, with high expression of IL-10, TGF-β, IDO and COX-2 mRNA. IRT5 retarded the progression of TNBS-induced colitis and was also efficacious in other immune-mediated disorders, such as atopic dermatitis and collagen-induced arthritis. Both the atopic ear and the inflamed colon of IRT5-treated mice were enriched with FoxP3-expressing Treg cells, likely as a result of increased tissue levels of CCL1 and CCL22, chemokines involved in Treg attraction.

The probiotic mixture VSL#3, which contains 8 different bacterial strains and is clinically beneficial in human IBD and pouchitis, has been reported to downregulate IL-12 and upregulate IL-10 production by human blood and colonic LP DCs in a dose-dependent fashion[84]. This change in DC functional polarization translated into the inhibition of *in vitro* generation of Th1 cells from allogeneic $CD4^+$ T cells.

A comprehensive analysis of previously published studies detailing the activity of different probiotics in animal models of colitis suggests that the colitis model used may affect the results[85]. An interesting study has evaluated the ability of 3 *Lactobacilli* strains (*plantarum*, *LGG* and *paracasei* B21060) to activate DCs either directly or indirectly through epithelial cells. While inducing similar degrees of DC phenotypic maturation, the different strains elicited differential cytokine release, with *L. paracasei* inducing lower levels of IL-12p70, TNF-α and IL-10. The lactobacilli also affected epithelial cell function, and supernatants of *L. paracasei*-treated epithelial cells drastically reduced the ability of DCs to activate T cells and drive their polarization towards a Th1 phenotype. Finally, the *in vitro* activity of probiotics was predictive of their *in vivo* efficacy in an acute model of colitis. Taken together, these studies indicate that probiotics interact both with immune cells and with non-immune cells and that the clinical use of individual bacterial strains should be proposed and recommended only after taking into account *in vitro* immunostimulatory or immunoregulatory activity.

Immune modulating drugs

Granulocyte-CSF (G-CSF) has remarkable immune modulating activities[86]. Indeed, G-CSF mobilizes DC2, differentiates tolerogenic DCs *in vitro* through IL-10 and IFN-α, and polarizes naïve T cells to a Tr1-like functional profile[31,87,88]. G-CSF has been administered to patients with CD in order to modulate immune reactivity and induce potential clinical benefit[89]. Nine patients with active CD received subcutaneous G-CSF for 28 d at 5 μg/kg of body weight. Six patients reported improvement in the CD activity index (CDAI) and achieved either a clinical response (4 patients) or remission (2 patients). The 3 non-responding patients had a longer duration of disease, had had bowel resections and one was the only CD patient with active fistulae. In responder patients, IL-10 production by isolated memory $CD4^+$ T cells was significantly higher at the end of G-CSF treatment compared with non-responders. Conversely, IFN-γ production in post-G-CSF peripheral blood samples was significantly higher in non-responders. G-CSF also affected the relative proportion of circulating myeloid DCs and pDCs, inducing a decrease in the myeloid DC-to-pDC ratio in responding patients. Notably, 4 patients in the responder group showed an increase in LP $CD123^+$ DCs[89]. In sharp contrast, accumulation of $CD123^+$ pDCs could not be evidenced in the LP of non-responders. Finally, the percentage of FoxP3-expressing cells within LP $CD25^+$ cells decreased significantly in non-responders at the end of treatment. In line with this, the fraction of $CD25^{hi}FoxP3^+$ cells increased in the LP of responding patients at the end of treatment, although these differences failed to achieve statistical significance. Collectively, this study provided proof-of-principle in favor of IL-10-mediated immune regulation by G-CSF in patients with IBD and suggested that treatment with this cytokine may translate into disease control. G-CSF at 3 μg/kg of body weight was also highly effective at controlling an UC-like syndrome in a 23-year-old patient with glycogen storage disease Ib[90]. G-CSF therapy was maintained for 16 years, with good control of gastrointestinal symptoms and dramatic improvement of colon histology.

There is evidence that TNF-α antagonism translates into changes in DC function. Although this has been primarily shown in patients with rheumatoid arthritis, it is likely that modifications of DC functions by TNF blockers may also impact on the clinical manifestations of IBD. Both etanercept and adalimumab were shown to downregulate CD83, CD80 and CD86 expression on monocyte-derived DCs and to reduce their T-cell stimula-

tory capacity[91]. Anti-TNF-treated DCs polarize T-cell responses *in vitro* and favor T-cell release of IL-10, IL-4 and IL-17. Although no correlation was found between the clinical response to TNF blockade and the functional modulation of DCs *in vitro*, DCs derived from patients with rheumatoid arthritis given TNF blocking agents enhanced T-cell production of IL-10, while decreasing the release of IL-4, IL-17 and IFN-γ. Infliximab may also suppress the antigen-presenting capacity of DCs derived from patients with psoriasis by reducing the expression of CD1a and costimulatory molecules, an effect that is not reversed by LPS[92].

Mesenchymal stromal cells

Mesenchymal stromal cells (MSC) are cells endowed with multi-lineage differentiation capacity and have been isolated from bone marrow, adipose tissue, amniotic fluid, placenta and umbilical cord blood. MSC affect both innate and adaptive immune responses and have reduced immunogenicity, thus being a promising therapeutic tool for inflammatory, autoimmune and degenerative diseases[93]. Adipose tissue-derived MSC have been shown to ameliorate experimental colitis through the promotion of IL-10 release with subsequent inhibition of activated macrophages and differentiation of Treg cells[94]. Importantly, the intrafistular injection of *in vitro*-expanded MSC (median number: 64×10^6 for each patient) has resulted into sustained complete closure of fistula tracks, reduction of perianal disease activity index, and with rectal mucosal healing in 10 patients with CD[95]. Intriguingly, the percentage of mucosal and circulating Treg cells significantly increased during treatment and remained stable until completion of the 12-mo follow-up period. T cells isolated from the inflamed mucosal areas released higher amounts of IL-10 when co-cultured with MSC *in vitro*. Based on previously published data on the ability of IL-10 to skew DC differentiation towards a tolerogenic profile[96,97], it is tempting to speculate that MSC therapy may target pro-inflammatory DCs *in vivo*, through the promotion of IL-10 release by colitogenic T cells.

Based on the experience reported in patients with GVHD[98], MSC have also been infused intravenously, at a dose of $1\text{-}2 \times 10^6$ cells/kg of body weight, in 10 patients with chronic active CD, refractory to all currently available medical therapeutic options[99]. Although MSC-based therapy did not induce clinical remissions as defined by a CDAI < 150, reductions of 70 points in CDAI were recorded in 3 patients. The biological effects of MSC intravenous infusion included a trend towards higher percentages of $CD4^+CD127^+$ *bona fide* Treg cells. *In vitro*, patient-derived MSC inhibited the proliferation of autologous peripheral blood mononuclear cells and decreased their production of TNF-α. Collectively, the studies published so far demonstrate that MSC from patients with CD can be expanded *in vitro* and may induce favorable therapeutic effects *in vivo*, including differentiation of Treg cells, and possibly functional inhibition of DCs within the inflamed gut.

CONCLUSION

It is now clear that DC activation is a contributing factor in generation of IBD, as indicated both by mouse models of gut inflammation and by human disease. The recent advances in the phenotypic and functional characterization of DC populations in humans have unveiled a remarkable and previously unappreciated heterogeneity within the DC compartment but have also led to the identification of potential targets for therapeutic manipulation. A thorough understanding and knowledge of DC subsets and functionality in humans is a prerequisite for delivering interventions aimed at correcting DC malfunctioning. Several other cell types with APC function cooperate to ensure appropriate immune responses in the gut. They include IEC, basophils, MSC and other non-immune cells[10,41]. Theoretical strategies to interfere with DC activity include vaccination with gene-modified DCs or cytokine-treated DCs to restore tolerance, growth factor administration, therapy with DC-modulating drugs, and use of probiotics. The impact of TNF blocking antibodies on DC functions needs to be further investigated. Some of these approaches have been successfully applied to animal models of gut inflammation and other autoimmune/inflammatory disorders such as multiple sclerosis and arthritis. In this respect, "tolerogenic vaccination" with cytokine-modulated DCs may hold promise for the treatment of intestinal inflammation. However, there is a theoretical concern that tolerogenic DCs suppress beneficial anti-infective and anti-tumor responses, in addition to unwanted immune reactivity. These issues must be carefully addressed before this approach is translated into the clinic. Studies in murine GVHD are somehow reassuring, having clearly indicated that the injection of cytokine-treated DCs preserves $CD8^+$-mediated cytotoxic responses against leukemia while blunting GVH reactivity[100,101]. Finally, the patient categories that may benefit from DC-based therapeutic approaches need to be identified. It should be emphasized that other cell-based interventions such as the intravenous infusion of MSC have not induced any clinical remission in severe refractory CD[99]. Conceivably, patients with IBD should be offered DC-centered treatments earlier in the disease course, following patient profiling and stratification on the basis of molecular predictors for complicated disease (genetic markers such as NOD2 homozygous or compound heterozygous, and anti-microbial antibodies) as well as clinical features at diagnosis[102]. There is evidence from both pediatric and adult IBD that treatment of short-duration CD with TNF antagonists is associated with better response and remission rates[103,104]. Whether DC-based approaches have the potential to slow disease progression and alter the natural history of IBD will hopefully be determined in the near future.

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Spleen: A new role for an old player?

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Abstract

The spleen could be considered a neglected organ. To date, it has been deemed an ancillary organ in portal hypertension or an organ localization in lymphoproliferative diseases, even though it has had significant attention in infectious diseases for some time. Now, it is thought to be central in regulating the immune system, a metabolic asset and involved in endocrine function with regard to nonalcoholic fatty liver disease. The main mechanisms involved in this complex network will be critically discussed in this article.

Key words: Endocrine function; Immune system; Metabolic asset; Nonalcoholic fatty liver disease; Spleen

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SCENARIO

Due to the view that the "spleen is useless", research on this organ has lagged behind that of other organs. Since 1952, when King and Schumacker reported overwhelming post-splenectomy infection[1], there has been a growing recognition of the importance of the spleen in the human body. On the other hand, physicians often encounter spleen enlargement, i.e., splenomegaly which is almost always a consequence of other disorders. Hypersplenism is a secondary process that can arise from splenomegaly of almost any cause. In recent years, following in-depth studies of spleen organization and structure, cell function, secretion and innervations, a better understanding of the function of the spleen has been gained. It was initially accepted that the spleen not only filters blood but is an important regulation center of the body's immune-metabolic-endocrine network. However, a number of questions have arisen: Is the spleen a player or a bystander, and what are the roles of some cytokines, adipokines/growth factors and neurotransmitters in this complex mechanism? In other words, what is the contribution of the spleen to non-alcoholic fatty liver disease, is it a further expression of Metabolic Syndrome[2]?

ANATOMY

The spleen, in healthy adult humans, is approximately 11 cm (4.3 in) in length. It usually weighs 150 g (5.3 oz)

Table 1 Histology of spleen

Anatomy	Composition
Red pulp	"Sinusoids" which are filled with blood "Splenic cords" of reticular fibers "Marginal zone" bordering on white pulp
White pulp	Nodules, called Malpighian corpuscles, containing "lymphoid follicles" rich in B-lymphocytes "periarteriolar lymphoid sheaths", plenty of T-lymphocytes

Table 2 Function of the spleen

- Red pulp
  - Extramedullary hematopoiesis if required
  - Facilitating an environment wherein erythrocytes rid themselves of solid waste material
  - Blood filter for foreign material and damaged and senescent blood cells
  - Storage site for iron, erythrocytes, platelets, plasmablasts and plasma cells
  - Rapid release of antigen-specific antibodies into the circulation produced by red pulp plasma cells
  - Defense against bacteria using iron metabolism by its macrophages
- White pulp
  - T cell zone (periarterial lymphatic sheath) and B cell zone (follicles)
    - Storage site for B and T lymphocytes
    - Development of B and T lymphocytes upon antigenic challenge
    - Release of immunoglobulins upon antigenic challenge by B lymphocytes
    - Production of immune mediators involved in clearance of bacteria such as complement, opsonins, properdin and tuftsin
  - Marginal zone
    - Phagocytosis of circulating microorganisms and immune complexes by MZ macrophages
    - Development of marginal zone B lymphocytes upon TI-2 antigenic challenge
    - Blood trafficking of B and T lymphocytes
    - Release of immunoglobulins upon antigenic challenge by splenic B lymphocytes

and lies beneath the 9th to the 12th thoracic ribs. The spleen is an intraperitoneal organ with a smooth serosal surface and is attached to the retro-peritoneum by fatty ligaments that also contain its vascular supply. The splenic surfaces are described relative to their locations and are termed the diaphragmatic (phrenic) and visceral surfaces. The visceral surface is divided into an anterior or gastric ridge and a posterior or renal portion. The splenic hilum is directed antero-medially. The splenic artery and vein emerge from the splenic hilum in the form of six or more branches; the splenic artery is remarkable for its large size and tortuosity. The splenic artery is slightly superior to the vein. The spleen is part of the lymphatic system. The germinal centers are supplied by arterioles called penicilliary radicles. The spleen is derived from mesenchymal tissue (Table 1).

SPLEEN FUNCTION

Immune function (through phagocytosis, but also through T cell-mediated immunity and B cell-mediated humoral immunity) is the most important function of the spleen (Table 2). A current paradigm states that monocytes circulate freely and patrol blood vessels but differentiate irreversibly into dendritic cells (DCs) or macrophages upon tissue entry. Recently, it was shown that bona fide undifferentiated monocytes reside in the spleen and outnumber their equivalents in the circulation. The reservoir monocytes assemble in clusters in the cords of the subcapsular red pulp and are distinct from macrophages and DCs. In response to ischemic myocardial injury, splenic monocytes increase their motility, exit the spleen en masse, accumulate in injured tissue, and participate in wound healing. These observations uncover a role for the spleen as a site for storage and rapid deployment of monocytes and identify splenic monocytes as a resource that the body exploits to regulate inflammation[3]. The spleen plays a complex role in tumor immunity, which changes in the different periods of cancer[4]. The initiation of T-cell immune responses requires professional antigen-presenting cells. Emerging data point towards an important role for macrophages (Mphi) in the priming of naïve T cells. In this study we analyzed the efficiency and the mechanisms by which Mphi derived from spleen (Sp-Mphi) or bone marrow (BM-Mphi) present lymphocytic choriomeningitis virus antigens to epitope-specific T cells. It was demonstrated that because of phagosomal maturation, Sp-Mphi downregulate their ability to cross-present cell-associated, but not soluble, antigens, as they are further differentiated in culture without altering their capacity to directly present virus antigens after infection. Authors proposed that Sp-Mphi are extremely efficient at direct and cross-presentation. However, if these cells undergo further M-CSF-dependent maturation, they will adapt to be more scavenger and phagocytic and concurrently reduce their cross-presenting capacity. Accordingly, Sp-Mphi can have an important role in regulating T-cell responses through cross-presentation depending on their differentiation state[5]. The spleen is one of the centers of activity of the reticulo-endothelial system and can be considered analogous to a large lymph node, as its absence leads to a predisposition toward certain infections. Other functions of the spleen are the production of opsonins[6], properdin[7], and tuftsin[8], as well as the creation of red blood cells. While the bone marrow is the primary site of hematopoiesis in the adult, the spleen has important hematopoietic functions up until the fifth month of gestation. After birth, erythropoietic functions cease, except in some hematologic disorders. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes and, as such, remains a hematopoietic organ. In horses, roughly 30% of red blood cells are stored in the spleen. These red blood cells can be released when needed[9]. In humans, the spleen does not act as a reservoir for red blood cells but it can store platelets in case of an emergency. Platelets are major carriers of serotonin (5-HT) in the blood[10]. 5-HT has been reported to modulate T cell and natural killer (NK) cell proliferation. This aspect

was clearly elucidated by studies on cultures of mouse and rat spleen cells. Results showed that serotonin up-regulates mitogen-stimulated B lymphocyte proliferation through 5-HT1A receptors, thus providing an important link between this neurotransmitter and the immune system[11]. Another study using RT-PCR methods to examine the mRNA expression of 5-HT receptors in the cells of lymphoid tissues of the rat (*ex vivo* isolated spleen, thymus, and peripheral blood lymphocytes) confirmed 5-HT receptors (5-HT1B, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT6, and 5-HT7) in mitogen-stimulated spleen cells. In contrast, 5-HT1A, 5-HT1D, 5-HT2C, 5-HT4, 5-HT5A, and 5-HT5B mRNAs were not detected in any of the examined cell populations[12]. The role of platelets and serotonin was recently highlighted as novel contributors in the mechanisms of liver regeneration after partial hepatectomy[13]. Furthermore, platelets are attracted to the liver following systemic inflammatory stimuli[14].

ASSESSMENT OF SPLEEN FUNCTION

Patients with impaired splenic function are difficult to identify[15]. IgM memory B cells are a potential parameter for assessing splenic function[16]; however, more studies are necessary for its validation. The detection of Howell-Jolly bodies does not reflect splenic function accurately[17], whereas determining the percentage of pitted erythrocytes is a well-evaluated method and seems a good first-line investigation for assessing splenic function[18]. When assessing spleen function, (99m)Tc-labeled, heat-altered, autologous erythrocyte scintigraphy with multimodality single photon emission computed tomography (CT)-technology is the best approach, as all facets of splenic function are evaluated[19].

THE BLOOD-SPLEEN-BARRIER

The blood-spleen-barrier (BSB) is a barrier composed of macrophages and endothelial cells of the marginal sinus. Their basement membrane is composed of reticular tissue (reticular cells and reticular fibers) and collagen fibers. It can regulate splenic filtration and its intrasplenic consequences including blood flow, cell homing and migration, hematopoietic and immune responses, and clearance of infectious organisms. Here, the cells of the barrier can trap circulating infectious organisms and monocytes on their cell surfaces, clearing them from the blood and providing a selective environment for monocyte differentiation into macrophages and subsequent phagocytosis of the microorganisms. The interactions between the circulating lymphocytes and the macrophages may regulate the entry of lymphocytes into the white pulp. Thus, the functions of the BSB are to filter antigens, to keep the microenvironment of the white pulp stable, and to present antigen information to white pulp through the effects of the mechanical barrier, which depends on the connection between cells and the phagocytosis of macrophages. Compared to other biological barriers in the human body, such as the blood-brain barrier and the blood-thymus barrier, the structure of the BSB is relatively loose without the tight junction between cells; however, the BSB has more constituents and ability to stop and phagocytize more xenobiotic materials than other barriers[20,21]. As compared to the normal spleen, the density of macrophages in the portal hypertension (PH) spleen was decreased, but the macrophages were mainly located in the marginal zone and distributed around the splenic corpuscle, with many villi and pseudopodium-like protrusions on the cell surface. The accrementition of collagen fibers was obvious around the splenic corpuscle and central artery. The increased reticulate fibers encircled the splenic corpuscle with more connection between the fibers. The vascular endothelial cells were in diffused distribution, without any regionality in PH spleen, but the vessel with enlarged lumina increased in red pulp[22].

THE OLD PLAYER

Except for malaria and genetic metabolic diseases (e.g., Gaucher disease), splenic enlargement can be caused by diseases such as PH, lymphoma and leukemia. PH is considered the most common cause of splenomegaly in Western countries. Previous findings showed that splenomegaly is secondary to PH with associated liver cirrhosis. In fact, the increase in the width of the celiac axis in cirrhotic patients with PH was closely related to the increased width of the splenic artery which in turn was related to enlargement of the spleen, and increased blood flow through the spleen. The increased size of the spleen is due partly to venous engorgement and partly to reticulo-enthothelial cell hyperplasia, and is accompanied by an increased total blood supply, although flow per 100 g tissue is often reduced. An increase in blood flow can not occur without dilatation of the entire splenic arterial tree (Pouseille's law) and in keeping with this are the studies of[23], using injected spleen casts, which showed an increased number of peripheral arterioles of 100 mm diameter. In addition to local factors, circulating vasodilator substances may sometimes have an additional effect. The cardiac output is often raised, the blood flow through skin and muscle is also increased, and there is evidence of an increased number of small peripheral arterioles in the lungs. All these findings show that a generalized vasodilatation may occur in some patients with cirrhosis. Increasing tortuosity of peripheral vessels is a well-known accompaniment of aging, but in cirrhotic patients there was no relationship between tortuosity and either age or length of history. The increased length and tortuosity of the splenic artery is probably a secondary effect of arterial dilatation, although there was no direct relationship either to total splenic blood flow or size of the spleen. This was particularly striking in patients with tropical splenomegaly who had enormously enlarged spleens, increased blood flows, but splenic arteries of normal length[24]. Currently, there is controversy on the immune function of enlarged spleen in patients with PH and hypersplenism. As compared to the normal spleen, the density of macrophages in the PH spleen was decreased, but the macrophages were mainly located in the marginal zone and distrib-

uted around the splenic corpuscle, with many villi and pseudopodium-like protrusions on the cell surface. The "accrementition", i.e., growth by addition of similar collagen fibers, was obvious around the splenic corpuscle and central artery. The increased reticulate fibers encircled the splenic corpuscle with more connection between the fibers. The vascular endothelial cells were in diffused distribution, without any regionality in PH spleen, but the vessel with enlarged lumina increased in red pulp. Those morphological changes of the BSB may be one of the pathological fundaments for the abnormality of immune function and the increased destruction of blood cells located in the spleens of patients with PH[22].

Lymphoma is the commonest malignant tumor of the spleen. Although a number of lymphomas and leukemias can involve the spleen and may present clinically with splenomegaly, only the B cell disorders SMZL and hepato-splenic γ/δ T cell lymphoma can be considered true primary splenic lymphomas[25]. It is important to detect splenic involvement because it can alter the management and for this reason Gadolinium-enhanced sequences are sensitive.

LIVER CIRRHOSIS, SPONTANEOUS SPLENORENAL SHUNT AND HYPERSPLENISM

Although significant advances are expected to be made in the assessment of PH-related complications, the prognostic role of spleno-renal shunts (SRS) has not been fully explored so far. Clarifying this aspect could help tackle the life-threatening events occurring in patients suffering from liver cirrhosis. A recent study on SRS[26] focused on the role of the spleen and showed a strict link between spleen size and the presence of SRS and the development of hepatocarcinoma.

An up-to-date study evaluated the effect of liver transplantation on spleen size, spontaneous SRS function, and platelet counts in patients with hypersplenism in 462 adult patients who underwent orthotopic liver transplantations (OLTX). Of these patients, CT or magnetic resonance imaging information was reviewed retrospectively in 55 patients. Volume measurements of the spleen and liver, spleen/liver volume ratio (S/L ratio), presence and size of SRS, and platelet counts were evaluated before and after OLTX. Spleen size and SRS size were significantly smaller after OLTX. However, patients with postoperative S/L ratio > 0.35 tended to have lower platelet counts after OLTX[27].

THE NOVEL PLAYER

Nonalcoholic fatty liver disease (NAFLD), the most common cause of steatosis, is associated with obesity, mainly visceral and insulin resistance. In the presence of more severe risk factors (major obesity, diabetes mellitus, metabolic syndrome, MS), simple hepatic steatosis or fatty liver (FL) may be complicated by liver inflammation (nonalcoholic steatohepatitis or NASH). NASH can lead to perisinusoidal fibrosis and cirrhosis. Fat-laden hepatocytes are swollen, and in steatohepatitis, further swelling occurs due to hydropic change (ballooning) of hepatocytes to cause sinusoidal distortion, as visualized by *in vivo* microscopy, reducing intrasinusoidal volume and microvascular blood flow. Involvement of other cell types (sinusoidal endothelial cells, Kupffer cells, stellate cells) and recruitment of inflammatory cells and platelets lead to dysregulation of microvascular blood flow. In animal models, the net effect of such changes is a marked reduction of sinusoidal space (approximately 50% of control), and a decrease in the number of normally perfused sinusoids. Such microvascular damage could accentuate further liver injury and disease progression in NASH. Hepatic steatosis is also exquisitely sensitive to ischemia-reperfusion injury, at least partly due to the propensity of unsaturated fatty acids to undergo lipid peroxidation in the face of reactive oxygen species. This has important clinical consequences, particularly limiting the use of fatty donor livers for transplantation[28]. NASH is a progressive liver disease characterized by Kupffer cell dysfunction which contributes to its pathogenesis. It is noteworthy that the reticular-endothelial system also plays a key role in the spleen. Colloid scintigraphy is a good method of reflecting Kupffer cell activity. A study on 22 patients with biopsy-proven NASH who underwent colloid liver scintigraphy, after intravenous injection of 185 MBq Tc tin colloid, showed that liver right/left lobe ratio was altered in all of these patients. Colloid shift to the spleen was observed in 55% of patients as well as prolonged blood pool clearance time[29].

The first group of researchers[30] who aimed to determine if there was an association between NAFLD and spleen enlargement, measured spleen volume using CT. The values were compared with the patient's demographic data, the liver-to-spleen (L/S) ratio of CT Hounsfield unit measurements, and the results of liver function tests. Diagnosis of fatty liver was made if the L/S ratio was less than 1.0. The mean spleen volume was 73.0 ± 24.4 cm^3 (range, 21.1-106.1) in normal subjects and 141.2 ± 54.1 cm^3 (range, 44.1-267.3) in patients with fatty liver ($P < 0.0001$). Multivariate linear regression analysis identified that only the L/S ratio ($P < 0.0001$) and age ($P < 0.01$) were significantly correlated to spleen volume. Using forward selection stepwise regression, the L/S ratio entered first (β = -0.634) and age second (β = - 0.293).

Obesity and insulin resistance are strongly associated with systemic markers of inflammation. Focusing on this aspect, authors have attempted to find a noninvasive method that could likely assess the presence of NASH and help to decide liver biopsy performance. Using histology as a gold standard to diagnose NAFLD, 43 patients with NASH and 40 with fatty liver were consecutively studied, their data were compared with those of 48 healthy control participants. The outcomes evaluated were ultrasonographic spleen longitudinal diameter coupled with the splenic artery resistive index, serum interleukin (IL)-6 and vascular endothelial growth factor

concentrations. The NASH group had higher spleen longitudinal diameter values ($P = 0.0001$) as well as significantly higher IL-6 and vascular endothelial growth factor concentrations than the other groups ($P = 0.0001$). The optimal cut-off value for spleen longitudinal diameter that best discriminated NASH from fatty liver patients was 116 mm (specificity 95% and sensitivity 88%); the sensitivity and specificity of this parameter was better than both IL-6 and vascular endothelial growth factor in the same setting (area under the receiver operating characteristic curve 0.920 *vs* 0.817 and 0.678, respectively). Splenic artery resistive index was similar between patients with NASH and those with fatty liver, but differed when compared with controls ($P = 0.0001$). IL-6 was highly specific in confirming the absence of NASH at normal values. In that series of patients, normal values of spleen longitudinal diameter and IL-6 were strongly associated with fatty liver[31]. Further confirmation of these findings comes from another study which highlighted that spleen enlargement may be a distinct feature of NASH, especially early-stage NASH[32].

A subsequent study[33] showed that spleen enlargement was found at significant levels (38%) in obese patients as determined by Cavalieri stereologic volume calculation, an unbiased stereological method. Finally, recent results clearly indicated that high fat diet caused splenomegaly *via* sinusoidal dilatation and intracellular or intercellular deposits in obese female rats[34]. Although in patients with NAFLD, liver biopsy remains the only reliable method to differentiate simple steatosis from NASH, the objective of the study was to evaluate the efficacy of non-invasive (99m)Tc-phytate scintigraphy in the diagnosis of NASH. Thirty-seven patients with suspected NAFLD at the time of liver biopsy also underwent (99m)Tc-phytate scintigraphy. Signal intensities of regions of interest in the liver and spleen were measured. The same authors also examined scintigraphic features in a nutritional model of NASH in rats fed a methionine- and choline-deficient (MCD) diet. The liver/spleen uptake ratio determined by scintigraphy was significantly decreased in patients with NASH in comparison with patients with simple steatosis. The liver/spleen ratio was an independent predictor distinguishing NASH from simple steatosis. The decrease was observed for all stages of NASH, including the early stage (stages 1 and 0). In animal studies, the liver/spleen uptake ratio was significantly decreased in rats after 8 wk of a MCD diet in comparison with control diet-fed rats. These authors concluded that non-invasive (99m)Tc-phytate scintigraphy is a reliable tool to differentiate NASH from simple steatosis[35]. The frequency of ischemic heart disease observed after splenectomy for trauma and the low cholesterol levels found in patients with hypersplenism are observations that suggest a possible role for the spleen in lipid metabolism and in the etiology of atherosclerosis[36,37]. Previous studies showed that obese subjects are more susceptible to cardiovascular disease, hypertension, cerebrovascular disease, and diabetes mellitus than are non-obese subjects. They have a higher incidence of infection and some types of cancer, suggesting impaired immune function. In humans, only a few studies have directly compared specific immune responses in obese and non-obese subjects. It is known that obesity induces decreases in both T lymphocyte response to concanavalin A and B lymphocyte response to pokeweed mitogen[38]. In addition, a negative correlation between percentage body fat and natural killer cell activity was found in both elderly women[39], and adult men[40]. Elderly people (> 60 years of age) are also at risk of an increased incidence of infection. Their peripheral blood lymphocytes show an impaired proliferative capacity and a decreased reactivity to mitogens[41]. Researchers found and reported that obesity suppresses lymphocyte functions, natural killer cell activity, and lymphocyte mitogenesis in men and women > 60 years of age[42]. This suggests that obesity is a risk factor for deteriorating cellular immune functions. However, the mechanism by which obesity decreases cellular immune functions remains to be elucidated. Expression of glucose transporter 1 (GLUT-1), analyzed by Western blot analysis, was lower in the splenic lymphocytes of obese compared with lean Zucker rats. In obese subjects it is associated with the decreased uptake of glucose into immune cells, which in turn is associated with the decreased expression of GLUT-1. This suggests that decreased proliferation of splenic lymphocytes in obese Zucker rats is associated with the impairment of glucose uptake, which is due to the decreased expression of GLUT-1[43]. An up-to-date study assessed the magnitude of antigen-specific immunity in a murine model of NAFLD. Because antigen-specific immunity was diminished in NAFLD mice, the underlying mechanisms were evaluated through analysis of the functions of antigen-presenting DC and other immunocytes. For 12 wk, NAFLD mice received a high-fat and high-calorie diet. NAFLD mice and control mice were immunized with hepatitis B vaccine containing hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg). Antibody to HBsAg (anti-HBs), HBsAg and HBcAg-specific cellular immune response and functions of whole spleen cells, T lymphocytes, B lymphocytes and spleen DCs of NAFLD and control mice were assessed *in vitro*. Levels of anti-HBs and the magnitude of proliferation of HBsAg and HBcAg-specific lymphocytes were significantly lower in NAFLD mice than control mice. The spleen cells of NAFLD mice produced significantly higher levels of inflammatory cytokines and exhibited significantly increased T cell proliferation compared with control mice. However, the antigen processing and presenting capacities of spleen DCs were significantly decreased in NAFLD mice compared with control mice. Palmitic acid, a saturated fatty acid, caused diminished antigen processing and presenting capacity of murine DCs[44]. Liver fat represents a balance between input, secretion, and oxidation of fatty acids. As humans spend the majority of a 24-h period in a postprandial state, dietary fatty acids make an important contribution to liver fat metabolism. Oxidation of dietary fatty acids, hepatic desaturation and elongation of palmitic acid occurs to a greater extent in abdominally obese men.

NEUROTRANSMITTER, HORMONES, VITAMIN D AND THE SPLEEN

Increasing evidence has placed hormones and neurotransmitters among potent immunomodulators, in both health and disease. 5-HT functions as a neurotransmitter in the nervous systems of simple as well as complex animals. 5-HT diffuses to serotonin-sensitive neurons, which control the animal's perception of nutrient availability. This system has been partially conserved during the 700 million years of evolution which separates *C. elegans,* a transparent nematode, from humans. When humans smell food, dopamine is released to increase the appetite. However, unlike that in worms, serotonin does not increase anticipatory behaviour in humans; instead the serotonin released while consuming activates 5-HT_{2C} receptors on dopamine-producing cells. This halts their dopamine release, and thereby serotonin decreases appetite. Drugs which block 5-HT_{2C} receptors make the body unable to shut off appetite, and are associated with increased weight gain[45], especially in people who have a low number of receptors[46]. The expression of 5-HT_{2C} receptors in the hippocampus follows a diurnal rhythm, just as the 5-HT release in the ventromedial nucleus, which is characterized by a peak in the morning when the motivation to eat is strongest[47]. In humans, serotonin levels are affected by diet. An increase in the ratio of tryptophan to phenylalanine and leucine will increase serotonin levels. Fruits with a good ratio include dates, papaya and banana. Foods with a lower ratio inhibit the production of serotonin. Research also suggests that eating a diet rich in carbohydrates and low in protein will increase serotonin by secreting insulin, which helps in amino acid competition[48]. However, increasing insulin for a long period may trigger the onset of insulin resistance, obesity, type 2 diabetes, and lower 5-HT levels. Researchers showed that expression of 5-HT(2A) receptors was up-regulated in hypertrophic 3T3-L1 adipocytes, which exhibited decreased expression of adiponectin and increased expression of PAI-1. 5-HT(2A) receptor antagonists and suppression of 5-HT(2A) receptor gene expression enhanced adiponectin expression. Activation of Gq (the G protein-coupled receptor is activated by an external signal in the form of a ligand or other signal mediator) negatively regulated adiponectin expression, and inhibition of mitogen-activated protein kinase reversed the Gq-induced effect. Moreover, the 5-HT(2A) receptor blockade reduced PAI-1 expression[49]. As food intake and energy balance are among the functions regulated by 5-HT in the brain, it would be interesting to discover its link with some adipokines. Recent studies have shown an interaction between the serotonergic system and leptin, a protein released from adipose tissue that inhibits feeding behavior and increases fuel expenditure. An up-to-date study found low brain serotonin immunoreactivity in all animals with high neuronal leptin accumulation in the raphe nucleus, independently of their age. In contrast, high brain serotonin immunoreactivity was accompanied by a low neuronal accumulation of leptin. These findings indicate that serotonin regulates leptin uptake by neuronal cell bodies of the dorsal raphe and hypothalamus, suggesting that at least part of the effects of serotonin may be mediated by the regulation of neuronal trafficking in the brain[50]. 5-HT promotes the release of growth hormone (GH) by a hypothalamic site of action[51]. Exogenous GH enhances thymic microenvironmental cell-derived secretory products such as cytokines and thymic hormones. Moreover, GH increases thymic epithelial cell (TEC) proliferation *in vitro*, and exhibits a synergistic effect with anti-CD3 in stimulating thymocyte proliferation, which is in keeping with data showing that transgenic mice overexpressing GH or GH-releasing hormone exhibit overgrowth of the thymus. GH also influences thymocyte traffic: it increases human T-cell progenitor engraftment into the thymus; augments TEC/thymocyte adhesion and the traffic of thymocytes in the lymphoepithelial complexes, the thymic nurse cells; modulate *in vivo* the homing of recent thymic emigrants, enhancing the number of fluorescein isothiocyanate positive cells in the lymph nodes and diminishing them in the spleen. In keeping with the effects of GH on thymic cells, is the detection of GH receptors in both TEC and thymocytes. Insulin-like growth factor (IGF)-1 is a potent hormone that stimulates growth and differentiation and inhibits apoptosis in numerous tissues. Preliminary evidence suggests that IGF-1 exerts differentiating, mitogenic and restoring activities in the immune system, however, the sites of synthesis of local IGF-1 are unknown. Identification of these sites would allow the functional role of local IGF-1 to be clarified. The presence of IGF-1 in non-immune cells suggests that it acts as a trophic factor, while its occurrence in subtypes of lymphocytes or antigen-presenting cells indicates paracrine/autocrine direct regulatory involvement of IGF-1 in the human immune response. Additionally, data indicate that IGF-1 is involved in several effects of GH in the thymus, including the modulation of thymulin secretion, TEC proliferation as well as thymocyte/TEC adhesion. This is in accordance with the demonstration of IGF-1 production and expression of IGF-1 by TEC and thymocytes. Also, it should be seen as an intrathymic circuitry, involving not only IGF-1, but also GH itself, as intrathymic GH expression is seen both in TEC and in thymocytes, and that thymocyte-derived GH could enhance thymocyte proliferation[52]. With regard to the implication of the IGF family in immune physiology and development, a recent study has focused on type 1 IGF receptor, a transmembrane tyrosine kinase homologous to the insulin receptor that mediates most of the biological effects of IGF-1 and IGF-2. Normal development and *ex vivo* activation of T and B cells are observed in chimeric *Rag2*-deficient C57BL/6 mice reconstituted with fetal liver cells from *Igf1r*$^{-/-}$ mice. However, this model revealed an unexpected decrease in the T-independent B cell response which is important in bacterial defense mechanisms[53]. The major role of IGF-2 is as a growth promoting hormone during gestation. To date, very few studies have investigated the function of IGF-2 in immune development and physiology.

This growth factor is the dominant peptide of the insulin family expressed in the thymus epithelium of different species. Thymic IGF-2 influences thymic development and T cell differentiation as evidenced by the analysis of IGF-2 transgenic dwarf mice, which develop thymic hyperplasia with an increased number of thymocytes (and $CD4^+$ T lymphocytes in particular). This increase in T cells is also observed in the spleen compartment of IGF-2 transgenic mice, but there is no significant effect on B cell development[54]. There is further evidence that IGF-2 may intervene in the control of T cell differentiation[55]. A recent study investigated the location of IGF-1 messenger RNA and protein on archival human lymph node samples by *in situ* hybridization, immunohistochemistry and double immunofluorescence staining using an IGF-1 probe and antisera specific for human IGF-1 and CD3 (T lymphocytes), CD20 (B lymphocytes), CD68 (macrophages), CD21 (follicular DCs), S100 (interdigitating DCs) and podoplanin (fibroblastic reticular cells). Numerous cells within the B- and T-cell compartments expressed the IGF-1 gene, and the majority of these cells were identified as macrophages. Solitary follicular DCs exhibited IGF-1. A few T lymphocytes, and no B lymphocytes, contained IGF-1 immunoreactive material. Furthermore, IGF-1 immunoreactive cells outside the follicles that did not react with CD3, CD20, S100 or podoplanin markers were identified as high-endothelial venule cells[56]. GH was used to counteract the catabolic metabolism in critically ill patients until it was demonstrated that administration of GH was associated with increased morbidity due to uncontrolled infections and sepsis[57]. The immunomodulatory effect of GH and its main mediator IGF-1 during systemic inflammation remain to be established. Authors investigated the effect of GH and IGF-1 on cellular immune functions in a murine model of sepsis and found that GH did not affect cellular immune functions or the survival rate in that model. In contrast, IGF-1 improved splenocyte proliferation and cytokine release independently of GH but did not affect the determined clinical parameters of septic mice[57]. Aging is under the control of a small number of regulatory genes. Mice genetically selected for high immune responses, in most cases, exhibit a longer life span and lower lymphoma incidence than do mice selected for low responses. The link between immunity and aging is further evidenced by the age-related alterations in the immune system, mostly of the T-cell population, in terms of replacement of virgin by memory cells, accumulation of cells with signal transduction defects, and changes in the profile of Th1 and Th2 type cytokines[58]. Also, B cells exhibit intrinsic defects, and NK cell activity is profoundly depressed by aging. *In vitro* experiments indicate that the production of IL-2, interferon (IFN)-γ, and IL-4 by mouse spleen cells changes with aging and may be upregulated by recombinant cytokines. These findings suggest possible cytokine interventions to prevent or treat age-related immune disorders, as they may affect the duration and the biological quality of life[59]. Excessive alcohol consumption continues to be a major public health problem, particularly in the adolescent and young adult populations. Generally, such behavior tends to be confined to the weekends, resulting in frequent binge drinking. Various authorities have emphasized the strict link between mechanisms inducing alcoholic and nonalcoholic liver diseases, thus it could be of interest to ask questions about alcohol toxicity, such as: is there a link between alcohol abuse and impaired immune system and what is the link? A study in peri-pubertal male rats compared the effect of the discontinuous feeding of a liquid diet containing a moderate amount of ethanol (6.2% wt/vol) to that of continuous ethanol administration or a control diet, taking as end points the 24-h variations in plasma prolactin levels and mitogenic responses and lymphocyte subset populations in the spleen. Animals received the ethanol liquid diet starting on day 35 of life, the diet being similar to that given to controls except that maltose was iso-calorically replaced by ethanol. Ethanol provided 36% of the total caloric content. Each week, the discontinuous ethanol group received the ethanol diet for 3 d and the control liquid diet for the remaining 4 d. After 4 wk the rats were killed. A significant decrease in splenic cell response to concanavalin A, and of splenic cell response to lipopolysaccharide was found in rats under the discontinuous ethanol regime, when compared with control- or ethanol-chronic rats. Under discontinuous ethanol feeding, mean values of splenic CD8(+) and CD4(+)-CD8(+) cells decreased, whereas splenic T cells, and splenic B cells were augmented. In rats chronically fed with ethanol, splenic mean levels of CD8(+) and CD4(+)-CD8(+) cells were augmented. Both modalities of ethanol administration disrupted the 24 h variation in immune function seen in controls. Mean plasma prolactin levels increased by 3.6-fold and 8.5-fold in rats chronically or discontinuously fed with alcohol, respectively. These results supported the view that the discontinuous drinking of a moderate amount of ethanol can be more harmful for the immune system than continuous ethanol intake, presumably by inducing greater stress as indicated by the augmented plasma prolactin levels observed[60]. Numerous studies have focused their attention on the role played by vitamin D in obesity, MS and NAFLD. The hormonal form of vitamin D, 1,25-dihydroxyvitamin D3, is well known for its immunosuppressive, anti-proliferative and pro-apoptotic activities. In a recent work, authors studied the effect of 1,25-dihydroxyvitamin D3 on *Toxoplasma gondii*-infected mice. They observed that 1,25-dihydroxyvitamin D3 reduces the survival rate of infected mice by up to 37% at day 10 post-infection compared to untreated infected mice ($P < 0.0001$). IFN-γ and IL-12p40 levels were significantly reduced by 1,25-dihydroxyvitamin D3 in infected mice sera indicating an inhibition of Th-1-type cytokines. CD4+ T lymphocyte and splenocyte counts were also reduced following 1,25-dihydroxyvitamin D3 treatment and a marked induction of apoptosis, accompanied by down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X(L), was observed. The above results indicate that 1,25-dihydroxyvitamin D3 induces splenocyte apoptosis and enhances host suscepti-

bility to toxoplasmosis[61]. Bone components participate in the regulation of hematopoietic stem cells (HSC) in the adult mammal. Vitamin D regulates bone mineralization and is associated with pleiotropic effects in many cell types including putative roles in hematopoietic differentiation. Researchers reported that deletion of the vitamin D receptor (VDR) in hematopoietic cells did not result in cell autonomous perturbation of HSC or progenitor function. However, deletion of VDR in the microenvironment resulted in a marked accumulation of HSC in the spleen that could be reversed by dietary calcium supplementation. These data suggest that VDR participates in restricting splenic hematopoiesis through maintenance of bone calcium homeostasis and are consistent with the concept that calcium regulation through VDR is a central participant in localizing adult hematopoiesis preferentially to bone marrow[62].

CONCLUSION

This special organ should be taken into account when interpreting the mechanisms of NAFLD and in its diagnosis, mainly when dealing with the more severe form, i.e., NASH, although recent research has challenged the benignity of FL[63].

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

JNKs, insulin resistance and inflammation: A possible link between NAFLD and coronary artery disease

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Abstract

The incidence of obesity has dramatically increased in recent years. Consequently, obesity and associated disorders such as nonalcoholic fatty liver disease constitute a serious problem. Therefore, the contribution of adipose tissue to metabolic homeostasis has become a focus of interest. In this review, we discuss the latest discoveries that support the role of lipids in nonalcoholic fatty liver disease. We describe the common mechanisms (c-Jun amino-terminal kinases, endoplasmic reticulum stress, unfolded protein response, ceramide, low-grade chronic inflammation) by which lipids and their derivatives impair insulin responsiveness and contribute to inflammatory liver and promote plaque instability in the arterial wall. Presenting the molecular mechanism of lipid activation of pro-inflammatory pathways, we attempt to find a link between nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular diseases. Describing the common mechanisms by which lipid derivatives, through modulation of macrophage function, promote plaque instability in the arterial wall, impair insulin responsiveness and contribute to inflammatory liver and discussing the molecular mechanism of lipid activation of pro-inflammatory pathways, the key roles played by the proliferator-activated receptor and liver X receptor α, nuclear receptors-lipid sensors that link lipid metabolism and inflammation, should be emphasized. Further studies are warranted of anti-inflammatory drugs such as aspirin, anti-interleukin-6 receptors, immune-modulators (calcineurin inhibitors), substances enhancing the expression of heat shock proteins (which protect cells from endoplasmic reticulum stress-induced apoptosis), and anti- c-Jun amino-terminal kinases in well-designed trials to try to minimize the high impact of these illnesses, and the different expressions of the diseases, on the whole population.

Key words: Non-alcoholic fatty liver disease; c-Jun amino-terminal kinase; Cardiovascular disease

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INTRODUCTION

The rapid increase in the prevalence of obesity is a major global health problem. Its associated complications are burdened by an increased risk of death by 20%-40% in overweight individuals and by 2-3-fold in obese individuals compared to normal controls, even though the

strength of the association between body mass index and heart failure events declines with age[1]. Obesity is a known risk factor for non-alcoholic fatty liver disease (NAFLD)[2], hypertension, stroke, gallbladder disease, osteoarthritis, obstructive sleep apnea, and other breathing problems as well and some forms of cancer (breast, colorectal, endometrial and kidney). Type 2 diabetes (T2D) and obesity, now collectively referred to as "diabesity", are interrelated, in that obesity is known to exacerbate the pathology of T2D and greater than 60% of diabetics are obese. Low grade chronic inflammation, strictly linked to overweight/obesity, causes insulin resistance (IR) that interacts with other complex mechanisms such as hypercholesterolemia, smoking, hypertension, hyperglycemia, type A behavioral patterns, hemostatic factors, hereditary differences in such diverse aspects as lipoprotein structure and that of their associated receptors, homocysteine processing/metabolism, and high levels of lipoprotein(a) to increase the risk of coronary heart disease (CHD). CHD is present not only in nondiabetic and normotensive obese adult subjects[3], but also in obese children[4]. IR is a condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells. Indeed, lipolysis, which is normally inhibited by insulin, is overstimulated in insulin-resistant states leading to an increase in free fatty acid (FFA) flux[5]. IR in muscle reduces glucose uptake whereas IR in liver reduces glucose storage, both effects serving to elevate blood glucose. High plasma levels of insulin and glucose due to IR often leads to metabolic syndrome (MS) and T2D. Plasma FFAs from white adipose tissue lipolysis have been shown to be the major contributor to triglyceride accumulation[6] observed in NAFLD, further expression of MS, which ranges from simple fatty liver (FL) through the more severe form, non alcoholic steatohepatitis (NASH) to cryptogenic cirrhosis. Sharing the same mechanisms of NASH, FL is no longer considered completely benign, accordingly to recent data[7]. Activation of the endoplasmic reticulum (ER) by stress has been reported in most models of hepatic steatosis in rodents and its contribution to hepatic fat deposition has been recently documented, with lipogenesis being the main metabolic pathway affected. ER stress-related activation, observed in adipose tissue of obese humans[8], could have metabolic consequences and participate in fat deposition in the liver. Activation of ER could directly induce an insulin-resistant state in adipocytes. Indeed, it has been shown that activation of the ER stress sensor kinase/endonuclease inositol-requiring protein 1 (IRE1), a component of the unfolded protein response (UPR) could stimulate c-Jun amino-terminal kinase [JNKs or stress-activated protein kinases (SAPKs)][9], which, by phosphorylating serine residues of insulin substrate receptor 1, is a key player in the development of IR[10]. The IRE1/box binding protein 1, a branch of the ER stress signaling pathway, has been recently shown to regulate and be regulated by innate immune signaling pathways in both the presence and absence of ER stress[11]. Disruption of ER homeostasis has been observed in liver and adipose tissue of humans with NAFLD and/or obesity. Importantly, the signaling pathways activated by disruption of ER homeostasis, the UPR, has been linked to inflammation and apoptosis, lipid biosynthesis, insulin effects, all of which are involved in the initiation/evolution of NAFLD. The ER is a crucial organelle for cellular homeostasis, in which the synthesis and the post-translational modifications of membrane and secreted proteins take place, as well as the synthesis of lipids and cholesterol for membranes formation. However, the ER quality control system can be compromised under a variety of conditions such as accumulation of unfolded protein, alteration of calcium homeostasis or disruption of the redox state. The UPR activates JNKs[9,12]. Up-to-date results provide evidence that heat shock proteins protect cells from ER stress-induced apoptosis. Obesity and associated disorders constitute a serious problem, for example NAFLD can lead to hepatocarcinoma, and the contribution of adipose tissue to metabolic homeostasis has become a focus of interest. Adipose tissue secretes FFAs and hormones, known as adipokines, and thus seems to play a major role in the development of NAFLD. Apoptotic cell death is a prominent feature in NASH. Indeed, toxic FFAs can activate the intrinsic apoptosis pathway in hepatocytes *via* c-JNK. JNK activates the proapoptotic protein Bim, resulting in Bax activation[13] and enhanced apoptosis, termed "lipoapoptosis".

JUN AMINO-TERMINAL KINASES

Jun N-terminal kinases (JNKs), also named SAPKs, are one of 3 members of the mitogen-activated protein kinase (MAPK) superfamily, which also includes the extracellular signal-regulated kinases (ERKs) or classical MAPKs and the p38 MAPK. JNKs bind and phosphorylate c-Jun on Ser63 and Ser73 within its transcriptional activation domain. MAPK kinases (MKK) are responsive to stress stimuli, mainly inflammatory signals, but also to a lesser extent, to ultraviolet irradiation, heat and osmotic shock, and are involved in apoptosis and T cell differentiation. This latter immunological aspect should not be overlooked.

JNKs consist of 10 isoforms derived from 3 genes: JNK1 (4 isoforms), JNK2 (4 isoforms), and JNK3 (2 isoforms). JNK1 and JNK2 are found in all cells of every tissue. JNK3 is found mainly in the brain, but is also found in the heart and the testes. JNK1 is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions and cytokine production mediated by activation protein-1 (AP-1) such as regulated upon activation, normal T-cell expressed, and secreted cytokine, interleukin-8 and granulocyte-macrophage colony-stimulating factor. Recently, JNK1 has been found to regulate Jun protein turnover by phosphorylation and activation of the ubiquitin ligase Itch (polyubiquitination marks proteins for degradation by the proteasome). JNKs can associate with scaffold proteins, JNK-interacting proteins as well as their upstream Jun N-terminal kinase kinase 1 and Jun N-terminal kinase

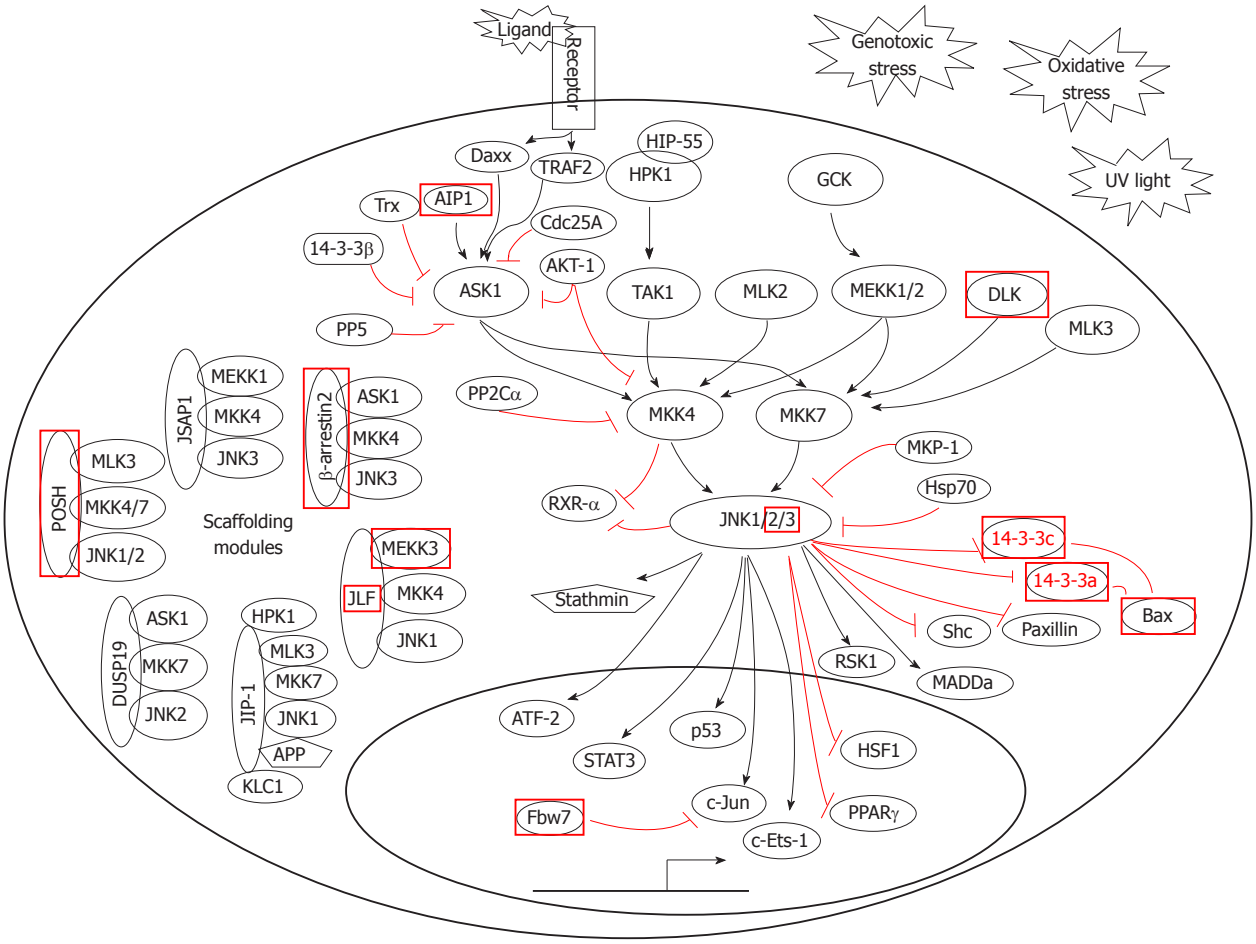

Figure 1 Jun N-terminal kinase signaling pathway. ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; MKK: MAPK kinase; MEKK: MAPK/ERK kinase kinase; MLK: Mixed lineage kinase; JNK: Jun N-terminal kinase; ASK: Apoptosis signal-regulating kinase; HSP: Heat shock protein; RSK: p90 ribosomal S6 kinase; HPK: Hematopoietic progenitor kinase; PPAR: Peroxisome proliferator-activated receptor; DLK: Dual leucine zipper-bearing kinase; RXR: Retinoid X receptor; ATF: Activating transcription factor; STAT: Signal transducers and activators of transcription; MADD: Mitogen-activated kinase activating death domain protein; TRAF: Tumor necrosis factor receptor-associated factor; MKP: MAPK phosphatase; TAK: Transforming growth factor-activated kinase; KLC: Kinesin light chain; DAXX: Death domain-associated protein.

kinase 2 following their activation. JNK, by phosphorylation, modifies the activity of numerous proteins that reside at the mitochondria or act in the nucleus. Thus, JNK activity regulates several important cellular functions. One way this activation may occur is through disruption of the conformation of sensitive protein phosphatase enzymes; specific phosphatases normally inhibit the activity of JNK itself and the activity of proteins linked to JNK activation. The JNK proteins lead to varied and seemingly contradictory cellular responses. In particular, JNKs have been reported to have a role in the induction of apoptosis, but have also been implicated in enhancing cell survival and proliferation. The opposing roles of JNKs have been attributed to the observation that JNKs activate different substrates based on a specific stimulus, cell type or temporal aspects[14].

JNK signaling pathway

JNKs are activated by MAPK kinases such as MKK-4, MKK-6 and MKK-7 (Figure 1). These kinases are in turn activated by the MAP3 kinases, such as apoptosis signal-regulating kinase 1 (ASK1) also known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5), mixed lineage kinases (MLKs) (MLK1, MLK2 and MLK3), MAP/ERK (extracellular signal-regulated kinase) kinase kinase 1 (MEKK1), MEKK4 and transforming growth factor (TGF)-β-activated kinase 1 (TAK1). JNK MAP3 kinase pathways are activated by MAP4 kinases that link to a variety of cell receptors which sense stress and inflammation, including death receptors (Fas), inflammatory cytokine receptors of tumor necrosis factor alpha (TNF-α) and TGF-β, G-protein-coupled receptors (GPCRs) and antigen receptors. Signals are communicated to JNK pathway MAP4 kinases by tyrosine kinase receptor associated adapter and effector molecules and/or by G-protein mediated signaling. GPCRs signal the JNK pathway through trimeric G-proteins to monomeric p21RhoGTPases, Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (CDC42). ASK1 links to stress receptors such as TNF receptor (TNFR) and Fas, and is activated by reactive oxygen species (ROS)-mediated dissociation

of thioredoxin, binding to TNFR associated factor 2 (TRAF2) or death domain-associated protein (DAXX) and oligomerization. ASK1 activates the JNK MAP2Ks, MKK4 (SEK1) and MKK6 (MKK3/MAPKK6). The JNK MAP3 kinases: TAK1, MEKK1 and MLK3 are activated by the tyrosine kinase (TK) receptor-activated MAP4 kinase, hematopoietic progenitor kinase 1 (HPK-1). HPK-1 associates with TK receptors through adaptor proteins, such as CT10-regulated kinase (Crk), Crk-like (CrkL) and growth factor receptor-bound 2 (Grb2). Activation of HPK1 requires multiple phosphorylation events including autophosphorylation and protein kinase phosphorylation by protein kinase D1 (PKD1). The JNK MAP3 kinases, MEKK1, MEKK4 and MLK3 are also activated by the p21Rho-GTPases, Ras-related C3 botulinum toxin substrate 1 also known as Rac1 and Cdc42 through the MAP4K, P21-activated kinase- 1 (PAK-1). This links the JNK pathways to a wide variety of GPCR, integrin and receptor pathways. In addition to participating in the stress response, the MAPKs c-Jun N-terminal Kinases JNK1 and JNK2 regulate the proliferation of normal and neoplastic cells. JNKs contribute to these processes largely by phosphorylating c-Jun and thus contributing to the activation of the AP-1 complex. Furthermore, JNKs control entry into mitosis. It has been observed that JNK activity and phosphorylation of c-Jun become elevated during the G/M transition of the cell cycle in immortalized fibroblasts and ovarian granulosa cells. Pharmacological inhibition of JNK causes a profound cell cycle arrest at the G/M transition in both cell types. This effect is specific as it occurs with 2 distinct small molecular compounds. Inactivation of JNK prior to mitosis prevents expression of aurora B and phosphorylation of histone-H3 at Ser 10. Silencing of JNK1 and 2 causes a similar effect, whereas overexpression of JNK1 and 2 causes the opposite effect. Inhibition of JNK delays activation of Cdc2 and prevents downregulation of cyclin B1, whereas Ras controls the activation of MAPKs. Authors have recently observed that in certain cells, the small guanosine triphosphate (GTP)-binding proteins Rac1 and Cdc42 but not Rho regulate the activity of JNKs[15]. Furthermore, because Rac1 and Cdc42 but not Rho, bind and activate a P21-activated kinase 1 (Pak1), it has been suggested that Pak1 is the most upstream component of the pathway linking these GTPases to JNK. However, in mammalian cells, Rho1p, a Rho homologue, and RhoA directly interact with a number of proteins, including kinases related to protein kinase C. Exploring the ability of Ras and Rho-related GTP-binding proteins to activate MAPK or JNK in a variety of cell lines, it was found that in the human kidney epithelial cell line, 293T, Cdc42 and all Rho proteins, RhoA, RhoB, and RhoC, but not Rac or Ras can induce activation of JNK. Furthermore, other researchers provided evidence that signaling from Rho proteins to JNK in 293T cells does not involve Pak1[16]. c-JNK activity is abnormally elevated in obesity. Furthermore, an absence of JNK1 results in decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity[17]. As previously mentioned, elevated FFAs and hepatocyte lipoapoptosis are the main features of NAFLD. However, the mechanism by which FFAs mediate lipoapoptosis is unclear. Recently, data have indicated that saturated FFAs induce JNK-dependent hepatocyte lipoapoptosis by activating the pro-apoptotic Bcl-2 family Bim and Bax-mediated apoptosis, which triggers the mitochondrial apoptotic pathway[18]. Additional support for involvement of JNK1 overactivation in conditions associated with IR and MS has been provided[19]. A positive correlation was found between the expression intensity of JNK1 and IR[20], and JNK1 contributes to the development of liver fibrosis by inducing chronic inflammation as ascertained in a mouse NASH model[21]. Methionine-choline-deficient feeding causes NASH coincident with the activation of c-JNK and caspase-12 in a murine model[22].

Further data indicated that the increased oxidative stress and its associated JNK activation, as well as an imbalance in pro- and anti-apoptotic proteins in the Bcl-2 family all contribute to marked hepatocyte apoptosis in a rat NASH model[23]. Examining fat biopsy samples from obese insulin-resistant nondiabetic individuals, UPR activation in subcutaneous adipose tissue was demonstrated, with JNK being a link between obesity, IR, and inflammation[8]. ER stress activates the proteolytic cleavage of the lipogenic transcription factor sterol regulatory element binding protein-1c leading to the induction of lipogenic enzyme expression. A role for X box-binding protein 1, an ER stress-activated transcription factor, has also recently emerged. ER stress, by inhibiting apoB100 secretion, has associated with impaired very low density lipoprotein (VLDL) secretion. In rodents, treatment with molecular or chemical chaperones that reduce ER stress markers have demonstrated effectiveness in the treatment of hepatic steatosis[24].

INSULIN RESISTANCE

SAPK/JNKs are activated by inflammatory cytokines, and JNK signaling is involved in IR and β-cell secretory function and survival. An up-to-date study suggested that FFAs stimulate functional autophagy of β cells, possibly through the RNA-dependent protein kinase (PKR)-JNK1 pathway independent of the ER or oxidative stress[25].

Post-transcriptional modifications altering activity of insulin signaling molecules are the most proposed mechanism for inhibition of the insulin pathway. Various kinases including stress activated protein kinase, c-JNK, and protein kinase C (PKC) can phosphorylate insulin receptor substrate (IRS) 1-2 at specific serine and threonine residues, leading to inhibition of insulin signaling[26]. A high fat diet is an established cause of systemic and adipose tissue IR. Activated JNK is a major contributor to FFA-induced cellular IR, and TNF-α is an autocrine/paracrine downstream effector of activated JNK that can also mediate IR[27]. TNF-α is over-expressed in adipose tissue of obese rodents and humans, and its concentra-

tion is reduced after weight loss. TNF-α inhibits insulin signaling in the liver by mechanisms which include the activation of serine kinases such as JNK-1 and induction of suppressor of cytokine signaling proteins[28]. An important consequence of IR in adipose tissue is to reduce the anti-lipolytic effect of insulin that in turn leads to elevated plasma FFA in obese and diabetic patients. Released FFAs from adipose tissue is taken up by liver and muscle cells. In the liver, FFAs induce gluconeogenesis and VLDL overproduction. Increased FFAs, especially metabolites such as acyl-CoAs, ceramides, and diacylglycerol, have been shown to inhibit insulin signaling by activating protein kinases such as PKC, JNK, and the inhibitor of nuclear factor-κB (IKK-β)[29].

Chronic high glucose concentrations and leptin induce IL-1β secretion from pancreatic islets, an event that is possibly promotes β-cell dysfunction and death. A recent study provided evidence that chronically elevated concentrations of leptin and glucose induced β-cell apoptosis through activation of the JNK pathway in human islets and in insulinoma (INS 832/13) cells. JNK inhibition by the dominant inhibitor JNK-binding domain of IB1/JIP-1 (JNKi) reduced JNK activity and apoptosis induced by leptin and glucose. Exposure of human islets to leptin and high glucose concentrations led to a decrease of glucose-induced insulin secretion, which was partly restored by JNKi. An interplay between the JNK cascade and the caspase 1/IL-1β-converting enzyme in human islets has been found. The *caspase 1* gene, which contains a potential activating protein-1 binding site, is upregulated in pancreatic sections and in isolated islets from T2D patients. Similarly, cultured human islets exposed to high glucose- and leptin-induced caspase 1 and JNK inhibition prevents this upregulation. Therefore, JNK inhibition may protect β-cells from the deleterious effects of high glucose and leptin in diabetes[30].

Obesity is closely associated with IR and is established as a leading risk factor for T2D, yet the molecular mechanisms of this association are poorly understood. JNKs can interfere with insulin action in cultured cells and are activated by inflammatory cytokines and FFAs, molecules that have been implicated in the development of T2D. As previously highlighted, it has been shown that JNK activity is abnormally elevated in obesity. Furthermore, an absence of JNK1 results in decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity in 2 different models of mouse obesity. Thus, JNK is a crucial mediator of obesity and IR and a potential therapeutic target[31]. Chronic oxidative stress results in decreased responsiveness to insulin, eventually leading to T2D and CHD. Activation of the JNK signaling pathway can mediate many of the effects of stress on IR through inhibitory phosphorylation of IRS-1. In contrast, exercise, which acutely increases oxidative stress in muscle, improves insulin sensitivity and glucose tolerance in patients with T2D. Authors used a cellular model of insulin-resistant muscle to induce either chronic or acute oxidative stress and investigate their contrasting effects on insulin and JNK signaling. Chronic oxidative stress resulted in increased levels of phosphorylated (activated) JNK in the cytoplasm, whereas acute oxidative stress led to redistribution of JNK-specific phosphatase MKP7 from the nucleus into the cytoplasm, a reduction in cytoplasmic phospho-JNK, and concurrent accumulation of phospho-JNK in the nucleus. Acute oxidative stress restored normal insulin sensitivity and glucose uptake in insulin-resistant muscle cells, and this effect was dependent on MKP7[32].

Finally, it is likely that JNK activity modulates pancreatic islet function and/or survival in numerous ways. First, there is convincing evidence for the involvement of JNK in islet cell inflammation and death mediated by cytokines[33]. Second, JNK activation may generate a state of β-cell dysfunction and defective insulin production, thereby contributing to the development of overt diabetes[34]. Third, administration of SP600125, a synthetic inhibitor of JNK, results in improved glucose-stimulated insulin production in isolated islets in the *db/db* model of obesity and diabetes[35]. Hence, there is a strong possibility that JNK may integrate defects in insulin secretion with peripheral IR in T2D through its actions in pancreatic β-cells as well as peripheral sites of insulin action. If this is the case, it is also likely that JNK may be important in the pathogenesis of type 1 diabetes, and recent studies have provided evidence to support a role for the JNK-2 isoform in this disease[36].

LIPOLYTIC MACHINERY, ADIPOCYTES AND NAFLD

Several lines of evidence implicate an inadequate response to lipid storage/catabolism of cellular fat stores as being important in NAFLD. Subcutaneous adipose tissue (AT) is composed mostly of small, differentiated adipocytes that absorb circulating FFAs due to their insulin-sensitivity. They form triglycerides (lipogenesis) and store them in cellular lipid droplets (LD) or lipid bodies (surrounded by a monolayer of lipase-regulating proteins) until FFAs are needed during fasting. They also secrete adiponectin, which by opposing hepatic lipogenesis and stimulating long chain fatty acid β-oxidation, protects the liver from harmful effects of lipid accumulation, such as IR[37]. In MS, failure of subcutaneous AT to store energy leads to swollen adipocytes that are stressed and de-differentiated. They continually release FFAs from triglycerides (lipolysis). Lipolysis is the biochemical pathway responsible for the catabolism of triacylglycerol (TAG) stored in cellular LD. The hydrolytic cleavage of TAG generates FFAs, which are subsequently used as energy substrates, essential precursors for lipid and membrane synthesis, or mediators in cell signaling processes. Consistent with its central importance in lipid and energy homeostasis, lipolysis mostly occurs in white and brown adipose tissue. Over the last few years, important enzymes and regulatory protein factors involved in lipolysis have been identified. These include an essential TAG hydrolase named adipose triglyceride lipase (ATGL) [a

patatin-like phospholipase domain-containing protein A2 (PNPLA2)], the ATGL activator comparative gene identification-58 (an α/β hydrolase containing protein 5), and the ATGL inhibitor G0/G1 switch gene 2. ATGL catalyzes the first step in adipocyte and muscle triglyceride hydrolysis. Together with the established hormone-sensitive lipase (lipase E) and monoglyceride lipase, these proteins constitute the basic "lipolytic machinery". Additionally, a large number of hormonal signaling pathways and lipid droplet-associated protein factors regulate substrate access and the activity of the "lipolysome"[38]. Activation of β-(AR) in adipocytes triggers acute changes in metabolism that can alter patterns of gene expression. A recent work examined the mechanisms by which activation of hormone sensitive lipase induces expression of inflammatory cytokines in adipocytes *in vivo* and model adipocytes *in vitro*. β3-adrenergic receptor (AR) activation in mice triggered expression of inflammatory genes CCL2, IL-6, and PAI-1, as well as ER stress markers GRP78 and CHOP[39]. Recent findings suggest that genetic variants in PNPLA3 predispose towards hepatic steatosis and, in the context of other environmental stressors, progression to irreversible liver failure. PNPLA3 is predominantly expressed in human liver and adipose tissue, possesses both lipolytic and lipogenic activity *in vitro*, and is localized on the surface of lipid droplets in hepatocytes. The 148M mutant protein has reduced lipolytic activity, with attendant increased cellular triglycerides[40], only recently confirmed[41]. Studies in animal models of NAFLD demonstrate that inhibition of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT)-1, the enzymes that catalyze the final step in triglyceride synthesis, results in improvement in hepatic steatosis and insulin sensitivity. Researchers recently confirmed that hepatic-specific inhibition of DGAT-1 with antisense oligonucleotides improved hepatic steatosis in obese, diabetic mice but, unexpectedly, exacerbated injury and fibrosis in that model of progressive NAFLD. When hepatocyte triglyceride synthesis was inhibited, FFA accumulated in the liver, leading to induction of fatty acid oxidizing systems that increased hepatic oxidative stress and liver damage. These findings suggest that the ability to synthesize triglycerides may, in fact, be protective in obesity[42]. This is a key point. MEK1/2 inhibition significantly increased both cellular and microsomal triglycerides mass, and mRNA levels for DGAT-1 and DGAT-2. In contrast to ERK, modulation of the phosphatidylinositol 3-kinases pathway or inhibition of the p38 MAP kinase, had no effect on lipoprotein density profile[43]. The biogenesis of LD induced by serum depends on group IVA phospholipase A(2) [cPLA(2)α/GIVA PLA(2)], a regulatory enzyme that releases arachidonic acid for production of prostaglandins and leukotrienes. Recent data suggest that cPLA(2)α regulates the transport of tight junction and adherens junction proteins through Golgi cell-cell contacts in confluent endothelial cells. Expression of specific activators of different MAP kinases show that phosphorylation of cPLA(2)α at Ser-505 is due to JNK. This was confirmed by pharmacological inhibition and expression of a dominant-negative form of the upstream activator MEKK1. LD biogenesis was accompanied by increased synthesis of ceramide 1-phosphate. Overexpression of its synthesizing enzyme ceramide kinase increased phosphorylation of cPLA(2)α at Ser-505 and the formation of LD, and its downregulation blocked the phosphorylation of cPLA(2)α and LD biogenesis. These results demonstrate that LD biogenesis induced by serum is regulated by JNK and ceramide kinase[44].

The effect of glucose and palmitate on the phosphorylation of proteins is associated with cell growth and survival. Fresh results suggest that short-term changes in MAPK and AKT signaling pathways, and c-fos and c-JNK expressions induced by glucose are abolished by palmitate through phosphatidylinositol 3-kinase inhibition *via* ceramide synthesis[45].

Activation of β-AR in mouse adipocytes triggered expression of inflammatory genes CCL2, IL-6, and PAI-1, as well as ER stress markers GRP78 and CHOP. Pharmacological inhibition of hormone sensible lipase (HSL) blocked induction of inflammatory genes, but not ER stress markers. Promoting intracellular accumulation of FFAs in 3T3-L1 adipocytes increased the expression of inflammatory cytokines, whereas inhibiting ceramide synthesis partly blocked PAI-1 expression, but not IL-6. Induction of inflammatory markers *in vivo* and *in vitro* was preceded by phosphorylation of p38 and JNK, and inhibition of HSL prevented activation of these kinases. Together, these results demonstrate that FFAs liberated by HSL activate p38 and JNK, and p38 mediates pro-inflammatory cytokine expression in adipose tissue[39].

As previously emphasized, accumulation of lipid metabolites within non-adipose tissues can induce chronic inflammation by promoting macrophage infiltration and activation. Oxidized and glycated lipoproteins, FFAs, free cholesterol, triacylglycerols, diacylglycerols and mainly ceramides have long been known to induce cellular dysfunction through their pro-inflammatory and pro-apoptotic properties. Emerging evidence suggests that macrophage activation by lipid metabolites and further modulation by lipid signaling represents a common pathogenic mechanism underlying lipotoxicity in atherosclerosis, obesity-associated IR and inflammatory diseases related to MS such as NAFLD and chronic kidney disease.

The sphingolipid ceramide is an important second signaling molecule that regulates diverse signaling pathways involving apoptosis, cell senescence, the cell cycle and differentiation. For the most part, effects of ceramide are antagonistic to growth and survival. Interestingly, ceramide and the pro-growth agonist, diacylglycerol (DAG) appear to be regulated simultaneously but in opposite directions in the sphingomyelin cycle. While ceramide stimulates signal transduction pathways that are associated with cell death or at least are inhibitory to cell growth (SAPK), DAG activates the classical and novel isoforms of the PKC family. These PKC isoforms are associated with cell growth and cell survival. Furthermore, DAG activation of PKC stimulates other signal transduction

pathways that support cell proliferation, e.g., MAPK pathways. Thus, ceramide and DAG generation may serve to monitor cellular homeostasis by inducing pro-death or pro-growth pathways, respectively. The production of ceramide is emerging as a fixture in programmed cell death. Ceramide levels are elevated in response to diverse stress challenges including treatment with pro-death ligands such as TNF-α, chemotherapeutic drug treatment or irradiation. Consistent with this notion, ceramide itself is a potent apoptogenic agent. Ceramide activates c-JNK and thus affects its transcription pathways. Ceramide activates protein phosphatases such as protein phosphatase 1 and PP2A. Ceramide activation of protein phosphatases has been shown to promote inactivation of a number of pro-growth cellular regulators including the kinases PKCα and Akt, Bcl-2 and the retinoblastoma protein. A new role has recently emerged for ceramide in the regulation of protein synthesis. Ceramide-induced activation of PKR, a protein kinase important in anti-viral host defense mechanisms and recently implicated in cellular stress pathways, results in the inhibition of protein synthesis as a prelude to cell death[46].

ENDOPLASMIC RETICULUM, CHAPERONES AND LONGEVITY

Heat shock proteins (HSPs) have proven to be effective tools for extending invertebrate lifespan, and in *C. elegans* daf-2 mutants, longevity resulting from loss of insulin/insulin-like signals is at least partly dependent upon elevated HSP expression. In mice, inhibition of the orthologous growth hormone/insulin-like growth factor I (GH/IGF- I) pathway has similar pro-longevity effects. A recent study, however, suggested that loss of GH/IGF- I signaling in long-lived mice did not broadly elevate HSP expression, but in fact decreased HSP expression in many tissue types, such as liver and kidney. The contribution of chaperones to the longevity of long-lived mice with altered GH/IGF- I signals may therefore differ from that described in *C. elegans* daf-2 mutants. This result, in combination with other recent findings, underscores the possibility that systemic overexpression of chaperones will have dissimilar effects on longevity in vertebrate and invertebrate systems[47].

MS AND CARDIOVASCULAR DISEASE RISK

MS is a constellation of common metabolic disorders that is strictly linked to CHD. It is now commonly accepted that low-grade chronic inflammation associated with obesity induces IR in the liver. Low-grade chronic inflammation is characterized by the production of abnormal cytokines and adipokines such as IL-6, TNF-α, IL-1, leptin and resistin. These factors inhibit insulin signaling in hepatocytes by activating SOCS proteins, several kinases such as JNK, IKK-β and PKC and protein tyrosine phosphatases such as PTP1B and PTEN that in turn impair insulin signaling at the insulin receptor and IRS level. Hepatic IR in turn causes impaired suppression of glucose production by insulin in hepatocytes leading to hyperglycemia. An important and early complication of hepatic IR is the induction of hepatic VLDL production, *via* changes in the rate of apoB synthesis and degradation and *de novo* lipogenesis, or increased FFA flux from adipose tissue into the liver. IR also stimulates the production of C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), both markers of an inflammatory state. All these subsequent metabolic abnormalities related to hepatic IR have been shown to directly or indirectly promote atherosclerosis. Hyperglycemia induces a series of alterations including endothelial dysfunction, cellular proliferation, changes in extracellular matrix conformation and impairment of low density lipoprotein (LDL) receptor-mediated uptake, decreasing the *in vivo* clearance of LDL. Small dense LDLs associated with high circulating VLDL levels have higher affinity for the intimal proteoglycans leading, to the penetration of more LDL particles into the arterial wall. CRP can also accelerate atherosclerosis by increasing the expression of PAI-1 and adhesion molecules in endothelial cells, inhibiting nitric oxide formation and increasing LDL uptake into macrophages.

Recently it has been shown that a small molecule pan-JNK inhibitor, dosed orally and compared to rimonabant and rosiglitazone, significantly impacted parameters such as adiposity, glucose levels, and insulin sensitization without any effect on liver enzymes, thus establishing the role of JNK as a useful target for metabolic syndrome linked to the pre-diabetic state[48]. A JNK1 specific antisense oligonucleotide was studied in ob/ob and diet-induced obese mouse models. Profound improvement in insulin sensitivity, glucose levels, plasma cholesterol level, and adiposity without a negative impact on liver function was observed. Decreased body weight and lowered adiposity were attributed to increased food combustion/metabolic rate and decreased lipogenesis[49].

Describing the common mechanisms by which lipid derivatives, through modulation of macrophage function, promote plaque instability in the arterial wall, impair insulin responsiveness and contribute to inflammatory liver and discussing the molecular mechanism of lipid activation of pro-inflammatory pathways [JNK, nuclear factor (NF)κB], the key roles played by the proliferator-activated receptor and liver X receptor α, nuclear receptors-lipid sensors that link lipid metabolism and inflammation, should be emphasized[50]. Atherosclerosis begins as local inflammation of artery walls at sites of disturbed blood flow. JNK is thought to be among the major regulators of flow-dependent inflammatory gene expression in endothelial cells in atherosclerosis. Researchers have shown that JNK activation by both onset of laminar flow and long-term oscillatory flow is matrix-specific, with enhanced activation on fibronectin

compared to basement membrane protein or collagen. Flow-induced JNK activation on fibronectin requires new integrin ligation and requires both MKK4 and p21-activated kinase. *In vivo*, JNK activation at sites of early atherogenesis correlates with the deposition of fibronectin. Inhibiting p21-activated kinase reduces JNK activation in atheroprone regions of the vasculature *in vivo*. These results identify JNK as a matrix-specific, flow-activated inflammatory event. These data elucidate a network of matrix-specific pathways that determine inflammatory events in response to fluid shear[51].

Visceral AT is known to confer a significantly higher risk of T2D and CHD. Epicardial AT has been shown to be related to cardiovascular disease and myocardial function. Epicardial AT expresses an inflammatory profile of proteins. Authors studied key mediators of the NFκB and c-JNK pathways in paired epicardial and gluteofemoral (thigh) AT from CHD and investigated circulating endotoxin levels in CHD and control subjects. Serums and AT biopsies (epicardial and thigh) were obtained from CHD and non-CHD patients. Inflammation was assessed in tissue and serum samples through western blot, real-time PCR, ELISAs, and activity studies. Western blotting showed epicardial AT had significantly higher NFκB, inhibitory-κB kinase (IKK)-γ, IKK-β, and JNK-1 and -2 compared with thigh AT. Epicardial mRNA data showed strong correlations between CD-68 (again the impaired immunity function) and toll-like receptor-2, toll-like receptor-4, and TNF-α. Circulating endotoxin was elevated in patients with CHD compared with matched controls. Epicardial AT from patients with CHD shows increased NFκB, IKK-β, and JNK expression compared with both CHD thigh AT and non-CHD epicardial AT, suggesting a depot-specific as well as a disease-linked response to inflammation[52].

METHODS TO DETECT JNK

The detection of protein kinases is possible in biological liquids such as blood serum or cell lysate. Sandwich ELISAs for detecting phosphoproteins have commonly been used to quantify kinase function and can be performed in 2 configurations. In the first configuration, polyclonal antibodies directed against the structural part of the protein and away from the phosphorylation site (panprotein) are coated onto the bottom of a microwell plate. A cell lysate containing the phosphorylated target protein is added to the well, allowed to bind and the excess lysate is removed by washing. A monoclonal antibody of either mouse or rabbit origin, specific for the phosphorylated form of the protein, is added followed by an enzyme-labeled secondary antibody specific for the monoclonal antibody species. A chromagen is added and the color is quantified spectrophotometrically. In the second configuration, the capture antibody is directed against the phosphor-antibody and the detection antibody is an antibody directed against the panprotein. The latter configuration is sometimes preferred as the amount of phosphor-protein present may be small compared to the total amount of the panprotein.

In this situation, the large amount of non-phosphorylated panprotein can outcompete the phosphorylated protein for binding to the microwells. This decreases the overall sensitivity of the assay for the phosphor-protein. Using a phosphor-specific capture antibody enriches for the desired target and significantly increases the sensitivity of the assay. Phospho-ELISAs can be used to assess kinase activity in cell lysates or, alternatively, to screen drug candidates targeting a purified kinase. Quantitative measurement of protein phosphorylation has become essential for the development of kinase-inhibiting drugs aimed at therapy of various metabolic diseases. Since kinases are a major source of drug targeting, complex but reliable assay technologies that quantify phosphorylation will continue to be in demand. Biochemical assays that rely on antibodies for assay function are limited by the availability of phosphor-specific antibodies with high affinity and specificity. While many phosphospecific antibodies exist, most are unsuitable for use in quantitative assays due to poor sensitivity or nonspecificity. Alternative methods such as mobility shift, IMAP (a variation of fluorescence polarization that employs nanoparticles bearing immobilized trivalent metal co-ordination complexes that bind specifically to phosphate groups), IQ (using as signal a peptide comprised of an aminoacid sequence recognized by the desired kinase that is synthesized with a fluorophore end-label) and light-speed assays (the signal is generated from a polystyrene microsphere that is coated with a modified fluorescent polyelectrolyte) do not rely on antibodies and allow assessment of targets for which no suitable antibodies exist. As a result, these formats will find wider use in the future[53].

CONCLUSION

As repeatedly emphasized, inflammation is the common mechanism underlying obesity, MS, NAFLD[54], longevity, CHD and perhaps some cancers. The chicken-and-egg dilemma of IR being cause or effect of inflammation is unsolved at present. Further studies are warranted of anti-inflammatory drugs such as aspirin, anti IL-6 receptors, immune-modulators (calcineurin inhibitors)[55], substances enhancing the expression of HSPs (which protect cells from ER stress-induced apoptosis), and anti-JNKs in well-designed trials to try to minimize the high impact of these illnesses, and the different expressions of the diseases, on the whole population[56].

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REVIEW

Natural orifice transluminal endoscopy surgery: A review

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Abstract

Minimally invasive surgery started spreading worldwide in 1987, when the first laparoscopic cholecystectomy was performed. Meanwhile, improvement of endoscopic equipment and instruments allowed gastroenterologists to attempt more aggressive endoluminal interventions, even beyond the wall barrier. The first transgastric peritoneoscopy, in 2004, brought to light the concept of natural orifice transluminal endoscopic surgery (NOTES). The idea of incisionless surgery is attractive and has become a new goal for both surgeons and other people interested in this field of investigation. The authors present a review of all developments concerning NOTES, including animal studies and human experience.

Key words: Transesophageal; Transgastric; Transvesical; Transvaginal; Transcolonic; Natural orifice transluminal endoscopic surgery; Minimally invasive techniques

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INTRODUCTION

Surgery has experienced a huge development in the past three decades after Dr. Philippe Mouret performed the first laparoscopic cholecystectomy in 1987. Since then, minimally invasive surgery has begun to spread worldwide[1]. This was largely in part due to patient demands for laparoscopic surgery's advantages - shorter hospital stays, less pain, and smaller, less disfiguring scars[2]. The improvement of available equipment and instruments allowed more and more surgical procedures to be made through a minimally invasive approach, rapidly becoming a standard practice in most procedures.

At the same time, progresses in gastrointestinal endoscopy have made it an indispensable and multifaceted instrument for diagnosis and therapy. Besides endoluminal procedures, gastroenterologists attempted some interventions beyond the wall barrier, such as pseudocyst drainage[3] and percutaneous endoscopic gastrostomy[4]. However, it was not until 2004 that Kalloo *et al*[5] published the first report of a true transluminal procedure, a transgastric peritoneoscopy in a porcine model, which brought to light the concept of natural orifice transluminal endoscopic surgery (NOTES). The idea of incisionless surgery was attractive and has now become a new goal for both surgeons and other people interested in this field of investigation.

The term NOTES describes novel endoscopic interventions on internal organs performed through natural orifices[6]. In this new approach, endoscopes enter the abdominal and thoracic cavities *via* any single or combination of natural orifices - mouth, urethra, vagina, and anus. Depending on the orifice, rigid or flexible equipment can be used. The lower "short-ways" (bladder, colon or vagina) allow the easy passage of rigid or flexible instruments into the abdominal cavity, but the upper "long-ways" (esophagus and stomach) require flexible equipment[7] (Figure 1).

The main goal for NOTES is avoiding skin incisions. Other theoretical advantages include: decreased post-operative pain, reduction/elimination of general anesthesia, performance of procedures in an outpatient or even office setting, and possibly cost reduction. Moreover, eliminating skin incision avoids associated complications such as wound infections and hernias, as well as reduction in hospital stay, faster return to bowel function, improved cosmetic outcomes, and increased overall patient satisfaction[2].

WHAT DID THE INVESTIGATION ACHIEVE SO FAR?

The first challenge in NOTES is getting good and clean access to the cavity we want to "scope" (Table 1). The first mention of natural orifice procedure dates back to the 1940s, when culdoscopies were performed using an endoscope passed through the recto-uterine pouch to view pelvic organs, as well as to perform sterilization procedures[8]. At that time, these procedures did not gain much popularity and were restricted to some gynecological procedures. Recently, however, they were recovered by NOTES development. In 2002, Gettman *et al*[9] published one pure transvaginal nephrectomy along a series of hybrid transvaginal nephrectomy in a porcine model.

Taking advantage of the great developments in gastrointestinal endoscopy, some pioneers began working on the transgastric approach to the abdominal cavity. The first published description of transgastric peritoneoscopy was in 2004 by Kalloo *et al*[5], in a porcine model. Since then, a number of successful transgastric procedures have been attempted and performed[10-20]. These initial studies also identified major limitations of the isolated transgastric approach, mainly in more complex procedures such as cholecystectomy, first described in 2005 by Park *et al*[21]. Lack of triangulation and platform stability were the main problems identified. Searching for solutions to these problems, researchers tried other ways of entering the abdominal cavity. Fong *et al*[22-24] published the first transcolonic peritoneoscopy followed by a series of transcolonic procedures. The access from below gives a good, direct view of the upper abdominal cavity. Having that in mind, Lima *et al*[25] published the first transvesical endoscopic peritoneoscopy. And subsequently our group used a combination of transgastric and transvesical approaches to solve the problem of triangulation, and managed to do a series of cholecystectomies and nephrectomies in porcine models[26,27].

To accomplish NOTES procedures in the thorax, Sumiyama *et al*[28] proposed the transesophageal access. Transvesical-transdiaphragmatic thoracoscopy[29], transgastric-transdiaphragmatic thoracoscopy[30], and transtracheal thoracoscopy[31] have been suggested as well. Although the transesophageal method has been preferred as a direct entry to the thorax and posterior mediastinum, this permitted several simple thoracic procedures in porcine models[32-38].

CURRENT CHALLENGES

Despite the enthusiasm for NOTES, there are still some hurdles to be overcome. The initial concern is the potential for intra-abdominal infection and spillage from the viscerotomy. Infection must first be prevented by using a clean access site. Most transgastric protocols also follow a 24 h liquid formula diet, intravenous antibiotics and stomach irrigation with sterile water and antibiotic solution. Despite these precautions, even a sterile overtube used to protect the endoscope from oral contamination becomes contaminated on oral insertion and can transport bacteria to both the stomach and the peritoneal cavity[2]. Surprisingly, Narula *et al*[39] reported no infections after gastrotomy in patients undergoing diagnostic transgastric peritoneoscopy without previous gastric decontamination. The authors considered that the same degree of contamination of the peritoneal cavity is expected as in any operation performed with an open viscus.

There is also some controversy about the need for endoscope sterilization. In a recent literature review, Spaun *et al*[40] concluded that, although difficult, it is possible to terminally sterilize flexible endoscopes. Steris System 1™ that uses 0.2% peracetic acid was the cheapest and fastest sterilization method and scored second in the risk of recontamination. Ethylene oxide gas sterilization has the lowest risk of recontamination, but is the slowest and most expensive method. The authors recommend sterile instrumentation for clinical NOTES until well-designed and randomized clinical trials are available and guidelines are published.

Concerning viscerotomy closure, gastrotomy has been the most studied and the methods under investigation could also be applied to the colon, esophagus or bladder, depending on the circumstances. Several methods have been proposed for stomach closure, including: conventional endoscopic clips, over-the-scope clip (OTSC) system, septal occluders, T-tags, T-bars for tissue opposing, as well as more complex suturing devices such as the Eagle Claw Ⅶ, NDO Plicator, USGI Endosurgical Operating System, and linear endoscopic staplers. Most of these devices still have limitations that need improving, but OTSC shows the most promising results[41]. More recently, the Padlock-G clip have been described as also showing promising results[42]. Colonic closure in animal studies has been performed using the

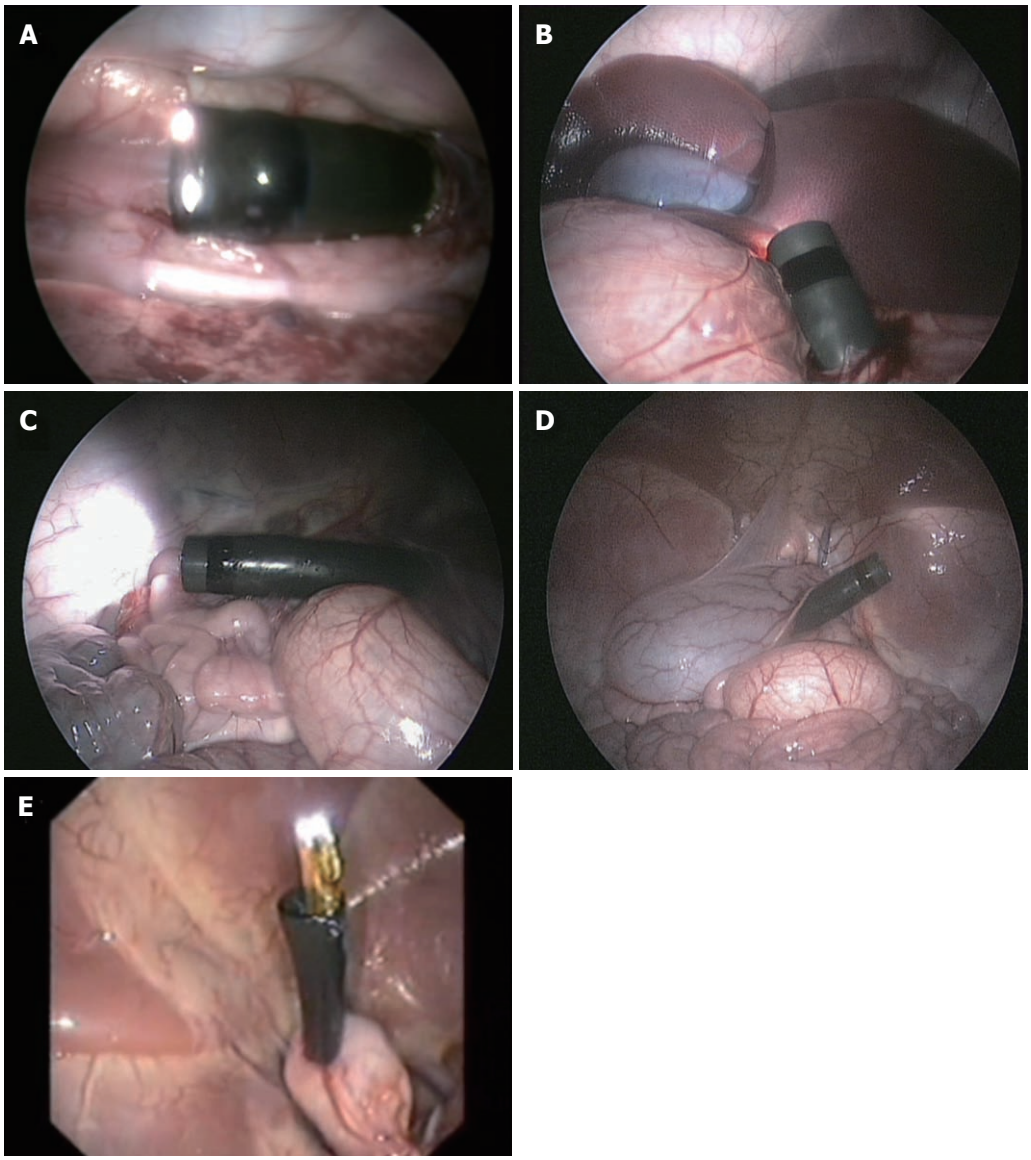

Figure 1 Internal view of natural orifice transluminal endoscopic surgery access (porcine model). A: Transthoracic view of transesophageal access; B: Transabdominal view of transgastric access; C: Transabdominal view of transcolonic access; D: Transabdominal view of transvaginal access; E: Transgastric view of transvesical access.

same techniques and devices as those used for gastrotomy closure. Transanal endoscopic microsurgery has been used for a long time, and has been useful for colonic closure in hybrid NOTES procedures in humans[43]. For vesicotomy closure, Lima *et al*[44] recently reported the first successful endoscopic closure using a suturing kit (T-fasteners with a locking clinch). Easy and safe closure has been the main advantage for transvaginal route acceptance. Closure after transvaginal access is readily and routinely performed by using standard surgical techniques. Even if closure were to fail, there would be little, if any, clinical significance, because of the extremely low risk of infection or hernia.

Concerning adequate exposure and visualization, pneumoperitoneum is a key component. Air insufflated in an uncontrolled manner through the endoscope results in wide fluctuations in intraperitoneal pressures, overdistension of the abdomen, and adverse hemodynamic effects. Insufflated air can also leak around the endoscope resulting in bowel overdistension[2]. Many authors are now using a Veress needle to inject carbon dioxide and safely control its pressure inside the abdomen[45]. Despite this, new insufflators are being adapted to both deliver and monitor carbon dioxide through the endoscope[46]. There is a great debate whether CO_2 or room air should be used. The effect of CO_2 with respect to laparoscopy has suggested an overall attenuated inflammatory response that may provide a further immunologic benefit. The acidic environment created has been the main contributing factor believed to facilitate this physiologic result. Conversely, "room air" laparoscopy has been shown to generate a greater inflammatory response, but a recent case-control study did not find a significant difference between the peritoneal inflammatory response of NOTES *vs* laparoscopy with carbon dioxide and air pneumoperitoneum[47].

As previously stated, maintaining spatial orientation and triangulation of instruments is challenging when using a flexible endoscope. Moreover, flexible endoscopes are difficult to stabilize inside the abdominal cavity and can only pass flexible instruments which are too flaccid for retraction. This challenge can be overcome with

Table 1 Major features of the different natural orifice transluminal endoscopic surgery access for thoracic and abdominal cavities

	Transesophageal	Transgastric	Transvesical	Transvaginal	Transcolonic
Rigid instruments	No	No	Yes	Yes	Yes
Available in both genders	Yes	Yes	Yes	No	Yes
Sterility	No	No	Yes	No	No
Size	Wide	Wide	Up to 6 mm	Wide	Wide
Closure	Endoscopic (in study)	Endoscopic (in study)	Endoscopic (in study)	Direct suture	Endoscopic (in study)
Specimen retrieval	Not reported	Possible	Not reported	Possible	Possible

adequate training, a combination of different routes, and with the constant development of new instruments. Transvaginal, transcolonic and transvesical routes allow the introduction of rigid equipment, and except in the transvesical route, the instruments can be used either through a rigid endoscope or in parallel with a flexible endoscope. Additionally, these access routes coming from the lower abdomen permit a good direct visualization of the upper abdomen. In some cases, one can use an additional transabdominal port. This has been named hybrid NOTES and has been seen as an intermediate step of great help in the training and development of NOTES[48]. Recently, magnets are being managed to provide the vigorous traction and countertraction required to advance NOTES procedures[49]. A new magnetic anchoring and guidance system allows concurrent use of multiple working instruments and control of an intra-abdominal camera. It has been used to perform transvaginal, single-port cholecystectomy[50]. Finally, one of the hurdles of NOTES is getting solid organs out of the thoracic and abdominal cavities. Excision of larger organs such as a kidney, or a gallbladder filled with stones through a small trocar orifice is a huge challenge. The transvaginal access has a big advantage in this matter and has been used for specimen retrieval in most NOTES procedures. On the other hand, transvaginal access is only an option in female patients.

HUMAN EXPERIENCE

In 2003, Rao and Reddy[51] performed the first NOTES procedure in humans. The authors carried out a transgastric appendectomy in a male patient presenting severe burn lesions in his abdominal wall using a conventional flexible endoscope with two working channels. Only in 2007, was there the first published human NOTES procedure. Marks *et al*[52] performed a transgastric rescue of a prematurely dislodge gastrostomy tube. The authors advanced a standard gastroscope through the previous gastrostomy, a performed peritoneoscopy, and suctioned away intra-abdominal free fluid. In that same year, another case reported the first human transvesical peritoneoscopy using a flexible ureteroscope during a standard laparoscopic robot-assisted prostatectomy[53].

The first natural orifice transluminal cholecystectomy in humans was performed in Strasbourg, France[54]. A 30-year-old woman with symptomatic cholelithiasis was submitted to cholecystectomy using a standard double-channel flexible gastroscope and standard endoscopic instruments. A 2-mm transabdominal needle port was used to insufflate carbon dioxide, to monitor the pneumoperitoneum, and to retract the gallbladder. Colpotomy was closed using conventional instruments. The patient had no post-operative pain and no scars, and was discharged on the second post-operative day. Shortly after that, the same technique was used by a team in Brazil, and by another in Italy[55,56].

In 2007, a group of investigators from Ohio, United States used transgastric peritoneoscopy after standard laparoscopy to diagnose pancreatic masses[57]. In 9 out of 10 patients, transgastric abdominal exploration corroborated the decision to proceed to open exploration made during traditional laparoscopic exploration. The average time of diagnostic laparoscopy was 12.3 min, compared to the 24.8 min taken for the transgastric route. Closure of the gastrotomy was obviated through its integration into the primary operation, whether that involved a resection with curative intent or palliation. No cross-contamination of the peritoneum or infectious complications was noted.

Other procedures using exclusively natural orifice transluminal procedures in humans have been performed - transgastric and transduodenal pancreatic necrosectomy[58], transvaginal incisional hernia repair[59], transvaginal liver, diaphragm, ovaries, and peritoneum biopsies[60], and transvaginal appendectomy[61]. This last one is especially important, as two of the three cases presented had an umbilical port inserted in order to complete appendectomy. As seen before in cholecystectomy, the use of a transabdominal port is essential to make natural orifice approaches feasible or at least easier at this time. Hybrid NOTES procedures are seen as a safe way to accomplish pure NOTES in the future. For this aim, hybrid procedures are developing in humans and achieving new goals like transvaginal nephrectomy[62], transrectal rectosigmoidectomy[63], sleeve gastrectomy[64], transvaginal liver resection[65], transvaginal splenectomy[66], transgastric cholecystectomy[67], transanal rectal cancer resection[43], intragastric stapled cystogastrostomy of a pancreatic pseudocyst[68], and adjustable gastric banding[69].

In 2009, de Sousa *et al*[70] published the first series of pure NOTES transvaginal cholecystectomies. The authors performed four cholecystectomies using two

endoscopes introduced simultaneously in the abdominal cavity through a transvaginal incision. Dissection was accomplished with conventional endoscopic instruments. Ligation of the cystic duct and artery was performed using endoscopic clips. Vaginal closure was achieved using the direct-vision suture technique. More recently, Bessler *et al*[71] described a different technique for pure NOTES cholecystectomy in a 35-year-old-woman. Instead of using two endoscopes, the authors used an extra-long 5-mm articulating retractor placed into the abdomen *via* a separate colpotomy made under direct vision using the flexible endoscope in a retroflexed position. This method overcame the retracting limitations that obliged the use of a transabdominal port.

Despite all the enthusiasm around NOTES, other clinical advantages besides the absence of skin incision remain to be fully proven. Although most studies claim that greater operative time would be compensated by shorter hospital stays, prospective control studies are lacking[72]. Hensel *et al*[73] reported a retrospective case-control study where hybrid transvaginal cholecystectomy group showed a lower need for analgesics, faster mobilization, more comfortable recovery and a shorter hospital stay than the conventional laparoscopy group.

Finally, patients' perspectives and expectations about NOTES are not yet fully understood. An interesting questionnaire-based study was derived to identify their preferences between different available surgical options upon a hypothetical scenario of an acute appendicitis[74]. Single port surgery (SPS) was the most popular method followed by conventional laparoscopy. Open surgery and NOTES were the least preferred. Choosing between SPS and NOTES only, 80.6% opted for SPS, 11.8% NOTES, and 5.6% declined surgery. The most popular route of access for NOTES was oral (37.7%). Another study asked women about their concerns and opinions regarding transvaginal surgery[75]. The majority of women (68%) indicated that they would want a transvaginal procedure in the future because of decreased risk of hernia and decreased operative pain (90% and 93%, respectively), while only 39% were concerned with the improved cosmesis of NOTES surgery. Of the women polled, nulliparous women and those under age 45 years were significantly more often concerned with how transvaginal surgery may affect healthy sexual life and fertility issues. Of the women who would not prefer transvaginal surgery, a significant number indicated concerns over infectious issues.

THE FUTURE OF NOTES

NOTES promises a new and innovative era of minimal access surgery based on traditional laparoscopic and endoscopic techniques. Researchers all over the world are investigating ways to improve NOTES procedures in order to make it easier and safer. With careful development of new equipment and techniques, NOTES may be a reasonable option to conventional laparoscopic procedures. It may even become the method of choice for selected surgical procedures in the future.

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ORIGINAL ARTICLE

Rebamipide promotes healing of colonic ulceration through enhanced epithelial restitution

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Abstract

AIM: To investigate the efficacy of rebamipide in a rat model of colitis and restitution of intestinal epithelial cells *in vitro*.

METHODS: Acute colitis was induced with trinitrobenzene sulfonic acid (TNBS) in male Wistar rats. Rats received intrarectal rebamipide treatment daily starting on day 7 and were sacrificed on day 14 after TNBS administration. The distal colon was removed to evaluate the various parameters of inflammation. Moreover, wound healing assays were used to determine the enhanced restitution of rat intestinal epithelial (RIE) cells treated with rebamipide.

RESULTS: Intracolonic administration of rebamipide accelerated TNBS-induced ulcer healing. Increases in the wet weight of the colon after TNBS administration were significantly inhibited by rebamipide. The wound assay revealed that rebamipide enhanced the migration of RIE cells through phosphorylation of extracellular signal-regulated kinase (ERK) and activation of Rho kinase.

CONCLUSION: Rebamipide enema healed intestinal injury by enhancing restitution of RIE cells, *via* ERK activation. Rebamipide might be a novel therapeutic approach for inflammatory bowel disease.

Key words: Rebamipide; Experimental colitis; Intestinal epithelial cells; Extracellular signal-regulated kinase; Rho kinase

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INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn' s disease (CD), is a chronic and recurrent intestinal inflammatory disorder whose precise pathogenesis remains unknown[1]. In patients with IBD, a variety of cells and soluble factors mediate extensive mucosal damage, and epithelial cell damage is frequently observed[2]. Generally, after sustaining mucosal injury, the intestinal epithelium rapidly reestablishes its integrity *via* restitution, proliferation, and differentiation of epithelial cells[3]. Among these three steps, restitution is thought to be most critical for intestinal mucosal healing[4]. Therefore, the promotion of restitution remains an important therapeutic target. Along these lines, various molecules involved in regenerating the intestinal epithelium are currently under consideration for clinical use[5].

Rebamipide is an amino acid derivative of 2(1H)-quinolinone, and is a gastric mucosal protective and ulcer-healing agent that has been widely used for treatment of acute and chronic gastritis and gastric ulcer. It is already known that rebamipide has anti-inflammatory properties including scavenging of free radicals, suppression of pro-inflammatory cytokine production, inhibition of inflammatory cell migration and adherence, and promotion of prostaglandin and mucus production[6]. Recently, rebamipide has been used as a gastric protective agent and for treatment of UC[7]. First, Makiyama *et al*[8] have reported that rebamipide enema had an anti-inflammatory effect in a patient with proctitis-type UC. They also have reported the efficacy of rebamipide enemas in active distal UC and proctitis in a prospective study[9]. Furthermore, it has been demonstrated that rebamipide enema is safe and useful in corticosteroid-refractory or -dependent patients with the active distal type of UC[10]. In addition, Matsumoto *et al*[11] have reported that rebamipide enema ameliorates disease activity in patients with left-side ischemic colitis. Thus, these clinical data suggest that rebamipide shows promise in terms of its potential for repairing intestinal injury. However, the detailed molecular mechanism of action of rebamipide against intestinal inflammation remains unclear.

Therefore, in the present study, we aimed to assess the effect of rebamipide in intestinal inflammation by using the trinitrobenzene sulfonic acid (TNBS)-induced colitis model, a well-accepted IBD model. Furthermore, we analyzed the possible mechanisms involved in rebamipide-mediated mucosal restitution by using a well-established model of intestinal epithelial wound healing *in vitro*[12,13].

MATERIALS AND METHODS

Reagents

All chemicals were prepared immediately before use. Rebamipide {2-(4-chlorobenzoylamino)-3[2-(1H)-quinolinon-4-yl] propionic acid} was a kind gift from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan). TNBS and 3, 3', 5, 5'-tetramethylbenzidine were obtained from Wako Pure Chemicals (Osaka, Japan). We used MEK1/2 inhibitor as an extracellular signal-regulated kinase (ERK) inhibitor (U0126; BIOMOL International LP, Plymouth Meeting, PA, United States) and Y27632 [(1)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] as the Rho kinase inhibitor (Biaffin GmbH and Co KG, Kassel, Germany). All other chemicals were of the highest quality commercially available.

In vivo study animals

Male Wistar rats weighing 180-200 g were obtained from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). The animals were housed at 22 ℃ in a controlled environment with 12 h of artificial light per day, and were allowed access to rat chow and water *ad libitum*. The animals were maintained and all experimental procedures were carried out in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals. All experimental protocols were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

Induction of colitis

TNBS-induced colitis was established using the method of Morris *et al*[14]. The rats were lightly anesthetized with pentobarbital following a 48-h fast, and then a rubber catheter (outer diameter, 2 mm) was inserted *via* the anus, such that the tip was 8 cm from the anus. TNBS dissolved in 50% ethanol (120 mg/mL) was instilled into the lumen of the colon *via* the catheter (volume, 0.25 mL). Following the instillation of TNBS at 30 mg per rat, the anus was occluded with a clip for 1 h.

Treatment protocol

All animals were randomized into groups that received rebamipide or physiological saline vehicle. We focused on the effects of rebamipide during healing after colonic mucosal injury. One percent rebamipide was intrarectally administered (2 mL/kg) twice daily starting on day 7 after induction of colitis, until day 14.

Evaluation of colonic damage

The rats were sacrificed on day 14 and the distal colon was removed and opened by longitudinal incision. The wet colon weight was measured immediately thereafter. As indices of inflammation, damage was estimated macroscopically as the sum of the mucosal score. The mucosal score was rated on a six-point scale (0-5) according to the criteria established by Morris *et al*[14] (Table 1). The degree of colitis was evaluated by an independent observer who did not have previous knowledge of the treatment. For the histological examination, formalin-fixed tissue was stained with hematoxylin and eosin. The colon histological score was evaluated using the histopathological grading system of Ameho *et al*[15] by an observer blinded to the treatment. This grading, which takes into account the degree of infiltration, the presence of erosion, ulceration, or necrosis, and the depth and

Table 1 Criteria for scoring gross morphological damage of the colon[12]

Mucosal score	Gross morphology
0	No damage
1	Localized hyperemia, but no ulcers
2	Liner ulcers with no significant inflammation
3	Liner ulcers with inflammation at one site
4	Two or more sites of ulceration and/or inflammation
5	Two or more major sites of inflammation and ulceration extending > 1 cm along the length of the colon

surface of the lesion, is scaled from 0 to 6.

In vitro study of intestinal epithelial cell line

Non-transformed rat intestinal epithelial (RIE) cells were grown in a 1:1 mixture of Dulbecco' s Modified Eagle' s Medium and Ham' s F12 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. The cells were incubated at 37 ℃ in a humidified atmosphere supplemented with 5% CO_2. RIE cells were trypsinized and seeded into 60-mm culture dishes. Experiments were performed when the cells reached confluency.

Wound assay

Wound assays were performed using a previously described method with minor modifications[16]. Confluent monolayers of RIE cells in 60-mm culture dishes were washed with phosphate-buffered saline, and cells were cultured for an additional 24 h in serum-free medium. Subsequently, cell monolayers were disrupted using an extra long 10-μL pipette tip (Pelican Life Sciences, San Diego, CA, United States), followed by a cycle of washing in serum-free medium to yield a cell-free zone in the culture dishes. The process of migration was monitored using an inverted phase-contrast microscope at 0, 6 and 12 h after induction of the artificial wound. Changes in the cell-free zone were analyzed with the ImageJ software (Wayne Rashband; NIH, Bethesda, MD, United States), and this analysis was performed by the same individual under blind conditions to prevent observer bias. To investigate the effects of rebamipide on RIE cell migration, the cells were co-incubated with rebamipide (2 mmol/L) after wound induction. Furthermore, to investigate involvement of the ERK signaling pathway and the Rho kinase pathway, cells were co-treated with U0123 (10 μmol/L) or Y27632 (1 μmol/L) after wounding.

Western blotting analysis

To determine whether rebamipide was involved in the ERK signaling pathway, proteins were obtained from RIE cells at 0, 5, 15 and 20 min after stimulation with 2 mmol/L rebamipide. The total proteins were mixed with SDS sample buffer. The samples were then subjected to 10% SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Atto Corporation, Tokyo, Japan). The membrane was blocked with 2% bovine serum albumin in Tris-buffered saline that contained 0.1% Tween (TBS-T) at room temperature for 30 min. Western blotting analysis was carried out using rabbit polyclonal anti-p44/42, phospho-p44/42 (1:1000), and actin antibody (1:1000) as an internal control at room temperature for 1 h. After three washes with TBS-T, the membrane was incubated with anti-rabbit IgG–horseradish peroxidase (1:3000; GE Healthcare UK Ltd., Little Chalfont, Bucks, United Kingdom) at room temperature for 45 min. The signals were visualized using an ECL kit (GE Healthcare) according to the manufacturer' s instructions.

Statistical analysis

Results are presented as the mean ± SE. Overall differences between groups were determined by one-way ANOVA. Whenever one-way ANOVA was significant, differences between individual groups were analyzed by Bonferroni' s multiple comparisons test. Differences of $P < 0.05$ were considered significant. All analyses were performed using the GraphPad Prism 4 program (San Diego, CA, United States) for a Macintosh computer.

RESULTS

Therapeutic effect of rebamipide on TNBS colitis

In rats exposed to TNBS, macroscopic findings in the colon demonstrated severe colitis with hyperemia, edema, thickening, ulceration, and necrosis. It has already been demonstrated that the lesion area reaches its maximum on day 2 or 3 after TNBS treatment, after which it decreases in a time-dependent manner[13,17]. In order to focus on the effects of mucosal healing by rebamipide enema, all rats were administered either placebo or rebamipide solution starting on day 7 after induction of TNBS, until day 14. On day 14, severe colitis with thickening of the mucosa and ulceration were still observed in the placebo group (Figure 1A). In contrast, rats treated with 1% rebamipide showed smaller erosions and mild edema in the colon (Figure 1A). Thus, the colonic mucosal damage score on day 14 had significantly increased due to TNBS administration in the sham-treated group. Increases in the mucosal damage score were significantly inhibited by treatment with 1% rebamipide (Figure 1B). Furthermore, the colonic wet weight was significantly increased in the TNBS colitis group. This increase was significantly decreased by treatment with 1% rebamipide (Figure 1C).

The therapeutic effects of 1% rebamipide enema were also confirmed by histological examination. Figure 2A shows the representative histological features of a normal colon (day 0) and those of the control group (day 14) and the rebamipide-treated group (day 14). TNBS administration induced marked thickening of the colonic wall, with transmural infiltration and aggregation of numerous inflammatory cells (Figure 2A), which is in contrast to the features of the normal colon, which does not show transmural infiltration or aggregation of inflammatory cells (Figure 2A). On the contrary, in rats treated with 1%

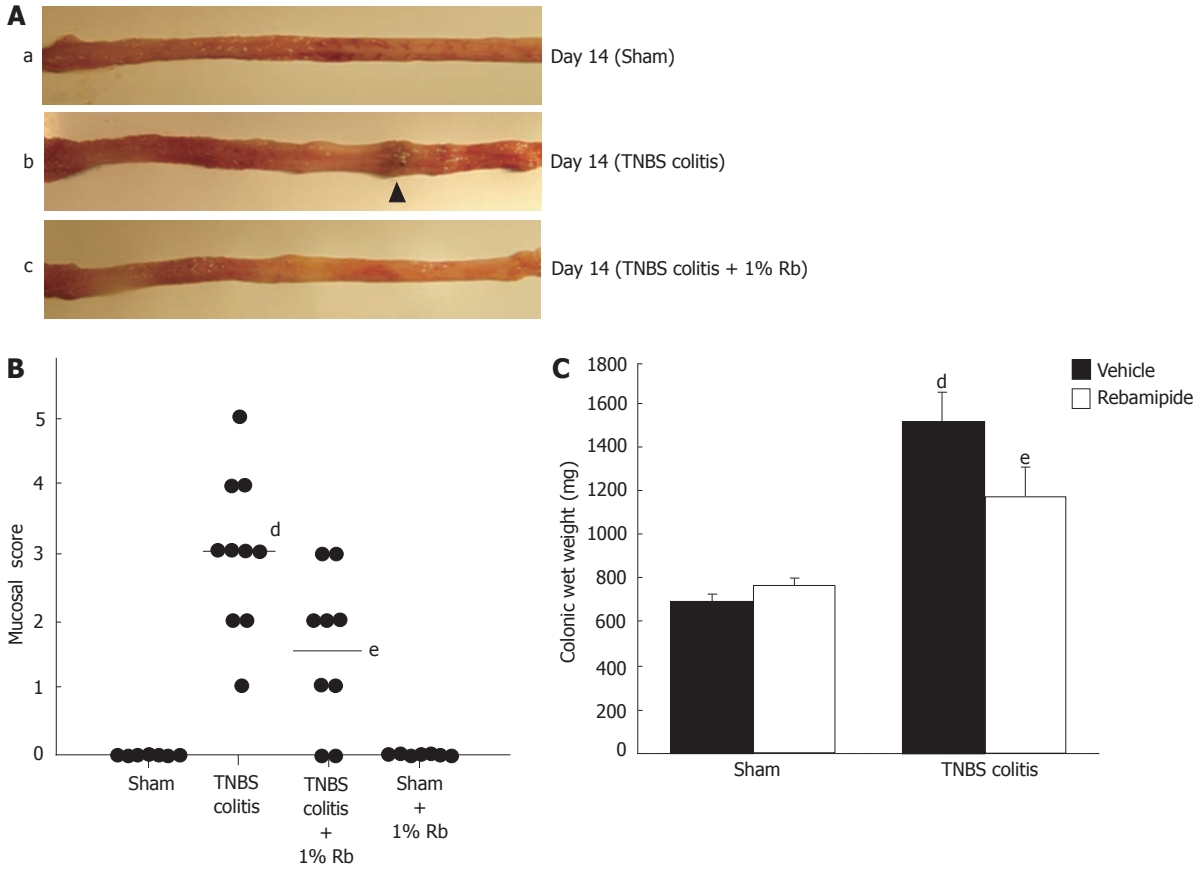

Figure 1 Effects of 1% rebamipide on macroscopic findings, mucosal damage score, and wet colon weight on day 14 after trinitrobenzene sulfonic acid-induced injury. A: Severe colitis was induced with hyperemia, edema, thickening, ulceration, and necrosis in trinitrobenzene sulfonic acid (TNBS)-colitis rats (b) compared to sham-operated rats (a). These changes were reduced in rats treated with 1% rebamipide (TNBS-colitis rats treated with 1% rebamipide) (c). B: A 1% rebamipide enema was administrated twice daily starting on day 7 after induction of colitis, until day 14. Rats were sacrificed on day 14, and the mucosal damage score was evaluated. Data are expressed as a scatter plot. $^{d}P < 0.01$ *vs* sham-treated rats. $^{e}P < 0.05$ *vs* TNBS-induced colitis rats receiving the vehicle. C: Rats were sacrificed on day 14 and the distal colon was removed, after which, the wet colon weight was immediately measured. Data represent the mean ± SE of seven rats. $^{d}P < 0.01$ *vs* sham-treated rats receiving the vehicle. $^{e}P < 0.01$ *vs* TNBS-induced colitis rats receiving the vehicle.

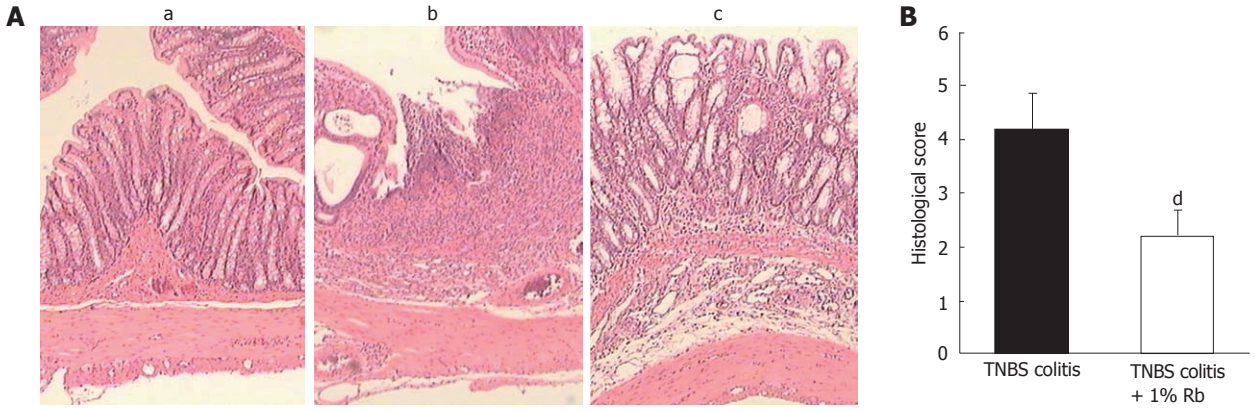

Figure 2 Effects of 1% rebamipide on histological findings in the colon on day 14 after trinitrobenzene sulfonic acid-induced injury. A: Histological appearance of colonic tissue in sham-operated rats (a), trinitrobenzene sulfonic acid (TNBS)-colitis rats (b), and TNBS-colitis rats treated with 1% rebamipide (c). Histological examination revealed that TNBS administration induced marked thickening of the colonic wall, which was associated with transmural infiltration of inflammatory cells. In contrast, both mural wall thickening and infiltration of inflammatory cells were inhibited in rats treated with 1% rebamipide. Hematoxylin and eosin staining (× 40). B: Histological score was evaluated. Data represent the mean ± SE of six rats. $^{d}P < 0.01$ *vs* TNBS-colitis rats receiving the vehicle.

rebamipide, inhibition of both mural wall thickening and inflammatory cell infiltration was observed (Figure 2A). More importantly, rebamipide enema promoted restitution of the colonic epithelium in the ulcerative area. The histological score was increased in the TNBS colitis group, and this increase was significantly inhibited by treatment

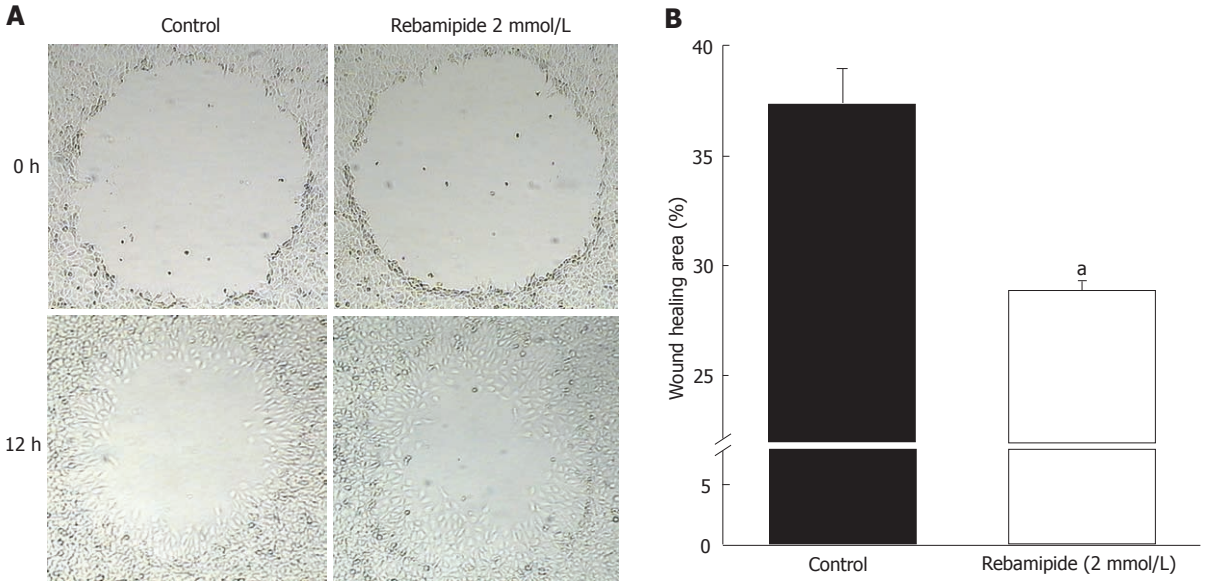

Figure 3 Restitution of rat intestinal epithelial cells around an artificially created wound in control and rebamipide-treated groups. A: Restitution of rat intestinal epithelial (RIE) cells was evaluated using a wound assay. The denuded area of RIE cells recovered in a time-dependent manner after wound induction. Restitution of the denuded area was promoted by rebamipide at 12 h after wound induction. B: To investigate the effects of rebamipide on RIE cell migration, cells were co-incubated with 2 mmol/L rebamipide after wound induction. Wound repair in 2 mmol/L rebamipide-treated cells occurred significantly earlier than it did in the controls. Datas represent the mean ± SE of four experiments. $^{a}P < 0.05$ *vs* controls.

with 1% rebamipide (Figure 2B).

Effects of rebamipide treatment as determined by wound assay using RIE cells

To investigate the effect of rebamipide on restitution of the intestinal epithelium, we performed a wound assay using RIE cells. The cell-free area at the wound site gradually decreased in a time-dependent manner, and complete recovery was observed 15 h after wound induction (data not shown). Restitution was significantly enhanced by rebamipide treatment (Figure 3A). Treatment with 2 mmol/L rebamipide accelerated wound healing compared to the control group (Figure 3B).

To investigate whether the promotion of restitution by rebamipide was involved in ERK signaling, RIE cells were stimulated with 2 mmol/L rebamipide by using a specific ERK inhibitor (U01263). As shown in Figure 4A, the ERK inhibitor blocked the promotion of wound healing by rebamipide. Moreover, to confirm the involvement of the ERK signaling pathway in the enhanced restitution associated with rebamipide treatment, we performed western blot analysis using phosphorylation-status-dependent and -independent antibodies against ERK1/2 (44 and 42 kDa) at 0, 5, 15 and 20 min after treatment with 2 mmol/L rebamipide. The western blots revealed ERK phosphorylation in RIE cells 5 min after rebamipide treatment (Figure 4B). Furthermore, to examine the role of Rho kinase in rebamipide-enhanced restitution, RIE cells were stimulated with rebamipide by using Y27632, a Rho kinase inhibitor (1 μmol/L). The inhibition of Rho kinase canceled the promotion of wound healing by rebamipide treatment.

DISCUSSION

In the present study, we demonstrated that rebamipide enema promoted wound healing in rats with TNBS-induced colonic ulceration. In this model, the area of colonic ulceration peaked on day 2 or 3 after TNBS treatment, and subsequent amelioration was observed in a time-dependent manner. In this study, rats were treated with 1% rebamipide enema starting on day 7 after TNBS injury induction, until day 14. Rebamipide clearly accelerated colonic wound healing under these conditions. These findings are consistent with previous results showing the beneficial effects of rebamipide enema in patients with active UC[7-10], as well as with the results of a study using another experimental colitis model treated with rebamipide enema[18-20].

Rebamipide is a gastric protective and ulcer healing agent that was developed in Japan. It is used clinically in Japan in combination with acid suppressive agents for gastric mucosal protection, acute and chronic gastritis treatment, and gastroduodenal ulcer healing. More interestingly, accumulating evidence suggests that rebamipide exerts protective and healing effects on other tissues. In fact, rebamipide has been shown to be effective in the treatment of patients with UC. In addition, the therapeutic effect of rebamipide may not be limited to the colon alone: indeed, this agent has been demonstrated by both clinical and basic research as effective for the treatment of stomatitis[21] and pulmonary[22], renal[23] and liver damage[24-26], and it also provides corneal protection[27-29].

The mechanisms responsible for amelioration of colitis by rebamipide have not been fully elucidated but may involve inhibition of reactive oxygen species produc-

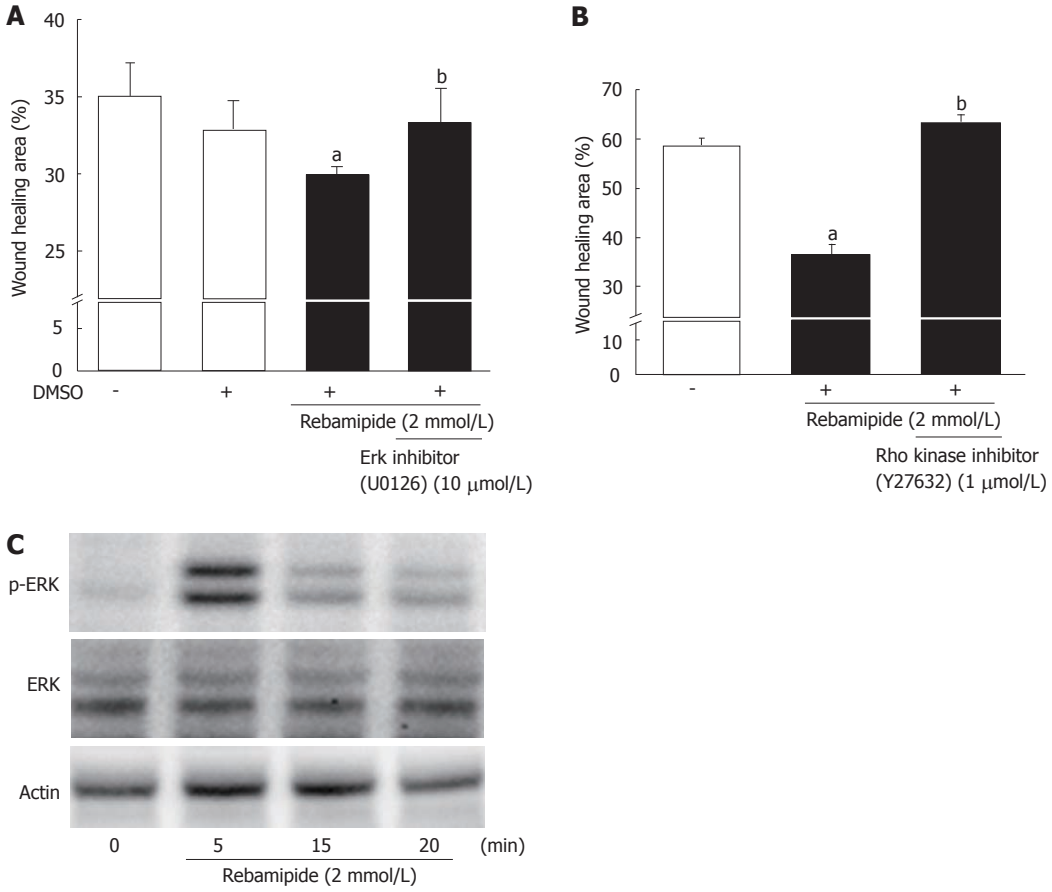

Figure 4 Involvement of extracellular signal-regulated kinase and Rho in rebamipide-treated rat intestinal epithelial cells. A: Rat intestinal epithelial (RIE) cells were treated with rebamipide (2 mmol/L) with or without an extracellular signal-regulated kinase inhibitor (U01263, 10 μmol/L) after wound induction. The wound healing area (12 h later) was then monitored. Data represent the mean ± SE of four experiments. [a]*P* < 0.05 *vs* controls. [b]*P* < 0.05 *vs* the 2 mmol/Lrebamipide-treated group. B: Expression of p44/42 and phospho-p44/42 in RIE cells incubated with rebamipide (2 mmol/L) was measured using western blot analysis. Actin antibody was used as an internal control. Representative data from three observations is shown. C: RIE cells were treated with rebamipide (2 mmol/L) with or without Rho kinase inhibitor (Y27632, 1 μmol/L) after wound induction. The wound healing area (6 h later) was then monitored. Data represent the mean ± SE of four experiments. [a]*P* < 0.05 *vs* controls. [b]*P* < 0.05 *vs* the 2 mmol/L rebamipide-treated group. DMSO: Dimethyl sulfoxide.

tion[19,20,30], suppression of neutrophil accumulation[31,32], increases in trans-epithelial electrical resistance[33], and induction of hepatocyte growth factor expression[34]. However, it remains unclear whether rebamipide enhances restitution of RIE cells. It is already known that rebamipide enhances gastric epithelial restitution, but the same has yet to be proven in intestinal epithelial restitution. In cases of intestinal mucosal injury, restitution is an important step in re-establishing mucosal integrity, and restitution is the most rapid post-injury response; in effect, restitution restores the continuity of the intestinal epithelial layer, primarily by redistribution of epithelial cells. This process is completed by the migration of local epithelial cells along the underlying matrix, which does not require epithelial cell proliferation[35,36]. In this study, to assess the effects of rebamipide on intestinal epithelial restitution, the round wound assay was evaluated using RIE cells. Our results showed that 2 mmol/L rebamipide treatment promoted the restitution of RIE cells.

With regard to the role of the ERK signaling pathway in epithelial restitution, several studies have been reported in which activation of the ERK signaling pathway played an important role in epithelial wound closure. In the present study, rebamipide promoted ERK phosphorylation. This result is in agreement with the results of Gazel *et al*[37] and Wang *et al*[38]. Moreover, rebamipide-promoted restitution was suppressed by U01263, a specific ERK inhibitor. These data indicate that accelerated restitution by rebamipide is at least partly mediated by the ERK pathway. Tanigawa *et al*[39] have demonstrated that rebamipide induces ERK phosphorylation in gastric cancer cells and inhibits cell growth through Smad signaling. Although they used gastric cancer cells, they also found that rebamipide induced phosphorylation of ERK.

Furthermore, we investigated whether rebamipide-promoted restitution was related to Rho kinase activation. Rho kinase has been identified as one of the effectors of the small GTP-binding protein Rho. Accumulating evidence has demonstrated that the Rho/Rho kinase pathway plays an important role in various cellular functions, including cell contraction, cell proliferation, gene expression, and especially cell migration[40,41]. With regard to restitution of IE cells, Santos *et al*[42] have found that

Rho protein is one of the essential elements of a mechanism by which growth factors induce cell migration to restore mucosal integrity, and Rao *et al*[43] have demonstrated that activation of Rho kinase results in increased phosphorylation of the myosin light chain, which leads to cell migration. In this investigation, we used Y27632, which has been widely used as a specific inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase family of protein kinases[44]. Co-treatment with Y27632 cancelled the effect of rebamipide on the restitution of RIE cells. These data indicate that rebamipide enhanced the restitution of RIE cells *via* ERK phosphorylation and Rho kinase activation. However, the detailed mechanism of rebamipide-induced ERK and Rho kinase activation remains unknown. Further investigations are needed to elucidate this mechanism.

In summary, the present study indicates that treatment with rebamipide can promote the healing of TNBS-induced intestinal injury, which is associated with acceleration of intestinal epithelial restitution. The present results suggest that rebamipide has great potential as a new therapeutic agent for the treatment of inflammation-associated intestinal injury.

COMMENTS

Background

Ulcerative colitis (UC) is a chronic and recurrent disorder of the colon and rectum. While the precise pathogenesis of UC remains unknown, medical management of patients with acute exacerbation of UC symptoms focuses on achieving remission by inhibiting intestinal inflammation and repairing mucosal injury. However, some patients with inflammatory bowel disease do not respond, or respond incompletely, to the existing treatments. Therefore, it is important to investigate new anti-inflammatory strategies.

Research frontiers

Rebamipide is a gastric mucosal protective and ulcer-healing agent, and has been used for treatment of UC. However, the detailed mechanism of action of rebamipide against intestinal inflammation such as UC remains unclear. In this study, the authors investigated the therapeutic efficacy of rebamipide in an experimental rat model of colitis and evaluated the restitution of intestinal epithelial cells treated with rebamipide *in vitro*.

Innovations and breakthroughs

The present study indicated that treatment with rebamipide could promote the healing of trinitrobenzene sulfonic acid-induced intestinal injury, which has been associated with acceleration of intestinal epithelial restitution through extracellular signal-regulated kinase and Rho kinase activation. The present results suggest that rebamipide has great potential as a new therapeutic agent for the treatment of inflammation-associated intestinal injury.

Applications

By understanding how rebamipide inhibits intestinal inflammation and promotes healing of the intestinal injury, rebamipide may represent a future therapeutic agent for treatment of patients with UC.

Terminology

Rho kinase has been identified as one of the effectors of the small GTP-binding protein Rho. Accumulating evidence has demonstrated that the Rho/Rho kinase pathway plays an important role in various cellular functions, including cell contraction, cell proliferation, gene expression, and especially, cell migration.

Peer review

This is an interesting, well-designed study with good documentation of results.

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ORIGINAL ARTICLE

Dickkopf3 overexpression inhibits pancreatic cancer cell growth *in vitro*

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Abstract

AIM: To elucidate the role of dickkopf3 (Dkk3) in human pancreatic cancer cell growth.

METHODS: Dkk3 mRNA and protein expression in human pancreatic cancer cell lines were detected by real-time reverse transcription polymerase chain reaction (real-time RT-PCR), Western blotting and immunofluorescence. Methylation of the Dkk3 promoter sequence was examined by methylation-specific polymerase chain reaction (MSP) and Dkk3 mRNA expression was determined by real-time RT-PCR after 5-aza-2′-deoxycytidine (5-aza-dC) treatment. The effects of Dkk3 on cancer cell proliferation and *in vitro* sensitivity to gemcitabine were investigated by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) after transfecting the Dkk3 expression plasmid into human pancreatic cancer cells. The expression of β-catenin, phosphorylated extracellular signal-regulated protein kinases (pERK) and extracellular signal-regulated protein kinases (ERK) was also examined by real-time RT-PCR and Western blotting after upregulating Dkk3 expression in human pancreatic cancer cells.

RESULTS: The results show that the expression levels of both Dkk3 mRNA and protein were low in all pancreatic cancer cell lines tested. The Dkk3 promoter sequence was methylated in the MIA PaCa-2 and AsPC-1 cell lines, which showed reduced Dkk3 expression. These two cell lines, which initially had a methylated Dkk3 promoter, showed increased Dkk3 mRNA expression that was dependent upon the dosage and timing of the DNA demethylating agent, 5-aza-dC, treatment ($P < 0.05$ or $P < 0.01$). When Dkk3 expression was upregulated following the transfection of a Dkk3 expression plasmid into MIA PaCa-2 cells, the ability of cells to proliferate decreased ($P < 0.01$), and the expression of β-catenin and pERK was downregulated ($P < 0.01$). Sensitivity to gemcitabine was enhanced in Dkk3 expression plasmid-transfected cells.

CONCLUSION: Our findings, for the first time, implicate Dkk3 as a tumor suppressor in human pancreatic cancer, through the downregulation of β-catenin expression *via* the ERK-mediated pathway.

Key words: Cell growth; Dickkopf3; *In vitro*; Overexpression; Pancreatic cancer

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INTRODUCTION

Pancreatic cancer is the sixth leading cause of cancer death in China[1]. The overall five-year survival rate is approximately 1%-3%, and the median survival period after diagnosis is only 4 to 5 mo. Pancreatic cancer remains one of the most aggressive human cancers, with an exceedingly poor prognosis because of its late onset of symptoms[2], rapid progression, frequent metastasis and insensitivity to chemotherapy and radiotherapy. Therefore, recognizing the factors associated with pancreatic cancer progression is critical for its treatment.

Dickkopf (Dkk) family proteins, including Dkk1/2/3/4, are secreted modulators of the canonical Wnt signaling pathway[3]. Dkk1, Dkk2 and Dkk4, antagonists of Wnt signaling[4,5], interact with Wnt coreceptors, low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and Kremen[6,7]. Dkk3 interacts with kremen1 and kremen2, but not with LRP5/6[8], and has been proposed to act as a tumor suppressor. Dkk3 is downregulated in some tumors, and it inhibits tumor growth[9-25]. For example, in cervical cancer and malignant glioma, Dkk3 regulates tumor cell growth and decreases β-catenin expression[16,23]. Dkk3 can induce cancer cell apoptosis by c-Jun-NH2-kinase (JNK) activation in testicular and prostate cancer cells[9,26]. The Dkk3 promoter sequence is methylated in several tumors, such as breast cancer, hepatoma, bladder cancer and malignant astrocytic gliomas[27-32]. In lung adenocarcinomas, however, Dkk3 inhibits cancer cell apoptosis by decreasing the intracellular level of reactive oxygen species and functions as an oncogene[33]. Dkk3 knock-out mice showed no enhanced tumor formation[34]. Recently, other studies have demonstrated that Dkk3 plays distinct roles in different cells[8].

To date, no study has investigated Dkk3 expression and its roles in human pancreatic cancer cell behavior. To better understand the role of Dkk3 in pancreatic cancer progression, we investigated Dkk3 expression and promoter sequence methylation in human pancreatic cancer cells. The effects of Dkk3 on cell proliferation and sensitivity to gemcitabine were simultaneously observed after expression was increased in MIA PaCa-2 cells, following transfection with the Dkk3 expression plasmid.

MATERIALS AND METHODS

Cell lines and cell culture

The human pancreatic cancer cell lines PANC-1, MIA PaCa-2, AsPC-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, Virginia, United States). AsPC-1 and BxPC-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, MO, United States) and PANC-1 and MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, MO, United States). All media were supplemented with 10% fetal calf serum (Tianjin Haoyang Biological Manufacture Co., LTD, China), 100 μg/mL streptomycin and 100 U/mL penicillin, and the cultures were grown at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Construction of and transient transfection with a plasmid expressing human Dkk3

Total RNA was extracted from PANC-1 cells using TRIzol reagent (Invitrogen, CA, United States), according to the manufacturer's protocol. The cDNAs were synthesized using the TaKaRa RNA polymerase chain reaction (PCR) Kit (TaKaRa, Japan). A full-length cDNA encoding human Dkk3 was cloned by PCR using 500 ng cDNA as a template and primers containing HindIII and BamHI restriction enzyme sites (Table 1). The PCR products were ligated into pcDNA3.1 (Invitrogen, CA, United States) to create the plasmid pcDNA3.1-Dkk3. MIA PaCa-2 cells were transfected with the pcDNA3.1 vector or pcDNA3.1-Dkk3 using FuGENE (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction

Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer's protocol. The cDNAs were synthesized using the TaKaRa RNA PCR Kit (TaKaRa, Japan). The optimal PCR conditions were 94 ℃ for 5 min; 35 cycles at 94 ℃ for 40 s, 61 ℃ (Dkk3)/52 ℃ (β-actin) for 40 s, 72 ℃ for 40 s; and 72 ℃ for 10 min. PCR products (5 μL) were separated by electrophoresis in a 2.0% agarose gel. Primer sequences for Dkk3 and β-actin are listed in Table 1.

RNA preparation and real-time reverse transcription polymerase chain reaction

Total RNA was isolated from the cells, with or without 5-aza-2'-deoxycytidine (5-aza-dC) treatment, using TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer's protocol. First-strand cDNA was synthesized from 500 ng of total RNA using the TaKaRa RNA PCR Kit (TaKaRa, Japan). PCR was conducted on a 7500 Real Time PCR System (Applied Biosystems, United Kingdom) in combination with the SYBR green PCR master mix (Applied Biosystems, United Kingdom). Melting curve analyses following amplification were performed to ensure product specificity. The relative expression levels of Dkk3 mRNA and β-catenin mRNA were normalized to mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA sample. ΔCt was calculated by subtracting the Ct of GAPDH mRNA from the Ct of the mRNA of interest. ΔΔCt was then calculated by subtracting the ΔCt of the control from the ΔCt of the sample. The fold change in mRNA was calculated according to the equation $2^{-\Delta\Delta Ct}$. Primer sequences for Dkk3, β-catenin and GAPDH are listed in Table 1.

Table 1 Oligonucleotide primers used in the study

	Sequence (5′ to 3′)	T_A (℃)	Cycles
PCR			
Dkk3 (full-length)	Forward: CCCAAGCTTATGCAGCGGCTTGGGGC Reverse: CGCGGATCCCTAAATCTCTTCCCCTCCCAGCAGT	53	35
Real-time RT-PCR			
Dkk3	Forward: ACAGCCACAGCCTGGTGTA Reverse: CCTCCATGAAGCTGCCAAC	60	40
β-catenin	Forward: AAAATGGCAGTGCGTTTAG Reverse: TTTGAAGGCAGTCTGTCGTA	60	40
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: GCCTTCTCCATGGTGGTGAA	60	40
RT-PCR			
Dkk3	Forward: AAGGCAGAAGGAGCCACGAGTGC Reverse: GGCCATTTTGGTGCAGTGACCCCA	61	35
β-actin	Forward: AAATCGTGCGTGACATTAA Reverse: CTCGTCATACTCCTGCTTG	52	35
MSP			
Dkk3 unmethylated	Forward: TTAGGGGTGGGTGGTGGGGT[32] Reverse: CTACATCTCCACTCTACACCCA[32]	59	34
Dkk3 methylated	Forward: GGGCGGGCGGCGGGGC[32] Reverse: ACATCTCCGCTCTACGCCCG[32]	59	34

T_A: Annealing temperature; Real-time RT-PCR: Real-time reverse transcription polymerase chain reaction; Dkk3: Dickkopf3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MSP: Methylation-specific polymerase chain reaction.

Bisulfite modification and methylation-specific polymerase chain reaction

Genomic DNA was isolated from pancreatic cancer cell lines using the TIANamp Genomic DNA kit (Tiangen Biotech Co., LTD, Beijing, China). One microgram of genomic DNA was bisulfite-modified using the CpGenome™ DNA Modification Kit (Chemicon, MA, United States) according to the manufacturer's protocol. Methylation-specific polymerase chain reaction (MSP) was performed at 95 ℃ for 5 min, followed by 34 cycles at 94 ℃ for 30 s, 59 ℃ for 30 s and 72 ℃ for 30 s. The final extension was at 72 ℃ for 10 min. Each PCR reaction was performed using 0.5 units of HotStarTaq Plus DNA Polymerase (Qiagen GmbH, Hilden, Germany). The primers are listed in Table 1. The specificity of the MSP primers in detecting the Dkk3 methylation status was demonstrated using unmethylated and methylated DNA as a template (EpiTect Control DNA Set; Qiagen GmbH, Hilden, Germany).

5-aza-dC treatment

Cells were seeded at a density of 4×10^4 cells/well in a six-well plate. After overnight incubation, the cells were treated with 10 μmol/L and 20 μmol/L of the DNA demethylating agent 5-aza-dC (Sigma-Aldrich, Steinheim, Germany) for 48 h or 72 h. Control cells were incubated with dimethyl sulfoxide and fresh medium.

Immunofluorescence and confocal microscopy

Cells grown on coverslips were washed and fixed with 4% paraformaldehyde, followed by washing with 0.2% Triton X-100. Coverslips were incubated with nonimmune animal serum to reduce nonspecific binding. The coverslips were subsequently incubated at 4 ℃ overnight with an anti-Dkk3 rabbit polyclonal antibody (1:100, Santa Cruz, CA, United States). Rhodamine-conjugated AffiniPure goat anti-rabbit IgG was used as the secondary antibody (1:200, Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China). Counterstaining was performed using 1 μg/mL 4',6-diamidino-2-phenylindole. Expression and localization of Dkk3 were observed under a confocal microscope (Leica, Mannheim, Germany).

Western blotting

The cells in culture were washed twice with ice-cold PBS, and proteins were extracted with M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, United States). Samples were centrifuged at 14 000 × *g* for 10 min. Aliquots of cell lysates containing 40 μg protein were separated on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore, MA, United States). The membranes were blocked with 10% skim milk and incubated with Dkk3 antibody (1:1500, Santa Cruz, CA, United States), β-catenin antibody (1:1500, BD Transduction Laboratories, San Diego, United States), phosphorylated extracellular signal-regulated protein kinase antibody (pERK antibody, 1:2000, Cell Signaling, MA, United States), extracellular signal-regulated protein kinase antibody (ERK antibody, 1:2000, Cell Signaling, MA, United States) and β-actin antibody (1:2000, Santa Cruz, CA, United States) at 4 ℃ overnight, followed by their corresponding secondary antibodies (1:2000, Zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) at room temperature for 2 h. The membrane-bound proteins were detected using the Pierce ECL Western blotting substrate (Pierce Biotechnology, Rockford, United States).

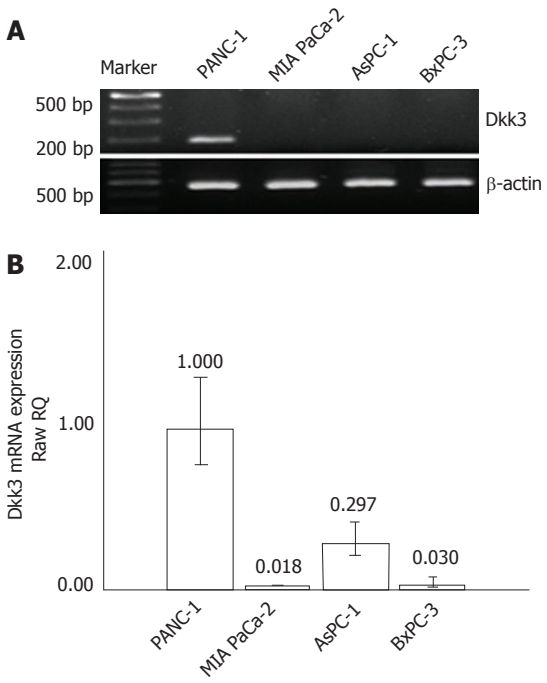

Figure 1 Dickkopf3 expression in human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, AsPC-1 and BxPC-3). A, B: Dickkopf3 (Dkk3) mRNA expression was detected by reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR. Dkk3 mRNA expression was low in all cell lines examined. Dkk3: Dickkopf3; RQ: Relative quantitation.

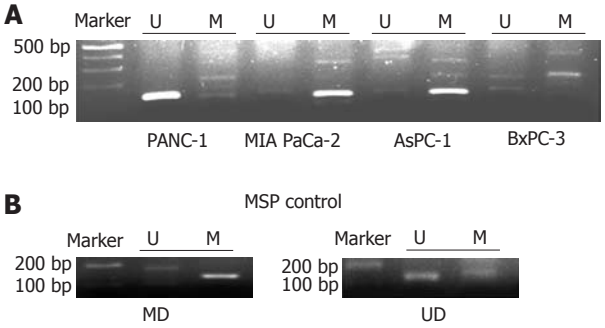

Figure 2 Dickkopf3 promoter methylation analysis in human pancreatic cancer cell lines. A: Methylation-specific PCR (MSP) was performed with bisulfite-treated DNA from pancreatic cancer cells. The Dickkopf3 (Dkk3) promoter was significantly methylated in MIA PaCa-2 and AsPC-1 cells; B: MSP controls demonstrate the specificity of the Dkk3 primers used. Methylated bisulfite-converted DNA exclusively yields amplification products with primers specific to methylated Dkk3 promoter sequences; unmethylated bisulfite-converted DNA yields exclusively amplification products with primers recognizing unmethylated Dkk3 promoter sequences. MD: Methylated bisulfite-converted DNA; UD: Unmethylated bisulfite-converted DNA; U: PCR products amplified with primers recognizing unmethylated Dkk3 promoter sequences; M: Amplification generated with methylation-specific primers.

Determination of dose-response curve

For determination of the dose-response curve, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. Six hours after transfection, cells were seeded in 96-cell plates in triplicate at a density of 3000 cells/well and were allowed to adhere. Gemcitabine (LILLY, France) was added to the medium 24 h after transfection. Cell proliferation was determined 72 h after gemcitabine addition using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, WI, United States), according to the manufacturer's protocol. The spectrophotometric absorbance of each sample was measured at 490 nm using the TECAN spectra (Thermo, Austria). Percent proliferation relative to the controls was calculated based on the MTS read-out; the IC_{50} value was defined as the concentration of drug that produced a 50% reduction in absorbance relative to the control.

Cell growth assay

For the cell growth assay, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. At 6 h after transfection, cells were seeded in 96-well plates in triplicate at a density of 1000 cells/well and were allowed to adhere overnight. At 24 h, 48 h and 72 h, cell proliferation was determined using MTS (Promega, WI, United States) according to the manufacturer's protocol. The spectrophotometric absorbance of each sample was measured at 490 nm using the TECAN spectra (Thermo, Austria).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Unless otherwise indicated, the level of significance for differences between data sets was assessed using *t* test and one-way analysis of variance. Data are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Dkk3 is downregulated in pancreatic cancer cell lines

Dkk3 expression was assessed in four human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, AsPC-1, BxPC-3). A low level of Dkk3 mRNA was observed in all cell lines, although Dkk3 expression in PANC-1 cells was slightly higher than in the other three cell lines (Figure 1). Dkk3 protein expression was too low to detect by Western blotting or immunofluorescence (data not shown).

Methylation of the Dkk3 promoter in pancreatic cancer cell lines

Through the use of MSP, we found that the Dkk3 promoter sequence was significantly methylated in MIA PaCa-2 and AsPC-1 cells, which were the cell lines with reduced Dkk3 expression. Conversely, the Dkk3 promoter sequence was unmethylated in the PANC-1 cells, which had slightly higher Dkk3 expression (Figure 2).

Demethylation of the Dkk3 promoter

Because methylation of the Dkk3 promoter sequence was detected in MIA PaCa-2 and AsPC-1 cells, we chose to treat these two cell lines with 10 μmol/L and 20 μmol/L, respectively, of the DNA methyltransferase inhibitor 5-aza-dC. After treatment with 5-aza-dC, for 48 h or 72 h, the cells were harvested to determine Dkk3 mRNA expression by real-time reverse transcription PCR. The results showed that these two cell lines with methylated Dkk3 promoters showed increased Dkk3 mRNA expression,

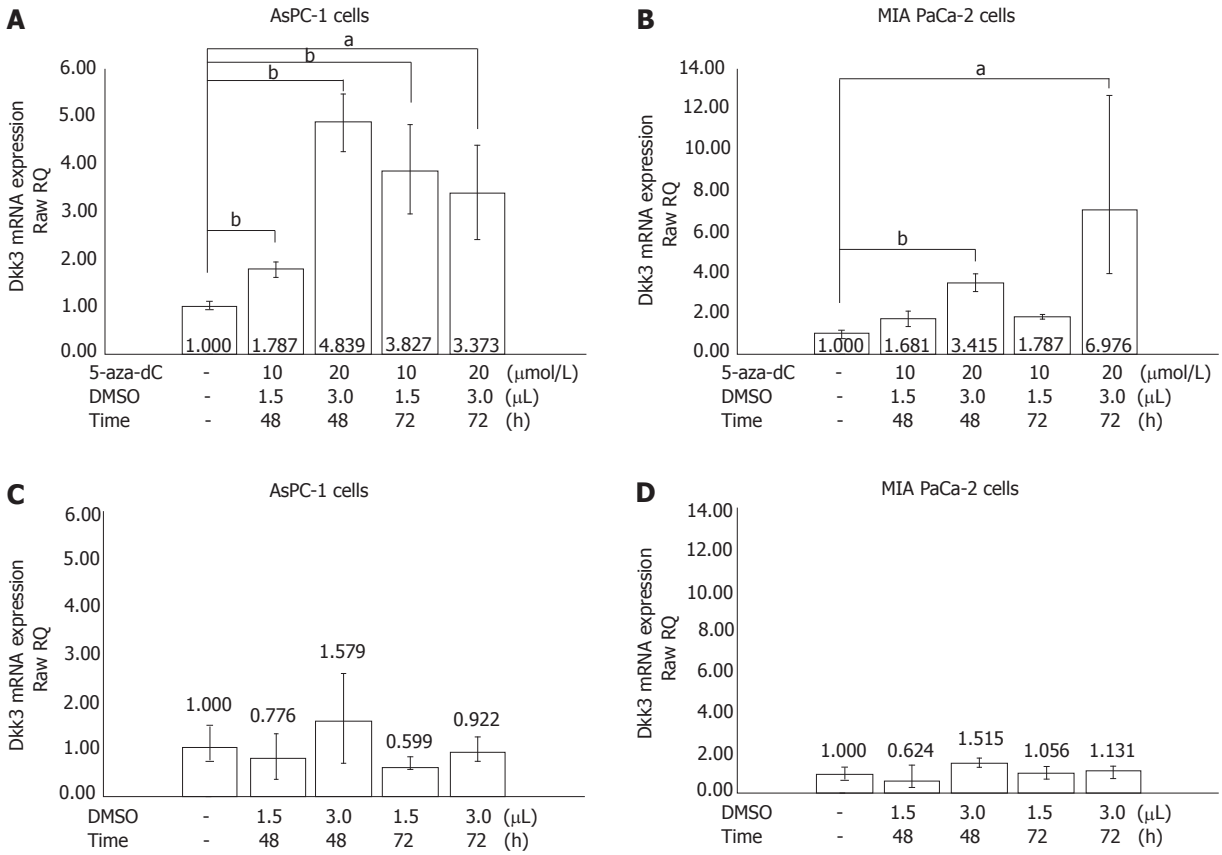

Figure 3 Dickkopf3 mRNA expression after demethylation *in vitro*. A, B: AsPC-1 and MIA PaCa-2 cells were treated with 10 μmol/L and 20 μmol/L of the DNA demethylating agent, 5-aza-dC, for 48 h or 72 h, respectively. The results show that these two cell lines, in which the Dickkopf3 (Dkk3) promoter was initially heavily methylated, had increased Dkk3 mRNA expression that was dependent on the dosage and timing of 5-aza-dC treatment ($^aP < 0.05$ *vs* untreated MIA PaCa-2 cells or untreated AsPC-1 cells, $^bP < 0.01$ *vs* untreated MIA PaCa-2 cells or untreated AsPC-1 cells); C, D: Control cells were incubated with dimethyl sulfoxide and fresh medium. RQ: Relative quantitation; **Dkk3: Dickkopf3; DMSO:** Dimethyl sulfoxide.

which was dependent on the dosage and timing of 5-aza-dC treatment ($P < 0.05$ or $P < 0.01$) (Figure 3).

Overexpression of Dkk3 suppresses pancreatic cancer cell growth and β-catenin expression

To study the roles of Dkk3 in the progression of pancreatic cancer, MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. After transfection, Dkk3 mRNA and protein levels significantly increased in the pcDNA3.1-Dkk3-transfected cells ($P < 0.01$), while no significant changes were observed in the pcDNA3.1-transfected cells (Figure 4A and B). At 48 h and 72 h after transfection, the β-catenin mRNA and protein expression levels were significantly decreased in the pcDNA3.1-Dkk3-transfected cells ($P < 0.01$) (Figure 4B and C). The protein expression of pERK was also decreased, but there was no significant change in total ERK expression (Figure 4B). The results of the MTS assay showed that in the pcDNA3.1-Dkk3-transfected cells, proliferation capacity was lower than in the pcDNA3.1-transfected cells ($P < 0.01$) (Figure 4D).

Sensitivity of Dkk3-overexpressing pancreatic cancer cells to gemcitabine

A dose-response curve was constructed, and the IC_{50} values were compared to determine the influence of Dkk3 overexpression in pancreatic cancer cells on the effect of gemcitabine on cell growth. MIA PaCa-2 cells were transfected with pcDNA3.1-Dkk3 or pcDNA3.1. Seventy-two hours after gemcitabine addition, the IC_{50} values for gemcitabine were 0.621 μmol/L for pcDNA3.1-Dkk3-transfected cells and 1.877 μmol/L for pcDNA3.1-transfected cells (Figure 4E). These results show that the IC_{50} value of the Dkk3-overexpressing cells was significantly lower than that of the control cells.

DISCUSSION

Dkk3 is expressed in many normal human tissues[35]. It was previously reported that Dkk3 expression is generally low in some tumors, such as sporadic epithelial ovarian cancer, cervical cancer, mammary tumors, malignant melanoma, hepatoma and kidney, pancreas, gastric and lung cancers[12,15,16,18,19,31,32]. Additional studies also revealed the association between Dkk3 expression and cancer metastasis or prognosis in gastric cancer, renal cancer and head and neck squamous cell carcinoma[36-38]. However, Dkk3 expression and its roles in pancreatic cancer remain unknown. In this study, we detected Dkk3 expression in human pancreatic cancer cells. We found that both Dkk3

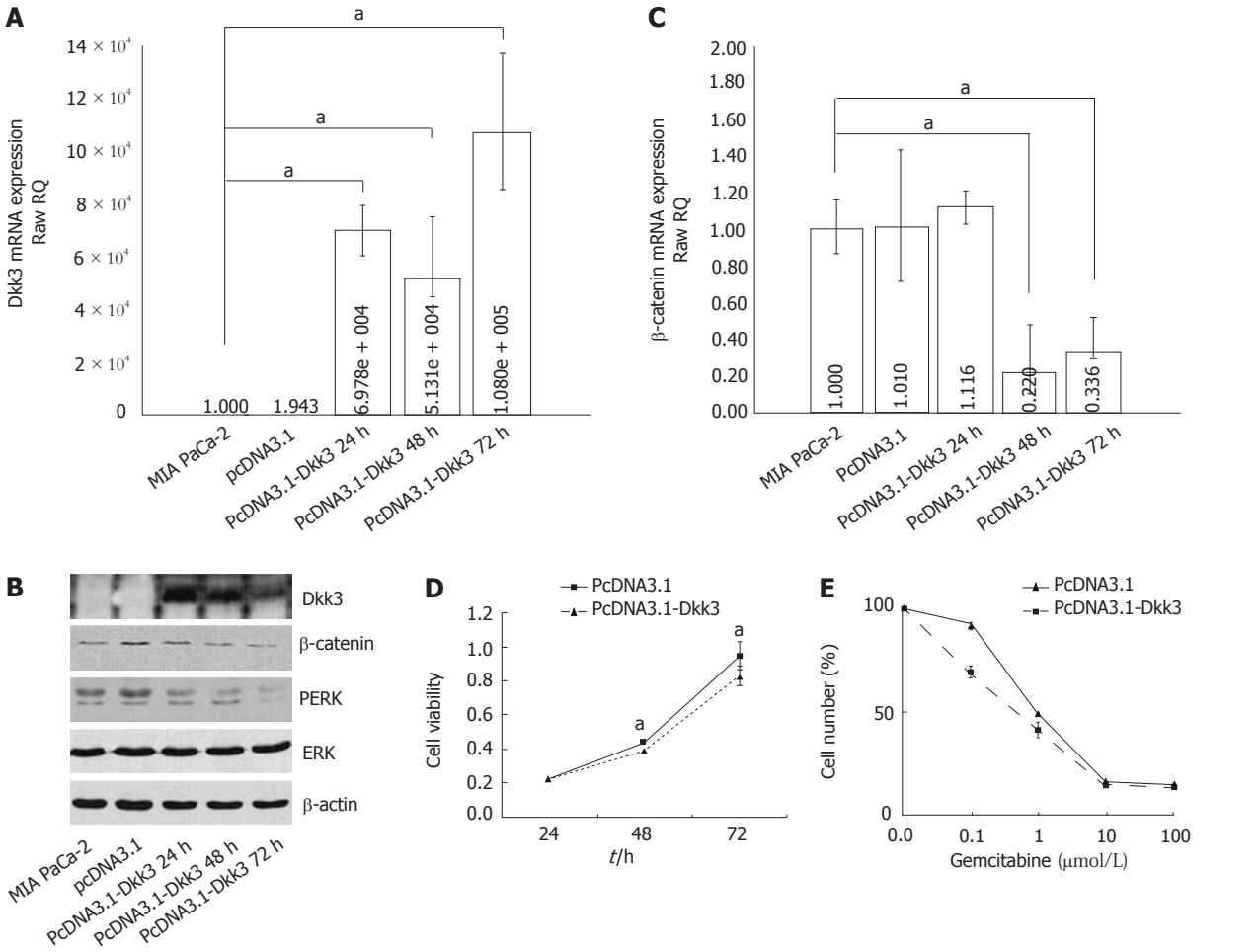

Figure 4 The effects of dickkopf3 overexpression on pancreatic cancer cells. MIA PaCa-2 cells were transfected with pcDNA3.1-dickkopf3 (Dkk3) or pcDNA3.1 vector. A, B: After transfection, Dkk3 mRNA and protein expression were examined by real-time reverse transcription polymerase chain reaction (real-time RT-PCR) and Western blotting. The results show that in the pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells, Dkk3 expression was significantly upregulated ($P < 0.01$, $^aP < 0.01$ *vs* MIA PaCa-2 cells). B, C: β-catenin expression was examined by real-time RT-PCR and western blotting. β-catenin expression was downregulated 48 h and 72 h after transfecting pcDNA3.1-Dkk3 into MIA PaCa-2 cells ($P < 0.01$, $^aP < 0.01$ *vs* MIA PaCa-2 cells). B: The expression of extracellular signal-regulated protein kinases (ERK) and phosphorylated extracellular signal-regulated protein kinases (pERK) was examined by western blotting. The expression of pERK was simultaneously downregulated, without a significant change in total ERK expression. D: MTS assay results showed that the proliferative ability of pcDNA3.1-Dkk3-transfected cells was lower than that of pcDNA3.1-transfected cells ($P < 0.01$, $^aP < 0.01$). E: Dose-response analysis of pcDNA3.1- or pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells with gemcitabine treatment. Seventy-two hours after gemcitabine addition, the IC50 values for gemcitabine were 0.621 μmol/L for pcDNA3.1-Dkk3-transfected cells and 1.877 μmol/L for pcDNA3.1-transfected cells. PERK: Phosphorylated extracellular signal-regulated protein kinases; ERK: Extracellular signal-regulated protein kinases; RQ: Relative quantitation; **Dkk3: Dickkopf3; DMSO:** Dimethyl sulfoxide.

protein and mRNA expression levels were low in all cell lines examined. Our results are partly in agreement with those of Takahashi N *et al*[39].

Methylation of the Dkk3 promoter has been observed in hepatocellular carcinoma, breast cancer, malignant astrocytic glioma, acute myeloid and lymphoblastic leukemia and gastrointestinal and bladder cancers[27-30,40-44]. Our MSP results showed that the Dkk3 promoter was methylated in MIA PaCa-2 and AsPC-1 cells, in which Dkk3 expression was low. After treatment with the DNA methyltransferase inhibitor 5-aza-dC, MIA PaCa-2 and AsPC-1 cells, which initially bore heavily methylated Dkk3 promoters, showed increased Dkk3 mRNA expression. In the present study, we demonstrated for the first time that decreased Dkk3 gene expression was associated with promoter methylation in two human pancreatic cancer cell lines (MIA PaCa-2 and AsPC-1). The inhibition of DNA methyltransferase activity by 5-aza-dC led to a reversion of methylation and upregulated expression of the previously downregulated gene.

Additional studies have recently demonstrated that Dkk3 has distinct roles in regulating the malignant behavior of cancer cells, depending on which cells are examined. For example, Dkk3 can reduce malignancy in mouse prostate cancer RM9 cells *in vitro* and *in vivo*[25]. Dkk3 can induce apoptosis or cell death in human bladder cancer, prostate cancer, breast cancer and lung cancer cells[9,20,24,45]. Dkk3 can inhibit tumor growth and metastasis in an orthotopic prostate cancer model[10]. While Jung *et al*[33] found that Dkk3 acts as an antiapoptotic molecule in lung adenocarcinoma, our results show that Dkk3 overexpression inhibited pancreatic cancer cell growth. The results revealed that in the pcDNA3.1-Dkk3-transfected MIA PaCa-2 cells, β-catenin mRNA and protein expres-

sion levels were both downregulated. Phosphorylation of ERK was decreased. These data demonstrate that Dkk3 suppressed MIA PaCa-2 cell growth by inhibiting β-catenin expression. Our results were consistent with the findings of Yue *et al*[45] in lung cancer. We hypothesize that Dkk3 acts as a Wnt signal transduction inhibitor in human pancreatic cancer cells.

Gemcitabine is the most commonly used chemotherapy drug for pancreatic cancer. Notably, our results show that gemcitabine's IC_{50} value for pcDNA3.1-Dkk3-transfected cells was significantly lower than that for the control cells. Dkk3 overexpression enhanced the sensitivity of pancreatic cancer cells to gemcitabine.

In summary, our results suggest that Dkk3 acts as a tumor suppressor in human pancreatic cancer cells by downregulating β-catenin expression *via* the ERK-mediated pathway. Dkk3 may be a valid adjunctive target of gemcitabine for the treatment of human pancreatic cancer.

COMMENTS

Background

Pancreatic cancer is the sixth leading cause of cancer death in China. The overall five-year survival rate is approximately 1%-3%. Pancreatic cancer remains one of the most aggressive human cancers. Recognizing the factors associated with pancreatic cancer progression is critical for its treatment.

Research frontiers

Dickkopf family proteins are secreted modulators of the canonical Wnt signaling pathway. Dickkopf 3 (Dkk3) is a member of the dickkopf family proteins. Dkk3 is downregulated in some tumors, and its overexpression inhibits tumor growth. The Dkk3 promoter sequence is methylated in several tumors. However, in lung adenocarcinomas, Dkk3 functions as an oncogene. Recently, other studies have demonstrated that Dkk3 plays distinct roles in different cells.

Innovations and breakthroughs

To date, no study has investigated Dkk3 expression and its roles in human pancreatic cancer cell behavior. In this study, the authors investigated Dkk3 expression and promoter sequence methylation in human pancreatic cancer cells. The effects of Dkk3 on cell proliferation and sensitivity to gemcitabine were simultaneously observed after expression was increased in MIA PaCa-2 cells, following transfection with the Dkk3 expression plasmid. According to the experimental results, the authors for the first time, confirmed that Dkk3 acts as a tumor suppressor in human pancreatic cancer cells by downregulating β-catenin expression *via* the ERK-mediated pathway. Dkk3 overexpression enhanced the sensitivity of pancreatic cancer cells to gemcitabine.

Applications

This study indicates that Dkk3 may be a valid adjunctive target of gemcitabine for the treatment of human pancreatic cancer.

Peer review

This is a paper that reports that Dkk3 is a tumor suppressor gene in pancreatic cancer. The findings are interesting, and overall writing is good.

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BRIEF ARTICLE

Balanced propofol sedation administered by nonanesthesiologists: The first Italian experience

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Abstract

AIM: To assess the efficacy and safety of a balanced approach using midazolam in combination with propofol, administered by non-anesthesiologists, in a large series of diagnostic colonoscopies.

METHODS: Consecutive patients undergoing diagnostic colonoscopy were sedated with a single dose of midazolam (0.05 mg/kg) and low-dose propofol (starter bolus of 0.5 mg/kg and repeated boluses of 10 to 20 mg). Induction time and deepest level of sedation, adverse and serious adverse events, as well as recovery times, were prospectively assessed. Cecal intubation and adenoma detection rates were also collected.

RESULTS: Overall, 1593 eligible patients were included. The median dose of propofol administered was 70 mg (range: 40-120 mg), and the median dose of midazolam was 2.3 mg (range: 2-4 mg). Median induction time of sedation was 3 min (range: 1-4 min), and median recovery time was 23 min (range: 10-40 min). A moderate level of sedation was achieved in 1561 (98%) patients, whilst a deep sedation occurred in 32 (2%) cases. Transient oxygen desaturation requiring further oxygen supplementation occurred in 8 (0.46%; 95% CI: 0.2%-0.8%) patients. No serious adverse event was observed. Cecal intubation and adenoma detection rates were 93.5% and 23.4% (27.8% for male and 18.5% for female, subjects), respectively.

CONCLUSION: A balanced sedation protocol provided a minimalization of the dose of propofol needed to target a moderate sedation for colonoscopy, resulting in a high safety profile for non-anesthesiologist propofol sedation.

Key words: Colonoscopy; Propofol; Sedation

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INTRODUCTION

Colorectal cancer (CRC) represents a major cause of morbidity and mortality in western countries[1]. Despite the fact that it has been shown to be highly effective in preventing CRC incidence, colonoscopy is usually perceived as an invasive and potentially painful procedure, resulting in a low uptake rate when compared with less invasive options, such as fecal tests or flexible sigmoidoscopy[2-4].

To improve acceptability and tolerability of colonoscopy, different protocols of sedation have been adopted[5]. Such regimens have been mainly restricted to benzodiazepines alone or in combination with opioids, because of the relatively high safety of these substances. Although these drugs result in a substantial improvement of patients' and endoscopists's experiences, some drawbacks have been observed. In particular, due to a relatively long half-life, a slow induction of sedation and a delayed discharging time with significant cost of monitoring have been reported[6]. Moreover, a significant proportion of patients are quite dissatisfied by the suboptimal degree of sedation provided by this protocol, and morbidity and mortality as a result of respiratory depression have also been reported[7,8].

Propofol represents a short-acting sedative agonist of gamma-aminobutyric acid receptor in the central nervous system, and it is mainly used for the induction and maintenance of deep sedation during surgical procedures[9]. Because of its short half-life (2-4 min) and high lipid solubility, propofol has the distinct advantages of a rapid induction of sedation and a fast recovery. When applied to gastrointestinal (GI) endoscopy, patient satisfaction with propofol has been shown to be equivalent or superior to that of benzodiazepines and/or narcotics[7]. Propofol, however, is a respiratory depressant with a narrow therapeutic range and without a reversal agent, resulting in a significant risk of inducing a too deep level of sedation, complicated by hypoventilation, apnea or cardiovascular depression. Moreover, it lacks a reversal agent. For this reason, propofol is largely administered by anesthesiologists or anesthesiologist nurses[10]. When considering the very high number of colonoscopies performed worldwide - 14 million every year in the United States alone[11] - anesthesiologist capacity is, however, likely to be insufficient to assure propofol sedation for this procedure.

It has been recently shown that propofol may be an effective and safe agent when used by non-anesthesiologists to target an adequate level of sedation[12]. A recent systematic review of the literature, including 646 080 cases, provided adequate evidence to the American Gastroenterological Association for them to support propofol administration by non-anesthesiologists (NAP), because of the extraordinary rarity of life-threatening episodes[13]. Most of these series were based on the use of propofol alone, during which NAP targeted a deep level of sedation. To further improve the safety profile, it has been suggested that a substantial reduction of the propofol dose may be achieved by administering this drug in association with other sedative agents, such as midazolam or meperidine[14,15]. This protocol has been defined as balanced propofol sedation (BPS), and, differently from NAP, it targets a moderate level of sedation.

No study has addressed the use of propofol by non-anesthesiologists for colonoscopy in Italy, and very few in Europe[13]. This is largely related to the product label of the drug which allows its administration only by physicians trained in general anesthesia. Due to the lack of an adequate anesthesiologist capacity and the low fee of reimbursement for a colonoscopy in the public system, virtually all the procedures are performed without propofol.

Only a few colonoscopy series have addressed the efficacy and safety of BPS for colonoscopy, most of them including only a few hundred of patients[14,15,17-23]. The purpose of this study was to prospectively assess the safety and the efficacy of endoscopist-administered BPS to target a moderate level of sedation for colonoscopy in a large series of consecutive patients.

MATERIALS AND METHODS

From February 2008 to December 2009, outpatients who presented to our unit for diagnostic colonoscopy were eligible for the study if they were between 18 and 75 years of age, American Society of Anesthesiology (ASA) class Ⅰ or Ⅱ, and capable of providing written informed consent for study participation. Exclusion criteria were inability to provide informed consent, history of allergic reactions or hypersensitivities to midazolam, propofol, eggs, or soybeans, high-risk head and neck anatomy (Mallampati score > 2) that could complicate airway rescue, sleep apnea syndrome, ASA class > Ⅱ.

The use of propofol by non-anesthesiologists in Italy is, at the time being, prevented by the specification in the product label that the use of this drug is exclusively allowed for anesthetists or intensive care unit physicians. For this reason, the administration of propofol within the present study has been performed under a study protocol that was supported by our Institution (Istituto Clinico Humanitas) and approved by the institutional review board. Nine endoscopists participated in this protocol, being authorized to administer propofol.

Patients underwent BPS administered by an endoscopist who was not involved in the endoscopic procedure. The physicians administering sedation were certified in advanced cardiac life support and had also successfully completed an intensively structured training program in propofol administration and laryngeal mask use under an anesthesiologist tutorship. The same anesthesiologist was always on call during the procedure time. Baseline vital signs (heart rate, blood pressure, oxygen saturation) were obtained in all patients before induction of sedation. Endoscopy-dedicated nurses also attended the procedure.

BPS was structured as follows: after a single dose of midazolam (0.05 mg/kg; Hameln pharmaceuticals gmbh, Hameln, Germany), a starter bolus of 0.5 mg/kg

Table 1 Scale for assessing Alertness/Sedation

Responsiveness	Score
Responds readily to name spoken in normal tone	5
Lethargic response to name spoken in normal tone	4
Responds only after name is called loudly and/or repeatedly	3
Responds only after mild prodding or shaking	2
Responds only after painful trapezius squeeze	1
Does not respond to painful trapezius squeeze	0

of propofol (Diprivan, Astra-Zeneca, Stockholm, Sweden) was administered. Repeated boluses of 10 to 20 mg of propofol were then administered on-demand with a 30-60 s interval for the entire duration of the procedure. Propofol bolus frequency and dose were titrated to the patient response, including vital signs and manifestations of restlessness or discomfort. The maximum dose allowed to be administered was 200 mg. Throughout the procedure, all patients received oxygen 2 L/min by nasal cannula. Continuous pulse oximetry, heart rate, electrocardiography, and end-expiratory carbon dioxide were monitored, with blood pressure being assessed at 5-min intervals. Level of sedation was evaluated according to the Scale for assessing Alertness/Sedation (MOAA/S), as reported in Table 1. In detail, deep sedation was defined as MOAA/S 1, moderate as MOAA/S 2-4, and minimal as MOAA/S 5. The following parameters were recorded: patient demographics, procedure indication and duration, midazolam dose, propofol dose, induction time, recovery time, cecal intubation rate, and polyp detection rate. The baseline values and changes in vital signs or oxygen saturation (SpO_2) from the baseline were also recorded. Adverse events were defined as hypoxia (i.e., a reduction in oxygen saturation < 90% for more than 20 s) requiring supplemental oxygen (O_2) by nasal cannula (NC) in excess of 2 L/min; and transient hypotension (< 90 mmHg) or bradycardia (< 60 beats/min) not requiring any active medical treatment. Serious adverse events were defined as hypoxia requiring positive pressure ventilation or laryngeal mask use; hypotension (< 90 mmHg) or bradycardia (< 60 beats/min) requiring medical treatment (i.e., infusion of liquid) other than propofol titration; and any event requiring the administration of a benzodiazepine antagonist (flumazenil). After the procedure, the patients were transported to the recovery room where blood pressure, SpO_2 and heart rate were measured continuously until discharge. Discharge was possible when blood pressure was within 20% of the initial value, SpO_2 > 90%, and the patient was able to drink and walk autonomously. Recovery time was measured from the time the patient entered the recovery area until departure by the recovery room nurse.

RESULTS

During the study period, 1593 eligible patients were included. Of these, 789 (49%) were male, the median age being 60 years (range: 22-75 years). Clinical indication for colonoscopy was evaluation of symptoms in 876 (55%) cases, screening or surveillance of a previous neoplastic lesion in 542 (34%), work-up of a positive fecal test in 96 (6%), and follow up of inflammatory bowel diseases in the remaining 79 (5%) cases.

Baseline mean heart rate and mean blood pressure were 71 ± 13 beats per min and 103 ± 16 mmHg, respectively. BPS was administered to all the patients. The median dose of midazolam was 2.3 mg (range: 2-4 mg), and the median dose of propofol administered was 70 mg (range: 40-120 mg). The median induction time of sedation (i.e., between the initiation of sedation and colonoscope insertion) was 3 min (range: 1-4 min). The deepest level of sedation was moderate in 1561 (98%) patients and deep in the remaining 32 (2%) cases. General anesthesia was not observed in any patient.

There was no serious adverse event related to any of the 1593 patients. The only adverse events observed with BPS were episodes of transient oxygen desaturation requiring O_2 supplementation by NC in excess of 2 L/min in 8 (0.46%; 95% CI: 0.2%-0.8%) patients. No patient required mask ventilation or endotracheal intubation. Although a transient decrease in blood pressure was common (446 patients, 28%), no episodes of sustained hypotension or bradycardia requiring active therapy were observed. No patient required administration of a benzodiazepine antagonist. Median recovery time was 23 min (range: 10-40 min).

The overall cecal intubation rate was 93.5%, corresponding to 1491 complete colonoscopies. Incomplete procedures were due to poor bowel cleaning in 72 (4.5%) patients and sigmoid strictures in 30 (2%) cases. The median procedural time was 11.3 min (range: 9-22 min), consisting of a median intubation time of 4 min (range: 3-9 min) and a median withdrawal time of 6.3 min (range: 4.2-11.9 min). Adenoma detection rate was 23.4% (27.8% for male, and 18.5% for female subjects). No major procedure-related complication occurred.

DISCUSSION

Our study showed that a BSP protocol, based on the co-administration of propofol with benzodiazepine, was a feasible, effective and safe approach for colonoscopy in a large series of consecutive patients. In particular, following a careful and rigid selection of the patients, BSP was successfully administered by non-anesthesiologist endoscopists without requiring anesthesiologist intervention in any of the cases. No BSP-related serious adverse event occurred in the study population, as outlined by the evidence that a midazolam-reversal agent was not needed in any patient. A transient oxygen desaturation was observed in only 0.5% of the study population, and it was treated conservatively in all cases.

The high safety profile of the BSP observed in our study appears to be strictly related to the very low dose

of propofol needed to target a moderate sedation, because of the additional effect of midazolam. Despite the fact that this was a non-randomized study in which a propofol-alone arm was not included, the median dose of propofol shown in the present study, corresponding to 70 mg per patient, appeared to be much lower than the 200-400 mg range described in previous propofol-alone series[16]. A similarly low propofol dose was also reported in previous series in which BSP was adopted[14,15,17-23]. When considering the potential legal implications related to NAP, the ability to minimize the dose of propofol needed appears as an attractive goal for the endoscopists. The very low rate of oxygen desaturation observed in our study may also be related to the systematic adoption of capnography to monitor our patients. It has been suggested that capnography may anticipate the diagnosis of propofol-induced hypoventilation as compared to the simple assessment of oxygen saturation[24].

It could be argued, however, that co-administration of midazolam could reduce the propofol-related advantages. In particular, the slow metabolization of benzodiazepines could result in a prolonged recovery time, reducing the efficiency of an endoscopic turnover system. The median recovery time in our series was consistently lower than 30 min. This value favorably compares with previous accounts of midazolam alone, in which a recovery time as long as 70 min was reported[25]. Such a difference in favor of the BSP regimen is presumably due to the relatively low dose of midazolam administered, the median being 2.1 mg per patient. Moreover, midazolam was administered only at the beginning of the procedure as a bolus, so that the drug started to be metabolized during the procedure itself, lasting on average 11 min.

Quality of colonoscopy procedures in our series appeared to reach the required standards, showing no interference of BSP in the diagnostic or operative procedures. In particular, the adjusted cecal intubation rate of 93.5% in a mixed setting with symptomatic and screening indications is remarkably superior to the 80.7% recently reported in an Italian survey, in which the use of propofol was not reported[26]. Of note, in a similarly designed Italian study, it was observed that the intubation rate in sedation-assisted colonoscopies was superior to that of those performed without sedation[27].

It could be argued that the results of our study were not unexpected; the safety of BSP having already been shown in previous studies. However, most of these series included only 100-200 patients[14,15,17-23], so that a greater confirmation of BSP safety in over 1500 subjects was needed. Moreover, this is the first Italian study in which NAP was applied to colonoscopy, and, more generally, to adults. This would appear to be of major importance when considering that the use of propofol in Italy is prevented by an unequivocal recommendation in the product label stating that only anesthesiologists are allowed to administer such a drug. The safety profile of BSP in our study should call for dedicated studies aiming to ascertain whether such a recommendation is really a protection for the patients and whether it is consistent with literature data or simply represents an obstacle preventing a safe propofol-assisted colonoscopy to most patients. Indeed, in Italy, due to the lack of anesthesiologist capacity, virtually all the colonoscopies are performed without propofol, using at best benzodiazepines and/or narcotics[25].

There are limitations to the present analysis. Our main target was to evaluate BSP efficacy in targeting a moderate level of sedation when administered by non-anesthesiologists, whilst we did not assess the level of satisfaction of patients or endoscopists with our sedation protocol. However, there is enough evidence regarding a higher satisfaction level with propofol as compared to midazolam[16]. Moreover, the short induction time clearly reflects a propofol type of sedation rather than the effect of midazolam. Secondly, we did not compare the propofol/midazolam BSP with other protocols, such as propofol alone or propofol with narcotics with or without midazolam. However, most of the propofol-related toxicity is associated with its narrow therapeutic window, so that it is unlikely that such a high safety profile would be achieved by protocols based on doses of propofol substantially larger than those reached in our experience. Thirdly, we did not blind the discharging nurse regarding the type of sedation, so that we cannot exclude a bias in the computation of the recovery time. Fourthly, we did not assess the alertness level after several hours from discharge, so that we cannot exclude a prolonged effect of midazolam bolus in our series. Fifthly, although our study included over 1500 subjects, we cannot exclude extremely rare events that have been associated with the use of propofol, such as neurologic injuries or even death. However, the lack of severe episodes of respiratory or cardiovascular depression reassures us about the safety of BSP. Moreover, no death has been reported up to now with the use of NAP in colonoscopy; all the cases having been associated with upper GI endoscopy or biliary maneuvers[13]. According to the study protocol, we systematically used a non-anaesthesiologist physician for monitoring propofol administration. It could be argued that this represents a waste of resources, requiring two endoscopists to perform one procedure. However, this simply reflects a prudent choice within the study protocol to prevent eventual litigation for an off-label use of the drug. It has already been shown that appropriately trained nurses may assist the endoscopist in propofol administration and sedation monitoring with a clear saving of resources. Finally, we did not use specific scales of recovery after the completion of the colonoscopy, considering discharge possible on the basis of blood pressure, SpO_2, and the patients' ability to drink and walk autonomously.

In conclusion, we report a large consecutive series showing the efficacy and safety of BSP for colonoscopy, when administered by non-anesthesiologists. When considering the controversy regarding NAP use for GI endoscopy, the very low dose of propofol allowed by the co-administration of midazolam appears to be a rational approach to maximize sedation efficacy and to minimize

propofol toxicity at the same time.

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COMMENTS

Background

Non-anesthesiologists propofol administration (NAP) represents an effective and safe alternative to sedation with benzodiazepines/narcotics for colonoscopy. NAP generally involves the administration of propofol alone to target a deep level of sedation. By associating propofol with other sedative agents, such as midazolam, a moderate level of sedation may be targeted, resulting in a substantial reduction of the propofol dose.

Research frontiers

Despite being validated in small controlled trials, such a balanced propofol sedation has never been tested in a large cohort.

Innovations and breakthroughs

In a large prospective study involving 1593 patients, a balanced propofol sedation consisting of the co-administration of propofol and midazolam resulted in a moderate level of sedation in 98% of colonoscopies. Recovery time also appeared to be favorably short. Such a balanced protocol of sedation appeared to be highly safe, the only serious event being a transient oxygen desaturation requiring further oxygen supplementation in less than 1% of the patients. The median dose of propofol administered was 70 mg, being less than 120 mg in the entire series. The overall cecal intubation and adenoma detection rates were 93.5% and 23.4%, respectively. No major procedure-related complication occurred.

Applications

A balanced administration of propofol by non-anesthesiologists may be safely implemented in dedicated centers.

Peer review

The paper assessed the efficacy and safety of a balanced approach using midazolam in combination with propofol administered by non-anesthesiologists in a large series of diagnostic colonoscopies. It is very interesting.

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BRIEF ARTICLE

Is it better to use two elastographic methods for liver fibrosis assessment?

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Abstract

AIM: To find out if by combining 2 ultrasound based elastographic methods: acoustic radiation force impulse (ARFI) elastography and transient elastography (TE), we can improve the prediction of fibrosis in patients with chronic hepatitis C.

METHODS: Our study included 197 patients with chronic hepatitis C. In each patient, we performed, in the same session, liver stiffness (LS) measurements by means of TE and ARFI, respectively, and liver biopsy (LB), assessed according to the Metavir score. 10 LS measurements were performed both by TE and ARFI; median values were calculated and expressed in kilopascals (kPa) and meters/second (m/s), respectively. Only TE and ARFI measurements with IQR < 30% and SR ⩾ 60% were considered reliable.

RESULTS: On LB 13 (6.6%) patients had F0, 32 (16.2%) had F1, 52 (26.4%) had F2, 47 (23.9%) had F3, and 53 (26.9%) had F4. A direct, strong correlation was found between TE measurements and fibrosis (r = 0.741), between ARFI and fibrosis (r = 0.730) and also between TE and ARFI (r = 0.675). For predicting significant fibrosis (F ⩾ 2), for a cut-off of 6.7 kPa, TE had 77.5% sensitivity (Se) and 86.5% specificity (Sp) [area under the receiver operating characteristic curve (AUROC) 0.87] and for a cut-off of 1.2 m/s, ARFI had 76.9% Se and 86.7% Sp (AUROC 0.84). For predicting cirrhosis (F = 4), for a cut-off of 12.2 kPa, TE had 96.2% Se and 89.6% Sp (AUROC 0.97) and for a cut-off of 1.8 m/s, ARFI had 90.4% Se and 85.6% Sp (AUROC 0.91). When both elastographic methods were taken into consideration, for predicting significant fibrosis (F ⩾ 2), (TE ⩾ 6.7 kPa and ARFI ⩾ 1.2 m/s) we obtained 60.5% Se, 93.3% Sp, 96.8% positive predictive value (PPV), 41.4% negative predictive value (NPV) and 68% accuracy, while for predicting cirrhosis (TE ⩾ 12.2 kPa and ARFI ⩾ 1.8 m/s) we obtained 84.9% Se, 94.4% Sp, 84.9% PPV, 94.4% NPV and 91.8% accuracy.

CONCLUSION: TE used in combination with ARFI is highly specific for predicting significant fibrosis; therefore when the two methods are concordant, liver biopsy can be avoided.

Key words: Transient elastography; Acoustic radiation force impulse elastography; Liver stiffness; Combined methods

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INTRODUCTION

Liver fibrosis evaluation in patients with hepatitis C virus (HCV) infection is essential for prognosis assessment and also for a decision regarding therapy. In many centers, liver biopsy (LB) is the "normal" means of fibrosis assessment. In the last few years, non-invasive methods for the evaluation of liver fibrosis have become more and more popular, especially in France and, subsequently, throughout the world.

Non-invasive methods for liver fibrosis assessment are: biological (serological) tests[1-5], ultrasound based (elastographic) methods, such as transient elastography (TE)[6-9], real time elastography[10-12] and acoustic radiation force impulse (ARFI) elastography[13-15] and magnetic resonance imaging (MRI) elastography[16,17]. Each method has certain advantages: only a few milliliters of blood are required for the serological tests, a special "ultrasound" examination is required for the elastographic methods and finally, a MRI examination reveals information about many abdominal organs and at the same time evaluates the liver stiffness (LS). All these methods have some disadvantages, the major one being that they are not 100% sensitive or 100% specific compared to the LB which is still considered the "gold standard".

Some authors have proposed to combine different noninvasive methods for liver fibrosis evaluation, hoping to increase the accuracy or maybe to decrease the number of LBs needed to solve unclear cases[18]. Some years ago, Castera[18] proposed to use only ALT for the evaluation of liver activity in patients with chronic hepatitis C, and for fibrosis to combine a FibroTest with a FibroScan. If these noninvasive tests are concordant, then LB can be avoided. In this study, when the FibroScan and FibroTest results agreed, significant fibrosis (F $\geqslant$ 2) was confirmed by LB in 84% of the cases, severe fibrosis (F $\geqslant$ 3) in 95% of cases, and cirrhosis (F = 4) in 94% of the cases.

The purpose of this study is to find out if, by combining 2 ultrasound based elastographic methods: ARFI elastography and TE, we can improve the prediction of fibrosis severity in patients with chronic HCV hepatitis.

MATERIALS AND METHODS

Patients

We performed a bicentric study in two university hospitals (Timisoara and Cluj-Napoca) that included 197 patients with chronic HCV hepatitis (anti HCV antibodies positive, with or without cytolysis for at least 6 mo, PCR HCV RNA positive). In all these patients, in the same session, LS was evaluated by means of TE (FibroScan®) and ARFI elastography, and LB was performed in order to assess the fibrosis stage. Patients with other causes of chronic hepatitis (HBV infection, chronic alcohol abuse, cholestatic chronic hepatitis, nonalcoholic steatohepatitis, autoimmune chronic hepatitis, haemochromatosis, Wilson's disease) were excluded from our study. Informed consent was obtained from each patient included in the study and the study protocol was approved by the local ethical committee.

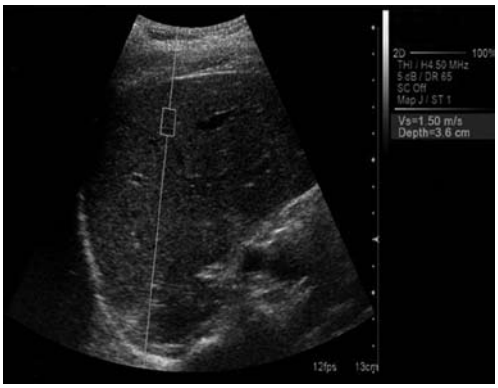

Figure 1 Acoustic radiation force impulse measurement in the liver.

TE

TE was performed in all patients with a FibroScan® device (EchoSens® - Paris, France) by experienced physicians (more than 500 TE), blinded to the results of LB and ARFI measurements. In each patient, 10 valid measurements were performed, after which a median value of LS was obtained, measured in kilopascals (kPa). Only patients in which LS measurements by means of TE had a success rate of at least 60%, with an interquartile range (IQR) < 30%, were included in our study. The success rate was calculated as the ratio of the number of successful acquisitions over the total number of acquisitions. IQR is the difference between the 75th percentile and the 25th percentile, essentially the range of the middle 50% of the data.

ARFI elastography

ARFI elastography was performed in all the patients with a Siemens Acuson S2000™ ultrasound system. The ultrasound probe automatically produces an acoustic "push" pulse that generates shear-waves which propagate into the liver. Their speed, measured in meters/second (m/s), is displayed on the screen. The propagation speed increases with fibrosis. The operator can select the depth at which the liver elasticity is evaluated by placing a "measuring box" (10 mm long and 5 mm wide) in the desired place (Figure 1). The patients were examined in left lateral decubitus, with the right arm in maximum abduction. Scanning was performed between the ribs in the right liver lobe in order to avoid cardiac motion (approximately in the place where we usually perform LB), 1 cm under the capsule, with minimal scanning pressure applied by the operator, while the patients were asked to stop breathing for a mo-

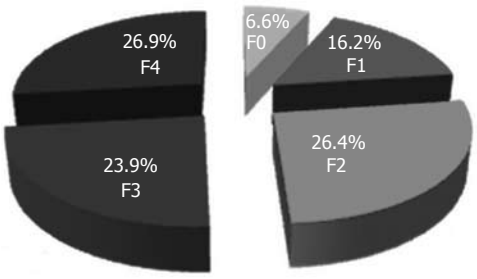

Figure 2 Severity of fibrosis in the studied group.

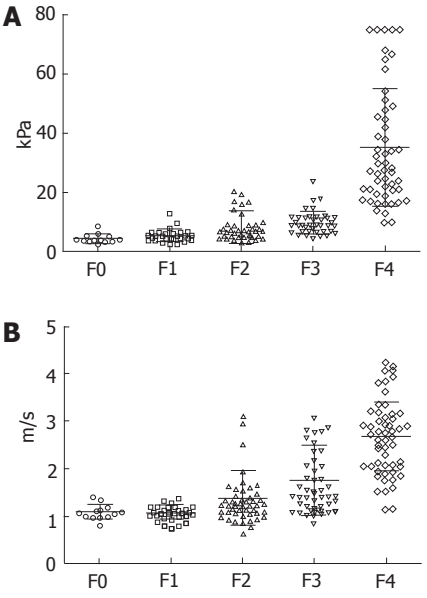

Figure 3 Liver stiffness measurements by means of transient elastography (A) and acoustic radiation force impulse elastography (B) for various stages of fibrosis.

ment, in order to minimize breathing motion.

We performed 10 measurements in every patient, and a median value was calculated, the result being measured in m/s. Only patients in which LS measurements by means of ARFI had a success rate of at least 60%, with an IQR < 30%, were included in our study. Operators were blinded to the results of LB and TE measurements.

LB

LB was performed in all the patients using echoguided TruCut technique, with a 1.8 mm (14 G) diameter automatic needle device-Biopty Gun (Bard GMBh), or echoassisted, using Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only LB fragments including at least 6 portal tracts were considered adequate for pathological interpretation and included in our study. The LBs were assessed, according to the Metavir score, by a senior pathologist (one in each center) blinded to the results of TE and ARFI measurements. Fibrosis was staged on a 0-4 scale: F0-no fibrosis; F1-portal fibrosis without septa; F2-portal fibrosis and few septa extending into lobules; F3-numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4-cirrhosis.

Statistical analysis

The data we obtained from our patients were collected in a Microsoft Excel file, the statistical analysis being performed using MedCalc and GraphPad Prism programs. The predictors for the stage of fibrosis (ARFI and TE measurements) were numeric variables, so the mean and standard deviation were calculated.

Associations between assay results and fibrosis stage according to the Metavir scoring system (range: 0-4, ordinal scale), were described using the Spearman rank correlation coefficient (r).

The diagnostic performances of ARFI and TE were assessed by using receiver operating characteristics (ROC) curves. ROC curves were thus built for the detection of significant fibrosis (F $\geqslant$ 2 Metavir) and cirrhosis (F = 4 Metavir). Optimal cut-off values were chosen to maximize the sum of sensitivity (Se) and specificity (Sp). Se and Sp were calculated according to standard methods. Exact confidence intervals of 95% were calculated for each predictive test.

RESULTS

Our study group included 197 patients, 119 women and 78 men, mean age 50 ± 9.8 years. On LB 13 (6.6%) patients had F0, 32 (16.2%) had F1, 52 (26.4%) had F2, 47 (23.9%) had F3 and 53 (26.9%) had F4 (Figure 2).

We obtained valid TE measurements in 187/197 patients (94.9%) and valid ARFI measurements in 191/197 patients (96.9%).

A direct, strong correlation was found between the values of liver stiffness evaluated by TE and fibrosis (r = 0.741) (Figure 3A), between the values of liver stiffness measured by ARFI and fibrosis (r = 0.730) (Figure 3B) and also between the values of liver stiffness evaluated by means both of TE and ARFI (r = 0.675).

The predictive values of TE and ARFI, alone, for F $\geqslant$ 2 and F4, respectively, are presented in Table 1.

By combining the two elastographic methods (values both for TE and ARFI above the mentioned cut-offs) the specificity increased, statistically significant as compared to ARFI (F $\geqslant$ 2: 93.3% *vs* 86.7%, P = 0.04; F = 4: 94.4% *vs* 85.6%, P = 0.007) but not as compared to TE (F $\geqslant$ 2: 93.3% *vs* 86.7%, P = 0.05; F = 4: 94.4% *vs* 89.6%, P = 0.12), of course with lower sensitivity (Table 2), with very good positive predictive value (PPV) (96.3%) for significant fibrosis (F $\geqslant$ 2 Metavir). By combining the two elastographic methods for F4, we obtained a very high negative predictive value (NPV), along with very good PPV and accuracy (Table 2).

The accuracy of the combined tests (TE + ARFI) was statistically significant better than ARFI alone for predicting cirrhosis (91.8% *vs* 83.4%, P = 0.02), but not as compared to TE alone (91.8% *vs* 91.4%, P = 0.96).

Table 1 Predictive value of transient elastography and acoustic radiation force impulse alone, for F ≥ 2 and F = 4 (%)

	F ≥ 2							F = 4						
	Cut-off	AUROC	Se	Sp	PPV	NPV	Accuracy	Cut-off	AUROC	Se	Sp	PPV	NPV	Accuracy
TE	6.7 kPa	0.87	77.5	86.7	94.8	54.9	79.6	12.2 kPa	0.97	96.2	89.6	78.1	98.3	91.4
ARFI	1.2 m/s	0.84	76.9	86.7	95.7	54.1	79.3	1.8 m/s	0.91	90.4	85.6	50.3	95.8	83.4

TE: Transient elastography; ARFI: Acoustic radiation force impulse; AUROC: Area under the receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

Table 2 Predictive value of transient elastography and acoustic radiation force impulse in combination for F ≥ 2 and F = 4 (%)

	F ≥ 2						F = 4					
	Cut-off	Se	Sp	PPV	NPV	Accuracy	Cut-off	Se	Sp	PPV	NPV	Accuracy
TE + ARFI	6.7 kPa and 1.2 m/s	60.5	93.3	96.8	41.1	68	12.2 kPa and 1.8 m/s	84.9	94.4	84.9	94.4	91.8
TE or ARFI	6.7 kPa or 1.2 m/s	86.1	71.1	90.9	60.3	82.7	12.2 kPa or 1.8 m/s	96.2	83.3	68	98.3	86.8

TE: Transient elastography; ARFI: Acoustic radiation force impulse; AUROC: Area under the receiver operating characteristic curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

DISCUSSION

Discussions regarding the replacement of LB in the evaluation of liver fibrosis with non-invasive methods are currently very active. Arguments in favor of maintaining LB are: it allows a correct evaluation of fibrosis as well as of the activity of the disease; it can provide arguments for the etiology (Mallory bodies, *etc*) or it can evaluate the presence and severity of hepatocytes' fatty infiltration. Arguments against this method are: it is an invasive one (there is a risk for complications, even if it is low); it is usually a stressful method for the patients; in some cases, good quality histological specimens are not obtained and, also, there are some questions regarding sampling variability.

In some countries, such as France, the non-invasive methods for fibrosis assessment have replaced the LB in a large number of cases. To become accepted worldwide, these non-invasive methods must be very accurate, in order to replace a well recognized method such as LB.

TE measures the liver stiffness of a fragment that is approximately a cylinder 1 cm in diameter and 4 cm long, 500 times bigger than the specimen obtained by LB. This examination is more or less blind, but the other method that we used for the elastographic evaluation of the liver, ARFI, is performed under clear ultrasonographic visualization of the area of interest (the operator being able to choose the area to be examined).

The criticism, especially of TE, is that it is not able to differentiate between contiguous stages of fibrosis (F0 *vs* F1, or F1 *vs* F2). On the other hand, from the point of view of the clinician, it is important to know if the patient has only mild fibrosis (probably with no need for treatment in HCV patients), or moderate or severe fibrosis. And for this purpose, TE is quite a good method (the following AUROCs were reported: 0.79 for F ≥ 2, 0.91 for F ≥ 3 and 0.97 for F = 4)[7].

The other elastographic method, ARFI technology, involves targeting an anatomic region to be interrogated for elastic properties, with the use of a region of-interest cursor, while performing real-time B-mode imaging. Tissue from the region of interest is mechanically excited by using short-duration (262 μs) acoustic pulses with a fixed transmit frequency of 2.67 MHz to generate localized tissue displacements. The displacements result in shear-wave propagation away from the region of excitation and are tracked by using US correlation-based methods[19]. The shear-wave propagation velocity is proportional to the square root of tissue elasticity. Results are expressed in meters per second (m/s). The technique is new and published data suggest that ARFI and TE have a similar predictive value for fibrosis assessment.

In a study performed by Friedrich-Rust *et al*[9] in which ARFI was compared to LB and blood markers in 86 patients with chronic hepatitis (HBV or HCV), the Spearman correlation coefficients between the histological fibrosis stage and ARFI, TE, FibroTest and APRI score were statistically significant: 0.71, 0.73, 0.66 and 0.45 respectively ($P < 0.001$).

In the study performed by Lupşor *et al*[14], 112 patients with chronic hepatitis were evaluated. All the patients underwent LB (fibrosis stage assessed according to the Metavir scoring system), ARFI and FibroScan. The mean ARFI values for different stages of fibrosis were: 1.079 ± 0.150 m/s (F0-F1), 1.504 ± 0.895 m/s (F2), 1.520 ± 0.575 m/s (F3) and 2.552 ± 0.782 m/s (F4). The mean values were statistically significant different only between F3 and F4. The following cut-off values were proposed for various stages of fibrosis: F ≥ 1:1.19 m/s; F ≥ 2:1.34 m/s; F ≥ 3:1.61 m/s; and F ≥ 4:2 m/s.

Since both types of elastographic evaluation are available in our Department, we tried to see if by combining them we can improve their predictive value for fibrosis assessment. Firstly, we evaluated their predictive value alone for significant fibrosis (F ≥ 2 Metavir) and cir-

rhosis (F = 4), after which we evaluated their predictive value in combination. If both methods were concordant (TE ≥ 6.7 kPa and ARFI ≥ 1.2 m/s), we obtained a high specificity (93.3%) for predicting significant fibrosis (F ≥ 2), also with a very good positive predictive value (96.8%), so in those cases there was no need to perform LB before initiating treatment. In our study group, 152/197 patients had significant fibrosis (F ≥ 2 Metavir) on LB. TE and ARFI were concordant for significant fibrosis in 92 of 152 patients. Therefore, we could avoid 60.5% of the LBs in our group of patients.

Also, by combining the two elastographic methods for predicting cirrhosis (F4) (TE ≥ 12.2 kPa and ARFI ≥ 1.8 m/s), the results were very good, with 94.4% Sp, 94.4% NPV and 91.8% accuracy, so the combined methods are excellent for confirming, but also for excluding the presence of cirrhosis. In our study group, 53 patients had cirrhosis (F = 4 Metavir on LB). TE and ARFI were concordant for liver cirrhosis in 45 from the 53 patients with F = 4 on LB (84.9%).

Other published data tried to combine different noninvasive methods for a better evaluation of liver stiffness. In a study published in 2005 by Castera *et al*[18], 183 patients with chronic HCV hepatitis were evaluated by LB, TE, FibroTest and APRI. The best performance was obtained by combining FibroScan and FibroTest, with areas under the ROC curve of 0.88 for F ≥ 2, 0.95 for F ≥ 3, and 0.95 for F = 4. When FibroScan and FibroTest results agreed, significant fibrosis (F ≥ 2) was confirmed by LB in 84% of the cases, severe fibrosis (F ≥ 3) in 95% of cases, and cirrhosis (F = 4) in 94% of the cases.

In another study published in 2010, Castera *et al*[20] evaluated two algorithms for liver fibrosis prediction: one combined TE and FibroTest (Castera) and the other APRI and FibroTest (SAFE biopsy). In all patients a LB was performed. Significant fibrosis (F ≥ 2 Metavir) was present in 76% of patients and cirrhosis (F4) in 25%. TE failure was observed in eight cases (2.6%). For significant fibrosis, the Castera algorithm saved 23% more liver biopsies than SAFE biopsy (71.9% *vs* 48.3%, respectively, $P < 0.0001$), but its accuracy was significantly lower (87.7% *vs* 97.0%, respectively; $P < 0.0001$). Regarding cirrhosis, the accuracy of the Castera algorithm was significantly higher than that of SAFE biopsy (95.7% *vs* 88.7%, respectively; $P < 0.0001$). The number of saved liver biopsies did not differ between the two algorithms (78.8% *vs* 74.8%, P = NS).

Shahenn[21] published a meta-analysis which compared the performances of TE and FibroTest for the prediction of liver fibrosis in patients with chronic HCV hepatitis. Thirteen studies were identified, 9 regarding FibroTest (1679 patients) and 4 regarding TE (546 patients). In heterogeneous analysis for significant fibrosis, the AUROC curves for FibroTest and TE were 0.81 and 0.83, respectively. At a threshold of approximately 0.60, the sensitivity and specificity of FibroTest were 47% (35%-59%) and 90% (87%-92%). For TE (threshold approximately 8 kPa), corresponding values were 64% (50%-76%) and 87% (80%-91%), respectively. However, the diagnostic accuracy of both tests was associated with the prevalence of significant fibrosis and cirrhosis in the study populations. For cirrhosis, the summary AUROCs for FibroTest and FibroScan were 0.90 and 0.95 (0.87-0.99).

In a study published in 2010 by Cross *et al*[22], 187 patients with chronic HCV hepatitis were evaluated by means of LB, TE and the King score. Liver fibrosis was scored using the Ishak score; significant fibrosis was defined as Ishak fibrosis stage F3-F6, and cirrhosis defined as Ishak fibrosis F5-F6. The AUROCs for TE, the King score and TE + King score for the diagnosis of Ishak F3-F6 were 0.83, 0.82 and 0.85, respectively and 0.96, 0.89 and 0.93, respectively, for the diagnosis of cirrhosis (F ≥ 5 Ishak). The negative predictive values for the diagnosis of cirrhosis, using the optimal cut-off results for TE (10.05 kPa), the King score (24.3) and the two combined (26.1), were 98%, 91% and 94%, respectively.

Our study tried to establish whether the combination of TE and ARFI could provide some advantages for the evaluation of significant fibrosis in patients with chronic hepatitis C in comparison with a single elastographic method. By combining the two elastographic methods (values both for TE and ARFI above the mentioned cut-offs), the specificity increased (of course with lower sensitivity), with very good PPV (96.3%) for significant fibrosis (F ≥ 2 Metavir). In our study group, 152/197 patients had significant fibrosis (F ≥ 2 Metavir) on LB. TE and ARFI were concordant for significant fibrosis in 92 of 152 patients. Therefore, we were able to avoid 60.5% of LB in our group of patients.

Also, by combining the two elastographic methods for predicting cirrhosis (F4) (TE ≥ 12.2 kPa and ARFI ≥ 1.8 m/s), the results were very good, with 94.4% Sp, 94.4% NPV and 91.8% accuracy, so the combined methods are not only able to confirm, but also to exclude the presence of cirrhosis.

In conclusion, LS measurements assessed by means of both TE and ARFI strongly correlate to histological fibrosis in HCV patients. TE used in combination with ARFI is highly specific (approximately 93%) for predicting significant fibrosis (F ≥ 2 Metavir), so that in patients with higher LS measurements than the proposed cut-offs for both methods, liver biopsy could be avoided. Also, in patients suspected of having severe fibrosis, if both methods are concordant, they are very good for confirming and excluding the presence of cirrhosis (94.4% Sp, 94.4% NPV).

COMMENTS

Background

Non-invasive methods for fibrosis assessment in chronic hepatitis, such as transient elastography (TE), are accepted more and more, tending to replace the invasive methods, especially in hepatitis C virus (HCV) chronic hepatitis. In the last few years, studies were published regarding the use of acoustic radiation force impulse (ARFI) elastography for fibrosis assessment in chronic hepatitis.

Research frontiers

Studies were published regarding the benefits of combining non-invasive methods for fibrosis evaluation (serological tests with or without TE), but not regarding a combination of elastographic methods (TE and ARFI).

Innovations and breakthroughs

The aim of this study was to find out if by combining ARFI and TE the prediction of fibrosis in patients with chronic HCV hepatitis can be improved, and the authors concluded that TE used in combination with ARFI is highly specific for predicting significant fibrosis; therefore when the two methods are concordant liver biopsy can be avoided.

Applications

In this study that included 197 patients with chronic C hepatitis, LS measurement by means of both TE and ARFI strongly correlated to the histological fibrosis. TE used in combination with ARFI was highly specific (93.3%) for predicting significant fibrosis (F $\geqslant$ 2 Metavir); therefore in patients with higher LS measurements than the proposed cut-offs for both methods, liver biopsy could be avoided (positive predictive value 96.8%).

Terminology

TE (FibroScan) is an ultrasound-based method that uses the transmission of low-frequency vibrations to create an elastic shear wave that propagates into the liver, followed by the detection wave propagation velocity, which is proportional to the tissue stiffness, with faster wave progression occurring through stiffer tissue. ARFI technology involves targeting an anatomic region to be interrogated for elastic properties, with the use of a region of-interest cursor, while performing real-time B-mode imaging. Tissue from the region of interest is mechanically excited to generate localized tissue displacements. The displacements result in shear-wave propagation away from the region of excitation and are tracked by using US correlation-based methods. The shear-wave propagation velocity is proportional to the square root of tissue elasticity.

Peer review

This is a well written paper that looks at TE (a well studied technology for liver fibrosis) and acoustic radiation impulse force (a technology for which there is much less clinical data). The data is well displayed.

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BRIEF ARTICLE

YKL-40 expression in CD14$^+$ liver cells in acute and chronic injury

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Abstract

AIM: To demonstrate that CD14$^+$ cells are an important source of the growth factor YKL-40 in acute and chronic liver damage.

METHODS: Rats were inoculated with one dose of CCl_4 to induce acute damage. Liver biopsies were obtained at 0, 6, 12, 24, 48 and 72 h. For chronic damage, CCl_4 was administered three days per week for 6 or 8 wk. Tissue samples were collected, and cellular populations were isolated by liver digestion and purified by cell sorting. YKL-40 mRNA and protein expression were evaluated by real-time polymerase chain reaction and western blot.

RESULTS: Acute liver damage induced a rapid increase of YKL-40 mRNA beginning at 12 h. Expression peaked at 24 h, with a 26-fold increase over basal levels. By 72 h however, YKL-40 expression levels had nearly returned to control levels. On the other hand, chronic damage induced a sustained increase in YKL-40 expression, with 7- and 9-fold higher levels at 6 and 8 wk, respectively. The pattern of YKL-40 expression in different subpopulations showed that CD14$^+$ cells, which include Kupffer cells, are a source of YKL-40 after acute damage at 72 h [0.09 relative expression units (REU)] as well as after chronic injury at 6 wk (0.11 REU). Hepatocytes, in turn, accounted for 0.06 and 0.01 REU after 72 h (acute) or 6 wk (chronic), respectively. The rest of the CD14$^-$ cells (including T lymphocytes, B lymphocytes, natural killer and natural killer T cells) yielded 0.07 and 0.15 REU at 72 h and 6 wk, respectively. YKL-40 protein expression in liver was detected at 72 h as well as 6 and 8 wk, with the highest expression relative to controls (11-fold; $P \leqslant 0.05$) seen at 6 wk. Macrophages were stimulated by lipopolysaccharide. We demonstrate that under these conditions, these cells showed maximum expression of YKL-40 at 12 h, with $P < 0.05$ compared with controls.

CONCLUSION: Hepatic CD14$^+$ cells are an YKL-40 mRNA and protein source in acute and chronic liver injury, with expression patterns similar to growth factors implicated in inflammation-fibrogenesis.

Key words: YKL-40; Kupffer cells; Liver cirrhosis; CD14$^+$ cells

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INTRODUCTION

YKL-40 glycoprotein (Chi3L1) is a growth factor named for its N-terminal amino acid sequence and molecular weight. It is related to 18-glycosylhydrolases, but lacks enzymatic activity[1,2]. Under physiological conditions, human YKL-40 is secreted in small quantities by synoviocytes and chondrocytes[3,4]. However, *in situ* experiments demonstrated increased tissue mRNA and protein levels in osteoarthritis and rheumatism as well as in patients with inflammatory joint disease, where activated monocytes and macrophages are the main source.

The biological function of YKL-40 is unclear because no receptors have been identified to date. However, it has been reported that YKL-40 binds to stabilin type 1 and heparin sulfate *in vivo*. Furthermore, YKL-40 stimulates the proliferation and migration of connective tissue cells and fibroblasts through activation of the mitogen-activated protein kinase signaling pathway, suggesting that YKL-40 modulates fibrogenesis and tissue remodeling[5,6].

Several studies have found elevated YKL-40 concentrations in sera of patients with liver diseases, such as hepatic fibrosis by hepatitis C virus[7]. Serum concentrations of YKL-40 correlated with extracellular matrix (ECM) products secreted by hepatic stellate cells (HSCs) and fibroblasts (e.g., PⅢNP, hyaluronan, MMP-2, and TIMP-1). It has been suggested that YKL-40 concentrations reflect the degree of liver fibrosis. However, extensive clinical evaluation is still required, and other inflammatory diseases have to be excluded as potential causes of YKL-40 elevations.

In hepatic tissue, immunohistochemical analyses show strong YKL-40 staining around fibrotic areas[3]; however, in this study it was not possible to discriminate the cells that produce YKL-40. Notably, HSCs from fibrotic liver tissue by *S. japonica* showed an increase of YKL-40 mRNA[8].

Currently, the kinetics and source of YKL-40 in the liver under damage conditions are unknown. This study addressed these issues as part of a broader effort to elucidate the role of this molecule in hepatic inflammation and tissue repair.

MATERIALS AND METHODS

Animals

Rats were treated according to the guidelines for reproduction, care and use of laboratory animals stated by the Norma Oficial Mexicana (NOM-066-ZOO-1999). Twenty-seven male Wistar rats weighing 250 g each were selected for treatment with CCl_4 and divided into nine groups of three animals each. One group was untreated and used as controls. Six groups were inoculated with a single intragastric dose of 0.5 mL/100 g of CCl_4 (Sigma, 319961, United States) mixed 1:1 with mineral oil (Sigma, M5409, United States) to establish acute injury. These animals were sacrificed at time 0, 6, 12, 24, 48 and 72 h. To establish chronic liver injury, two groups were inoculated three times per week with an i.p. injection of 0.1 mL/100 g CCl_4: mineral oil at ratios of 1:6, 1:5, 1:4 (one week for each), then 1:3 until the animals were sacrificed at week 6 or 8.

Tissue samples

Procedures were performed under ether anesthesia. Livers were washed with PBS (Gibco, 70013-032, United Kingdom) at 4 ℃, sectioned into small 100 mg pieces and stored in tubes at -70 ℃. Samples for RNA extraction were stored in 500 μL of TRIzol (Invitrogen® BRL 15596-026, United States) at -70 ℃.

RNA extraction

Liver tissue and cells were homogenized in 500 μL of TRIzol (Invitrogen® BRL United States). Chloroform (Sigma, C2432, United States) was added, and the samples were centrifuged for 15 min at 10 000 *g* and 4 ℃. Total RNA was precipitated with isopropanol (Sigma, 19516, United States). The RNA pellet was washed with 75% ethanol and dissolved in RNase-free water. The final concentrations and quality of the RNA were determined by spectrophotometry.

Reverse-transcriptase polymerase chain reaction

Approximately 1 μg of total RNA was reverse-transcribed to cDNA in a 20 μL reaction using murine leukemia virus reverse transcriptase M-MLV (Invitrogen®BRL, 28025-013, United States). The mixture was prepared with 1 μg of RNA, 125 ng/μL of random primers (Invitrogen® BRL, 48190011, United States), 1 μL of 10 mmol/L dNTP mix and 12 μL of sterile distilled water. The mixture was heated for 5 min at 65 ℃ and quick chilled on ice. Four μL of 5X First Strand Buffer, 2 μL of 0.1 mol/L DTT and 1 μL of RNaseOUT (Invitrogen® BRL, 10777019, United States) were added, and the reaction was incubated at 37 ℃ for 2 min. Finally, 200 U/μL of M-MLV were added, and the total reaction was incubated at 25 ℃ for 10 min and then at 37 ℃ for 50 min. The reaction was inactivated by heating at 70 ℃ for 15 min[9].

Real–time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed

with an ABI Prism 7300 thermocycler (Applied Biosystems, United States). Each 20 μL reaction contained 2 μL of cDNA and TaqMan Universal PCR master mix (Applied Biosystems, 4364338, United States). The primers and probe set sequences were specific for 18S rRNA (constitutive gene; Applied Biosystems, FG18S RNA, United States) and YKL-40 (inducible gene; Applied Biosystems, Rn0149065, United States). All reactions were run in duplicate at universal thermocycler conditions for TaqMan® Gene Expression Assays (2 min at 50 ℃, 10 min at 95 ℃ and 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 min). Results were analyzed with ABI Prism software[9].

YKL40 expression in cell subpopulations

Male Wistar rats were anesthetized, and livers were perfused *via* the portal vein with calcium-free Gey's solution (Sigma, 69779, United States) supplemented with 100 U/mL of heparin. A second perfusion was done with calcium-free Gey's solution containing 100 U/mL of heparin, 0.06% collagenase (Fluka, 27678, United States) and 0.01% DNase (Roche, 13035000, Sweden). Liver tissues were extracted, homogenized and placed in a bottle containing Gey's solution, 0.005% collagenase and 0.001% DNase, and stirred gently for 30 min at 37 ℃. The resultant suspension was filtered in 106 Nylon mesh, and 4 mL of MEM (Invitrogen® BRL, 11095, United States) supplemented with 10% fetal bovine serum (Invitrogen® BRL, 1082-139, United States) were added. The homogenates were centrifuged at 50 G for 2 min at 4 ℃ to precipitate hepatocytes. The supernatant was recovered and centrifuged at 400 G for 7 min at 4 ℃. The pellet was resuspended with Gey's solution plus 25% albumin (Sigma, A7906, United States) and centrifuged at 350 G for 10 min at 4 ℃ to obtain non-parenchymal cells (NPCs). NPCs were washed, resuspended with Gey's solution, placed on a lymphoprep (Axis-Shield, LYS3773, Oslo, Denmark) gradient and centrifuged at 1800 *g* for 25 min. The white ring of mononuclear cells was recovered. Cells were washed and then divided into aliquots of 1×10^6 per tube for sorting. Next, cells were incubated with a CD14 primary antibody (Santa Cruz Biotechnology, M305, United States, 1:25) for 30 min in the dark at 4 ℃. Cells were then washed and incubated with a FITC secondary antibody (Jackson ImmunoResearch, 71813, United Kingdom, 1:2000) for 30 min at 4 ℃ in the dark. Marked cells were washed and sorted (BD FACSAria) by CD14 protein surface marker expression.

Western blotting

Protein extraction from whole liver tissue and cellular populations was performed with the NE-PER (Pierce, 78833, United States) extraction reagent according to the manufacturer's instructions. Protein concentrations were quantified with the BioRad protein assay (BioRad, 500-13, 14, 15; United States). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done at 12%, and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane at 30 V overnight at 8 ℃. The PVDF membrane was washed twice in tris buffered saline (TBS) and blocked with 5% of non-fat milk used for 1 h at room temperature with constant stirring. The membrane was then incubated for 1 h at room temperature with primary antibodies against YKL-40 (Santa Cruz Biotechnology, sc-31722, United States, 1:200) or [-Actin actin (Santa Cruz Biotechnology, sc-47778, United States, 1:500)]. Membranes were then washed twice with tris buffered saline Tween-20 (TBST) and incubated for 30 min with an HRP-conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology, sc-2020, United States, 1:4000) to detect YKL-40 or goat anti-mouse for [-actin (Roche, 11520709001, Sweden, 1:500)]. Membranes were then washed twice in TBST and TBS. The signal was detected with the BM chemiluminescence Western Blotting kit (Roche, 1520709, Sweden) according to the manufacturer's instructions. Relative expression units (REU) were calculated from densitometric values of YKL-40 and β-actin (Kodak MI SE 4.5 software, Kodak, United States).

In vitro overexpression of YKL-40

Alveolar macrophage cells (ATCC, NR8383) were cultured in RPMI 1640 medium (Invitrogen® BRL, 21870084, United States) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic (Invitrogen® BRL, 15240062, United States). Upon reaching 70% confluence, cells were stimulated with LPS (Sigma, L4005, United States, 100 ng/L) for 3 h or 12 h; the medium was then discarded, and the cells were lysed in TrizolTRIzol reagent for mRNA extraction and evaluation of YKL-40 mRNA expression by real-time PCR.

Statistical analysis

The statistical analysis was performed with SPSS 10.0 software (SPSS Inc., United States), and significance was calculated by ANOVA. Data were expressed as mean ± SD. We considered $P \leqslant 0.05$ to be significant.

RESULTS

YKL-40 mRNA expression

Real-time PCR was performed on liver tissue samples after acute or chronic injury to evaluate changes in YKL-40 mRNA expression. In all experiments, healthy animals served as the control group. At 6 and 12 h after CCl_4 administration, YKL-40 expression was increased 2- and 10-fold, respectively, compared to controls ($P \leqslant 0.05$). Maximum expression (26-fold) relative to healthy tissue samples occurred at 24 h. At 72 h, YKL-40 mRNA expression levels declined to a level close to that of the 0-h and healthy groups (Figure 1).

In the chronic liver injury model, YKL-40 mRNA expression was significantly increased relative to controls at both 6 and 8 wk (8 and 10 REU, respectively, $P < 0.05$) (Figure 1).

YKL-40 protein expression

We next investigated the presence of YKL-40 protein in whole liver tissue from healthy, acute and chronic

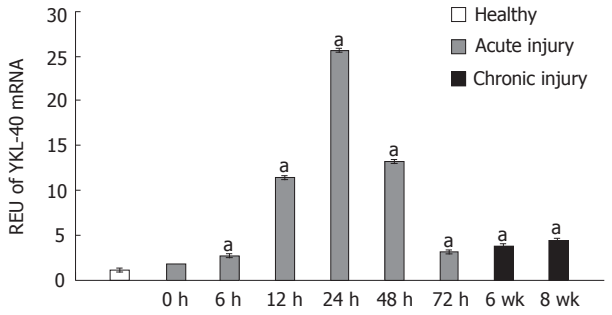

Figure 1 YKL-40 mRNA expression in whole liver tissue samples with acute and chronic injuries induced by CCl4. After acute injury, YKL-40 mRNA increased in a gradual and constant pattern and then fell to control levels. The maximum 26-fold increase was reached at 24 h in the acute damage model. For the chronic injury model, at 6 and 8 wk, YKL-40 mRNA levels were increased 7- and 9-fold compared to controls, respectively. $^{a}P \leq 0.05$. REU: Relative expression units.

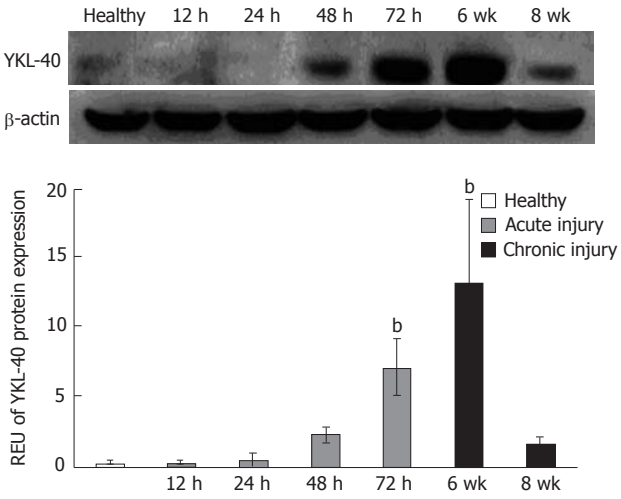

Figure 2 YKL-40 protein levels show a gradual increase. The maximum peak was at 72 h in the acute damage model and 6 wk in the chronic injury model, with 2.01 and 6.35-fold increases, respectively. $^{b}P \leq 0.05$ respect to the control group. REU: Relative expression units.

injury samples. In livers with acute injury, we observed that YKL-40 protein levels peaked at 72 h, with an increase of 2.0-fold compared to controls. At 12 and 24 h, YKL-40 levels were similar to those of healthy livers. In contrast, in the chronic injury model the maximum peak was observed at 6 wk with a significant decline apparent by 8 wk ($P < 0.05$) (Figure 2).

CD14^{+} cells are a source of YKL-40 protein

Based on their high levels of YKL-40 protein expression in whole liver samples, the 72-h group from the acute model and the 6-wk group from the chronic model were chosen for evaluation of protein expression in isolated cells. It has been reported that CD14^{+} cells population, which includes Kupffer macrophages, synthesize the largest amount of cytokines and growth factors. Additionally, they are found in abundance in the liver, accounting for about 20%-25% of NPCs[10] and 80%-90% of macrophages in the whole body. Therefore, we evaluated YKL-40 protein expression in this subpopulation.

Hepatocytes, CD14^{+} cells and CD14^{-} cells isolated from healthy animals did not show significant YKL-40 protein expression, with no statistical significance between groups (Figure 3A). However, when these cells types were isolated from CCl_4-treated animals, YKL-40 protein expression was elevated up to 4-fold compared with healthy animals. The highest level (0.09 REU) was detected in CD14^{+} cells in the acute damage model (Figure 3B). In the chronic injury model, both CD14^{-} and CD14^{+} cells were significant sources of YKL-40, with 0.15 and 0.11 REU, respectively ($P < 0.05$ compared to control cells) (Figure 3C).

Lipopolysaccharide induces YKL-40 mRNA expression in rat alveolar macrophages

Previous studies reported that CCl_4 induces damage to the intestinal tissue architecture[11]. CCl_4 promotes translocation of bacteria and associated compounds like Lipopolysaccharide (LPS) towards the liver by portal blood flow. Here, LPS is taken up by Kupffer cells, which then synthesize cytokines and growth factors. To demonstrate this effect, rat alveolar macrophage cells were stimulated with LPS for 3 or 12 h. Our results showed that YKL-40 mRNA levels were increased 9- and 11-fold, respectively, compared with control cells without LPS stimulation ($P \leq 0.05$). However, no significant difference was detected between the 3- and 12-h groups (Figure 4).

DISCUSSION

Several studies have found a correlation between serum YKL-40 levels and liver fibrosis stages[10,12,13]. However, other studies have reported contradictory findings[14-18]. Nevertheless, in the first set of studies, procollagen Ⅲ peptide and hyaluronic acid showed better correlations with fibrosis than YKL-40, likely because these molecules are scar components. YKL-40 is presumably a growth factor that indirectly contributes to fibrosis by stimulating proliferation of the cells that produce ECM proteins. For this reason, we considered it important to study the expression kinetics and source of YKL-40 in models of acute and chronic liver injury.

Although liver fibrosis and cirrhosis are characterized by inflammatory infiltration, a process in which a great number of cells participate, Johansen *et al*[3] showed that the liver was a possible source of YKL-40. This study noted that strong YKL-40 immunostaining could be detected around fibrotic areas in liver tissue samples where fibrosis had been induced by alcohol and viral hepatitis, but it was impossible to distinguish the cellular source[3].

We used the CCl_4 damage model because it resembles alcohol damage[14] and because the kinetics of damage is well characterized. Our results show that YKL-40 mRNA levels began to increase at 12 h, with a maximum peak at 24 h (Figure 1). In the CCl_4 fibrosis model, increased mRNA levels of growth factors such as TGF-β and PDGF at 48 and 72 h after intoxication suggest their participation in the fibrogenic process through their bio-

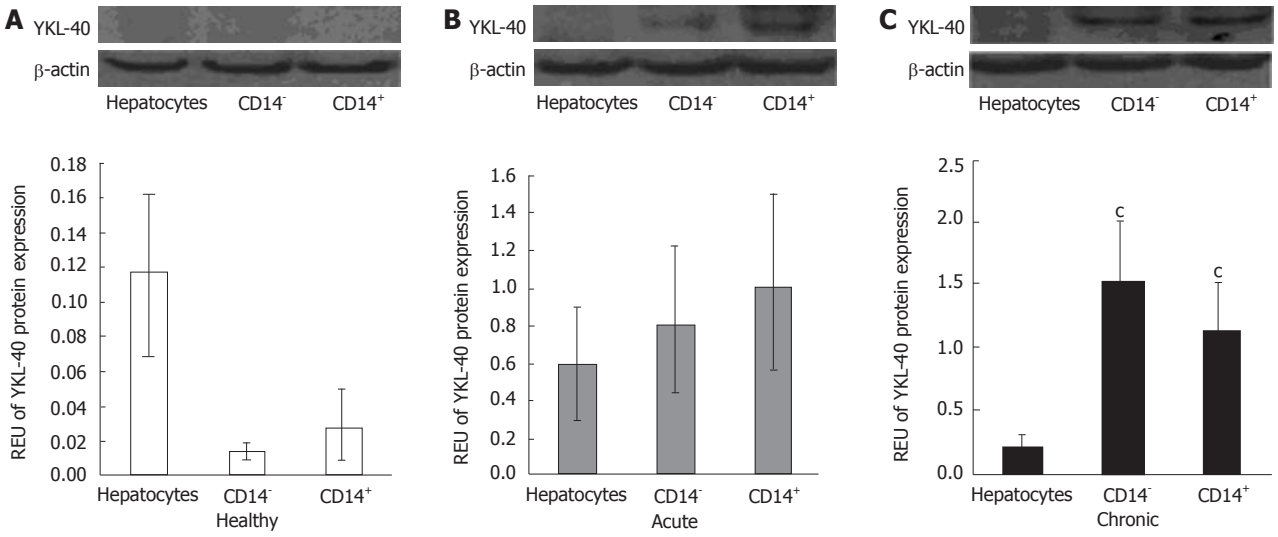

Figure 3 YKL-40 protein expression in cellular populations isolated from liver tissue. A: In healthy livers, expression of YKL-40 protein was lower in $CD14^+$ and $CD14^-$ subpopulations. B: In cells from livers exposed to acute damage, YKL-40 protein levels were higher in $CD14^+$ cells. C: In the chronic injury model, hepatocytes showed low levels of YKL-40, with significant difference between $CD14^+$ and $CD14^-$ cells against hepatocytes ($^cP < 0.05$). REU: Relative expression units.

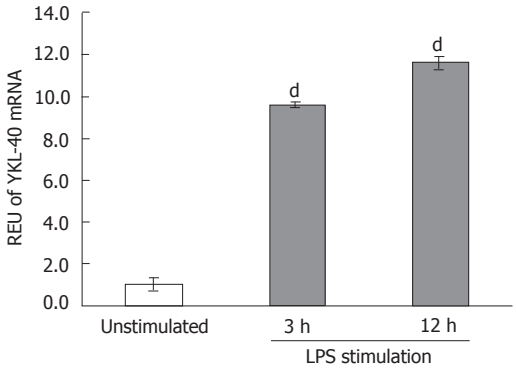

Figure 4 YKL-40 mRNA expression was induced by lipopolysaccharide in cell culture. After 3 and 12 h of stimulation with lipopolysaccharide, YKL-40 mRNA levels were increased by 9- and 11-fold, respectively, in a rat alveolar macrophage cell line ($^dP < 0.05$). Unstimulated cells were used as controls. REU: Relative expression units. LPS: Lipopolysaccharide.

logical activities of ECM synthesis and HSC-fibroblast proliferation respectively[15,19]. Like PDGFβ, YKL-40 might activate the proliferating cell signaling pathway PI3K-AKT[20].

Kupffer cells were the natural candidate source of YKL-40 because previous studies of joint, lung, kidney and skin inflammatory diseases[2,5] identified macrophages as the main producer of this protein. In our study, $CD14^+$ cells were a source of YKL-40, exhibiting 0.9 and 0.11 REU in the acute and chronic damage models, respectively. However, it is important to note that $CD14^-$ cells also produced YKL-40 protein, with 0.7 and 0.15 REU in the acute and chronic injury models, respectively. This population includes immune cells such as natural killer (NK) and natural killer T (NKT) cells, which represent about 37% and 26% of non-parenchymal cells respectively. These cells are the first line of defense in liver infections and consequently have immunoregulatory properties: they can synthesize cytokines (e.g., IFN-γ, TNF-α, IL-4, IL-10 and IL-13) (NK cells) or induce direct cellular destruction by TLR or CD1D molecules (NKT cells)[11].

LPS might also be a trigger of YKL-40 gene expression. Carbon tetrachloride and alcohol injure the upper gastrointestinal tract, forming lesions in gastric and duodenal mucosa. This promotes increased intestinal permeability to endotoxins, notably LPS, peptidoglycan, flagellin and zymosan, which are found in liver blood influx as well. These antigens are taken up by monocytes and Kupffer cells[12,13], which express TLR 1-8. LPS is known to be a ligand for TLR-4, a toll-like receptor widely expressed in monocytes and macrophages. This ligand-receptor interaction induces larger quantities of TNF-α, TGF-β and IL-10 cytokines[12,13]. Considering that the type of intestinal and liver damage induced by CCl_4 is similar to that of ethanol consumption, we stimulated alveolar macrophages *in vitro* with 100 ng/L and found increases in YKL-40 mRNA levels of about 9- and 12-fold at 3 and 12 h, respectively. These results indicate that LPS could act as a direct stimulus to induce YKL-40 in macrophages during the establishment of damage.

COMMENTS

Background

Hepatic diseases are a major cause of death in the world; some have, as a singular characteristic, the presence of fibrosis. Hepatic fibrosis is difficult to diagnose because a hepatic biopsy is needed, and the condition of patients frequently makes it impossible to perform a biopsy. For several years, researchers have been looking for fibrosis markers in the blood in order to avoid having to perform a hepatic biopsy. One of these is YKL-40, a protein produced by several tissues under conditions causing stress and damage, such as alcohol consumption. YKL-40 has been used as a fibrosis marker with controversial results, particularly since it is not known precisely which cells produce this protein.

Research frontiers

The focus of this article is to elucidate which cells in the liver are producing YKL-4 and to try to understand the role of YKL-40 in the process of repairing

fibrotic hepatic tissue and the maintenance of a healthy liver.

Innovations and breakthroughs

Until the present moment, there has not been a study that describes in which cells and at what time YKL-40 is produced. Several research protocols in humans have been made: in 2003 Nojgaard detected YKL-40 and successfully related this protein with hepatic fibrosis, however in recent years other research has shown contradictory results. Johansen in 2000 published that, in liver biopsies, cells were producing YKL-40; however, with the methodology used it was impossible to distinguish which cells types were actively producing YKL-40. This work was initiated because the authors thought that it was necessary to know more about YKL-40 production and to focus on the hepatic cellular source.

Applications

The hepatic cells that produce YKL-40 are $CD14^+$ and $CD14^-$ at different times. This helps the authors to better understand how serum YKL-40 could be increased at different stages of disease, and attempt to better understand YKL-40 as a blood fibrosis marker and how this molecule participates in the process of hepatic health and illness.

Terminology

YKL-40: Protein produced by cells that helps to augment cell growth and to stimulate proteins that form the extracellular environment. CD14: Molecules in the cell membrane useful to classify and distinguish cells.

Peer review

A research study about a model of hepatic injury in the rat that is relevant since nowadays liver cirrhosis/fibrosis is a health problem worldwide. Many treatment modalities have been suggested to prevent the development of fibrosis but, unfortunately, none of them have uniformly yielded promising results. Therefore, a better knowledge of the pathogenic mechanisms involved in the inflammatory response developed during acute and chronic liver diseases makes possible the use of more effective therapeutic approaches in these patients.

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BRIEF ARTICLE

Ghrelin attenuates gastrointestinal epithelial damage induced by doxorubicin

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Abstract

AIM: To examine the influence of ghrelin on the regenerative potential of gastrointestinal (GI) epithelium.

METHODS: Damage to GI epithelium was induced in mice by two intravenous injections of doxorubicin (10 and 6 mg/kg). Some of the doxorubicin-treated mice received a continuous subcutaneous infusion of ghrelin (1.25 μg/h) for 10 d *via* implanted mini-osmotic pumps. To label dividing stem cells in the S-phase of the cell cycle, all mice received a single intraperitoneal injection of 5′-bromo-2′-deoxyuridine (BrdU) one hour before sacrifice. The stomach along with the duodenum were then removed and processed for histological examination and immunohistochemistry using anti-BrdU antibody.

RESULTS: The results showed dramatic damage to the GI epithelium 3 d after administration of chemotherapy which began to recover by day 10. In ghrelin-treated mice, attenuation of GI mucosal damage was evident in the tissues examined post-chemotherapy. Immunohistochemical analysis showed an increase in the number of BrdU-labeled cells and an alteration in their distribution along the epithelial lining in response to damage by doxorubicin. In mice treated with both doxorubicin and ghrelin, the number of BrdU-labeled cells was reduced when compared with mice treated with doxorubicin alone.

CONCLUSION: The present study suggests that ghrelin enhances the regenerative potential of the GI epithelium in doxorubicin-treated mice, at least in part, by modulating cell proliferation.

Key words: Gastrointestinal cell proliferation; Gastrointestinal mucosal damage; Ghrelin

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INTRODUCTION

Ghrelin is a novel 28-amino acid peptide which was simultaneously discovered by two independent research

groups more than 10 years ago[1,2]. It was initially found to be a secretory product of a subset of enteroendocrine cells predominantly present in the stomach with a decreasing gradient toward the small intestine and the colon[1,2]. It was also found to be produced in small amounts by several other organs such as the brain, pancreas, pituitary, kidney, lung and placenta[3].

Functional studies revealed that ghrelin is involved in a wide variety of biological activities including growth hormone release[4], stimulation of food intake and body weight gain[5,6], gastrointestinal (GI) motility[7], and modulation of cardiovascular function[8]. Furthermore, it has been shown that ghrelin may also control proliferation and differentiation programs of neuronal and mesenchymal stem cells[9] and modulate cell proliferation of some tissue progenitors[10,11] and cell lines[12-14].

In the stomach, while ghrelin has been shown to modulate the secretory activity of parietal cells[15,16], it is not known whether this hormone is involved in the regulation of epithelial cell proliferation. It was shown earlier that parietal cells are the source of some instructive signals and their ablation in mice modulates the proliferation and differentiation program of gastric epithelial stem/progenitor cells[17] and with age cause gastric carcinoma[18]. Therefore, modulation of the secretory activity of these cells might also affect the proliferation/differentiation program of the epithelial progenitors. Surprisingly, when ghrelin knockout mice were examined, no abnormalities were reported in the GI mucosa[19]. Recently, there has been increasing evidence to suggest a role for ghrelin in protection against gastric mucosal damage[20,21]. The mechanism of mucosal protection was mainly attributed to the release of nitric oxide[22].

The aims of this study were to determine whether ghrelin can be used to protect against GI mucosal damage induced by doxorubicin, and to test whether ghrelin protection, if any, is associated with modulation of cell proliferation in the progenitor cell zone of the GI epithelium.

MATERIALS AND METHODS

Animals

In this study, female BALB/c mice (2-3 mo old) were evaluated after being housed in sterile microisolator cages with sterile bedding, food, and water ad libitum. The animals were kept under a 12-h light/dark cycle and at room temperature (22-24 ℃). The protocols described in this study were approved by the Animal Research Ethics Committee of the Faculty of Medicine, UAE University.

Chemotherapeutic treatment

To establish the experimental protocol of this study, we initially injected age-matched mice (n = 40) with a single dose of 5-fluorouracil (100 mg/kg body weight) or doxorubicin (10 mg/kg). Mice were then sacrificed at different time periods varying from 1 to 16 d. Gastroduodenal tissues were collected to identify any changes in mucosal integrity.

Experimental protocol

Mice (n = 9) were divided into three equal groups. Mice in the first group received ghrelin (Sigma, St. Louis, MO, United States) through Alzet micro-osmotic pumps (Durect Co, Cupertino, CA, United States) implanted subcutaneously which released ghrelin at a rate of 1.25 mg/h for 14 d. The pumps were prepared for implantation according to manufacturer's instructions and our previously published procedure[23]. On the 8th and 9th day of ghrelin perfusion, mice received two intravenous injections of doxorubicin (10 and 6 mg/kg, respectively). In the second group, mice were subcutaneously infused with saline instead of ghrelin and then received two intravenous injections of doxorubicin on two consecutive days as in the first group. The third group of mice served as controls and, instead of ghrelin and doxorubicin, received only saline by infusion pump and by intravenous injections, respectively. To label dividing cells in the S-phase of the cell cycle, mice in all 3 groups received a single intraperitoneal injection of 5' -bromo-2'-deoxyuridine (BrdU, 120 mg/kg) one hour before sacrifice. At day 4 post-doxorubicin (or saline) second injection, the stomach along with the duodenum were removed under ether anesthesia and processed for morphological and immunohistochemical analysis.

Morphological analysis

To examine the histopathological changes that occurred in the wall of the GI tract, the stomach and proximal part of the duodenum were dissected from all mice under anesthesia and immediately processed for conventional histological examination[23]. The tissues were fixed immediately in Bouin solution, dehydrated in ethanol, and infiltrated/embedded in paraffin. Five-micron-thick sections were mounted on slides and stained with periodic acid schiff and hematoxylin.

BrdU immunolabeling

To examine cell proliferation and estimate the number of cells in the S-phase of the cell cycle, paraffin tissue sections from all mice were processed to determine the localization of cells which incorporated BrdU[23]. Briefly, tissue sections were first deparaffinized, hydrated, and incubated with 3% hydrogen peroxide to block endogenous peroxidase. The anti-BrdU antibody used was goat polyclonal[24]. Biotinylated anti-goat immunoglobulin G was used as a secondary antibody which was identified by avidin-peroxidase and then di-aminobenzidine as a coloring agent. Immunoprobed tissue sections were scanned using the Olympus microscope to quantify BrdU-labeled cells. In each mouse, the numbers of BrdU-labeled cells per gastric gland or crypt-villus unit were averaged.

Statistical analysis

Data are presented as mean ± SD. Differences between groups were evaluated using the Student t test. $P < 0.05$ was taken as statistically significant.

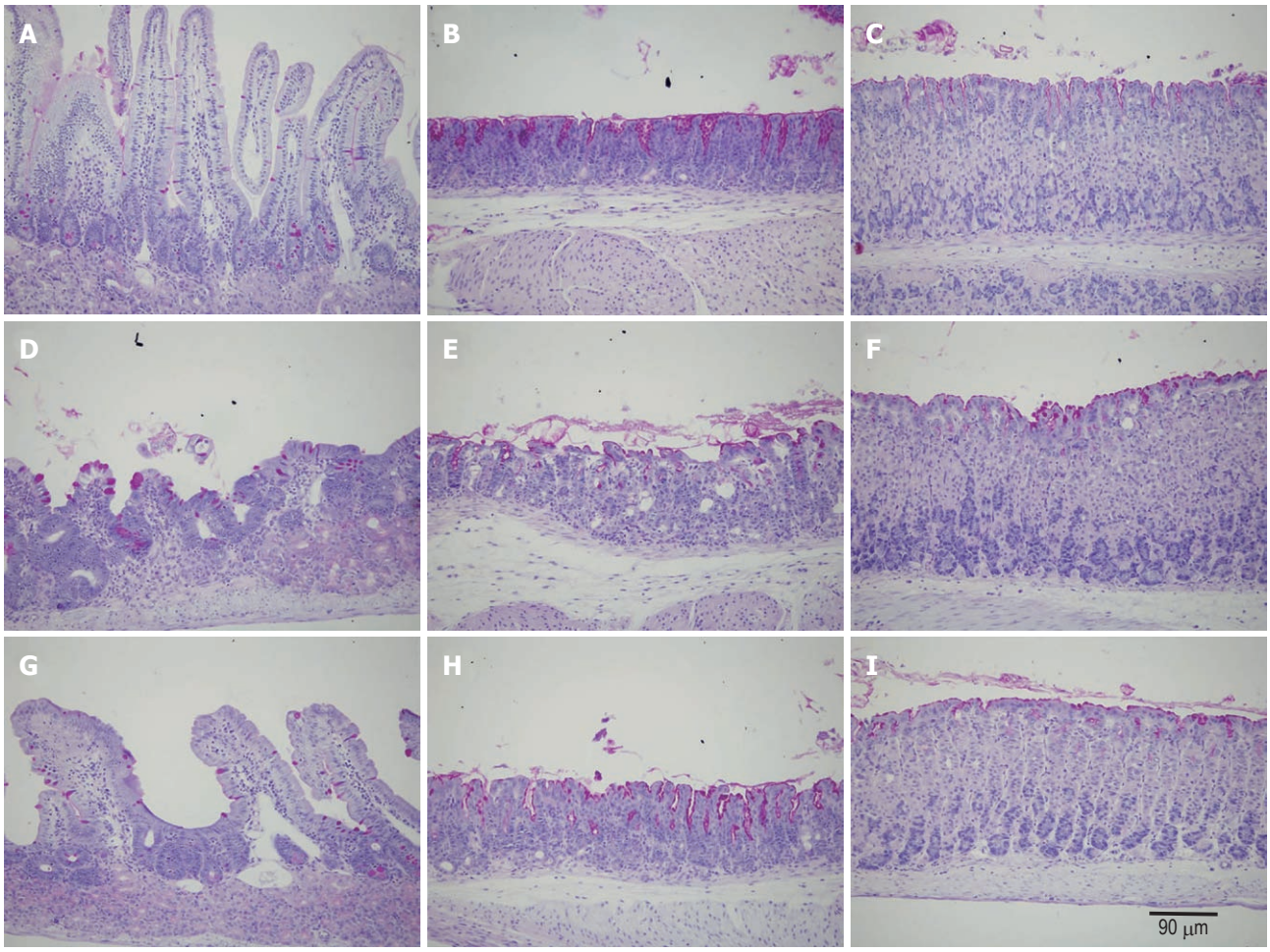

Figure 1 Light micrographs showing tissue sections obtained from the duodenum (A, D, G), pyloric antrum (B, E, H) and gastric corpus (C, F, I) of control (A-C), doxorubicin-treated (D-F) and ghrelin-plus-doxorubicin-treated (G-I) mice. All tissue sections were stained with periodic acid schiff and hematoxylin. Note that while there are apparent mucosal changes in D and E due to doxorubicin treatment, the tissues in G and H are more or less similar to control.

RESULTS

A single injection with chemotherapeutic agent induces mild effects in the GI mucosa

The damaging effect of the chemotherapeutic agents on GI mucosa was followed by microscopic examination of tissue sections. The dose regimen used for 5-fluorouracil showed inconsistent damaging effects in the mucosal tissues in the form of occasional vacuolation of the lining GI epithelial cells. Doxorubicin treatment induced a slightly more pronounced and consistent effect. Therefore, it was decided to use two intravenous injections of doxorubicin for the experimental protocol.

Protection of GI mucosa by ghrelin against the damaging effects of doxorubicin

Microscopic examination of gastroduodenal mucosa of control mice revealed the expected histological features of intact long oxyntic glands of the corpus region, short mucous glands of the pyloric antrum, and the very long crypt-villus units of the duodenum (Figure 1) as previously described[25]. In the second group of doxorubicin-treated mice, while little changes were observed in the oxyntic glands in the form of a few scattered cells with vacuolated cytoplasm, the antral glands showed more aggressive changes which induced dilatations of the glandular lumen. Massive mucosal changes in the duodenum were observed (Figure 1D-F). The villi appeared blunt or much shorter and broader than those of control mice. In addition, the integrity of the villus epithelium was not intact. Signs of vacuolation and cell damage were evident throughout. In the ghrelin-plus-doxorubicin-treated mice, the glands in the corpus and antral regions appeared similar to those of control mice. Even the duodenal villi appeared intact, long and populated mainly by absorptive and goblet cells as in control mice (Figure 1G-I).

Modulation of BrdU-labeling in doxorubicin- and ghrelin-treated mice

To correlate the morphological changes with cell proliferation, we used the BrdU labeling method. BrdU was made available to cells in the S-phase of the cell cycle one hour before sacrifice. Gastroduodenal tissue sections were then immunolabeled using anti-BrdU antibody (Figure 2). In control mice, BrdU-labeled cells in the corpus and antral mucosae were located at the pit-gland junction close to the luminal surface (corpus) or the gland bottom (antrum). Counts revealed the presence of

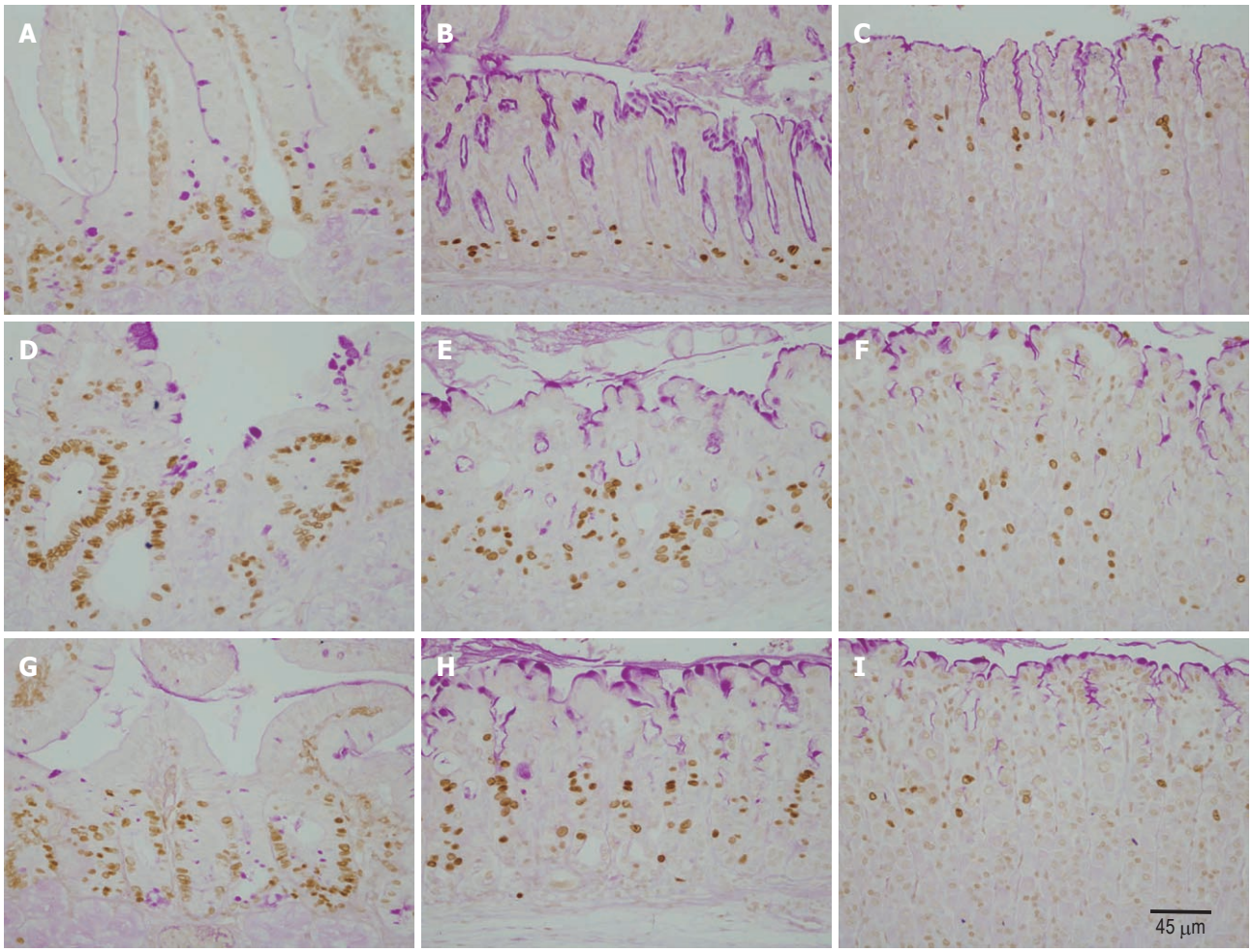

Figure 2 Immunohistochemical analysis of S-phase cells using anti-5'-bromo-2'-deoxyuridine antibody and tissue sections obtained from the duodenum (A, D, G), pyloric antrum (B, E, H) and gastric corpus (C, F, I) of control (A-C), doxorubicin-treated (D-F) and ghrelin/doxorubicin-treated (G-I) mice. Note that BrdU-labeled cells (brown nuclei) of doxorubicin-treated tissues in D, E and F appear more expanded when compared with control tissues in A, B and C. BrdU-labeled cells in G and and H are still more expanded than in the control, but less prominent than in D and E.

3.5 ± 0.18 and 5.5 ± 0.18 cells per gland in the corpus and antrum, respectively (Figure 3). In the duodenum, dividing BrdU-labeled cells were in the lower portion of the crypts and averaged 10.7 ± 0.85 (Figure 3).

In doxorubicin-treated mice, the labeling pattern of BrdU-immunoreactive cells was altered in the corpus, antrum and duodenum. There was a general increase in the number of BrdU-labeled cells in the gastroduodenal mucosa. In addition, the distribution of BrdU-labeled cells was expanded and tended to be more scattered rather than localized to the gastric isthmus (Figure 2E, F). Counts revealed an increase in the number of BrdU-labeled cells up to 31.2 ± 4.07, 12.1 ± 0.59, 4.3 ± 0.18 in the duodenum, antrum and corpus, respectively. Each of these values was significantly higher than its corresponding value in control mice ($P > 0.01$).

Ghrelin-treated mice which also received doxorubicin showed an usual pattern in the distribution of dividing cells, however, the number of BrdU-labeled cells in the antrum and duodenum remained at a higher level than the control (Figures 2G, H, 3). These findings were confirmed when all mice were examined and BrdU-labeled cells were quantified. When compared with control, the data showed a significant increase in the number of BrdU-labeled cells in the pyloric antrum and duodenum (9.6 ± 0.38 and 27.6 ± 2.75, respectively). However, when these values were compared with those obtained from the second group of mice (treated only with doxorubicin), there was a decrease in S-phase labeled cells, but this was only significant in the antrum ($P > 0.02$).

DISCUSSION

Despite the tremendous effort in modern drug discovery and development, dyspepsia and GI mucosal damage remain frequent complications affecting life quality in cancer patients receiving chemotherapy. The available data demonstrate that ghrelin could be a potential protective agent against these complications. In rats treated with cisplatin, it was demonstrated that ghrelin can be used to prevent delayed gastric emptying, early satiety, anorexia, nausea and vomiting, all characteristic of cancer-associated dyspepsia syndrome[26]. In the present study we induced GI mucosal damage using doxorubicin and

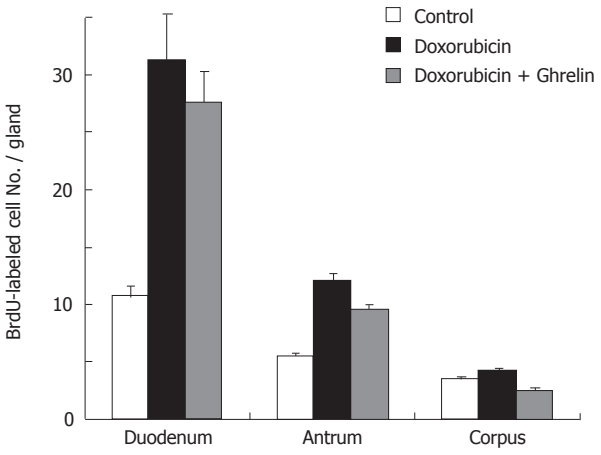

Figure 3 Analysis of 5'-bromo-2'-deoxyuridine-labeled cell counts in the duodenum, antrum and corpus of control, doxorubicin-treated and ghrelin/doxorubicin-treated mice.

demonstrated that ghrelin administration can also prevent the damaging effects of this chemotherapeutic agent.

Ghrelin appears to be a potent GI mucosal protective agent. Its effect on maintaining GI mucosal integrity was achieved using different experimental animal models. A recent series of studies showed that administration of ghrelin to rodents attenuated gastric mucosal lesions induced by ethanol[20], stress[21], ischemia-reperfusion[27,28] and even HCl administration[29]. The ghrelin effects on ethanol and stress models were mediated by release of nitric oxide as they were prevented by blocking nitric oxide synthase activity[22]. The effects of ghrelin on the HCl-model was mediated not only by growth hormone secretagogue receptor, but also histamine H3 receptor, suggesting the involvement of histamine release in ghrelin-induced protection[29].

It has been suggested that ghrelin is involved in several cell biological processes *via* different modes of actions: endocrine, paracrine and autocrine. We speculate that ghrelin exerted its GI mucosal protection *via* a paracrine effect. Examination of the GI mucosae of mice treated with two intravenous injections of doxorubicin revealed degenerative changes in their epithelial cell lining. However, mice which received ghrelin by continuous subcutaneous infusion showed minimal effects and appeared more or less similar to control mice when treated with the same dosage regimen of doxorubicin. The available data from the present study may suggest a mechanism which involves modulation of the cell cycle and induction of cell differentiation to substitute for the damaging effect of doxorubicin.

Several lines of evidence suggest that ghrelin modulates the proliferation of various cell types. It has been demonstrated that ghrelin stimulates proliferation of osteoprogenitor cells in bone tissue[30] and neuronal progenitor cells in the spinal cord[31]. Since cell proliferation and epithelial renewal are regarded as one of the protective mechanisms against GI mucosal damage, the question arises whether ghrelin also modulates proliferation of GI epithelial cells and hence could protect their integrity against noxious agents (such as chemotherapy). To answer this question we injected all mice used in our experiments with BrdU to label dividing cells during the S-phase of the cell cycle using immunohistochemistry. As expected, control mice showed BrdU-labeled cells in the isthmus regions of gastric glands and at the bottom of intestinal crypts. Doxorubicin-treated mice showed many more BrdU-labeled cells in both the gastric glands and intestinal crypts, probably to compensate for the damaged cells. However, in the presence of excess ghrelin, the damaging effects of doxorubicin were minimal and the number of BrdU-labeled cells was reduced, perhaps due to enhancement of cell differentiation. The other possibility is that ghrelin enhances cell differentiation and the increased proliferating cells observed in doxorubicin-treated mice were instructed by ghrelin to differentiate and migrate to restore the normal organization of the epithelium.

The protective effect of ghrelin against tissue damage is not restricted to the GI mucosa. Recent studies suggested that ghrelin promotes neuroprotective effects *via* stimulation of the regenerative potential of hippocampal neuroprogenitor cells to form new neurons. Incidentally, the expression of GHS-R was also demonstrated in the hippocampus[10,11].

In conclusion, this study demonstrates the protective effect of ghrelin against GI mucosal damage induced by doxorubicin and provides an addition justification for its potential use during chemotherapy in cancer patients to improve their quality of life.

COMMENTS

Background

Normal gastrointestinal (GI) mucosa is characterized by its regenerative potential following damage. However, cancer patients receiving chemotherapy develop severe GI complications due to mucosal damage. Ghrelin has been suggested to play a protective role against these mucosal damaging effects.

Research frontiers

It is important to define the factors involved in GI mucosal protection and regulation of stem cell proliferation and differentiation. Here the authors provide evidence in support of the role of ghrelin in GI mucosal protection.

Innovations and breakthroughs

This study provides evidence that ghrelin protects against GI mucosal damage caused by doxorubicin.

Applications

The findings of this article could help in designing new modalities for GI mucosal protection and regeneration in cancer patients undergoing chemotherapy.

Peer review

This is an interesting and elegant experimental paper examining the effect of ghrelin on the intestinal tract of doxorubicin-treated mice. The investigators showed that ghrelin protects against doxorubicin-induced epithelial damage. In addition, they showed it modulates epithelial proliferation.

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BRIEF ARTICLE

Management of acquired bronchobiliary fistula: A systematic literature review of 68 cases published in 30 years

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Abstract

AIM: To outline the appropriate diagnostic methods and therapeutic options for acquired bronchobiliary fistula (BBF).

METHODS: Literature searches were performed in Medline, EMBASE, PHMC and LWW (January 1980-August 2010) using the following keywords: biliobronchial fistula, bronchobiliary fistula, broncho-biliary fistula, biliary-bronchial fistula, tracheobiliary fistula, hepatobronchial fistula, bronchopleural fistula, and biliptysis. Further articles were identified through cross-referencing.

RESULTS: Sixty-eight cases were collected and reviewed. BBF secondary to tumors (32.3%, 22/68), including primary tumors (19.1%, 13/68) and hepatic metastases (13.2%, 9/68), shared the largest proportion of all cases. Biliptysis was found in all patients, and other symptoms were respiratory symptoms, such as irritating cough, fever (36/68) and jaundice (20/68). Half of the patients were treated by less-invasive methods such as endoscopic retrograde biliary drainage. Invasive approaches like surgery were used less frequently (41.7%, 28/67). The outcome was good at the end of the follow-up period in 28 cases (range, 2 wk to 72 mo), and the recovery rate was 87.7% (57/65).

CONCLUSION: The clinical diagnosis of BBF can be established by sputum analysis. Careful assessment of this condition is needed before therapeutic procedure. Invasive approaches should be considered only when non-invasive methods failed.

Key words: Bronchobiliary fistula; Digestive endoscopy; Endoscopic retrograde cholangio-pancreatography; Magnetic resonance cholangio; Percutaneous transhepatic cholangio; Iatrogenic damage; Congenital diaphragma defects; Hepatobiliary imino-diacetic acid scan

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INTRODUCTION

Bronchobiliary fistula (BBF) is a rare disorder, first reported by Peacock in 1850[1]. It consists of abnormal interconnection between the biliary tract and bronchial trees. Unlike congenital bronchobiliary fistula, acquired BBF is usually regarded as a consequence of local infection, such as hydatid or amebic disease of the liver, hepatic abscess, trauma, obstruction of biliary tract and neoplasm[1-58]. However, management of this condition can be very difficult and is often associated with a high rate of morbidity and mortality. Up to now, there has

been no widely accepted management strategy in this field. We searched the literature to outline the appropriate diagnostic methods and therapeutic options for acquired BBF.

MATERIALS AND METHODS

Data searching strategy

Literature searches were performed in Medline, EMBASE, PHMC and LWW (January 1980-August 2010), using the following keywords: biliobronchial fistula, bronchobiliary fistula, biliary-bronchial fistula, tracheobiliary fistula, hepatobronchial fistula, bronchopleural fistula and biliptysis. Two reviewers searched the literature independently in order to identify articles appropriate for inclusion in this review. Further articles were identified through cross-referencing. As a result, 68 cases were collected and reviewed.

Study criteria

Case reports and case series associated with acquired BBF and full texts of case reports in English were included. Cases did not provide basic information such as age, gender, primary diseases and etiological, clinical manifestation, therapeutic method, outcome and follow-up duration were excluded.

The clinical outcome was defined as cured when clinical symptoms such as biliptysis, fever and jaundice disappeared without use of drainage tube or with closure of the fistula. In addition, an adequate follow-up period (at least 2 mo) was necessary in patients without tumors. Treatment failure was defined as persistence of symptoms, and death due to bronchobiliary fistula or complications from the treatment.

RESULTS

General data

A summary of published case reports in recent 30 years concerning acquired BBF is shown in Table 1[1-58]. Sixty-eight cases were retrieved. Information regarding the demographics, clinical data and other variables were not reported in a few cases, thus the denominator varies in the following proportion of cases. Forty-two (61%) of the patients were men. The median age of onset was 48.3 years (range, 14-87 years) (Table 1). Fistula involving both lungs has not been reported so far. Only one case reported by Weis[2] showed that the BBF orifice was opened in the left lung (Figure 1).

Pre-existing conditions

According to the data, BBF secondary to tumors (32.3%, 22/68) including primary tumors (19.1%, 13/68) and hepatic metastases (13.2%, 9/68) shared the largest proportion. Bile duct obstruction (30.8%, 21/68), including biliary stenosis (17.6%, 12/68) and cholangiolithiasis (13.2%, 9/68) took the second position. Although liver tumor or chronic pancreatitis is associated with different degrees of biliary obstruction, it is considered as an independent etiology. Hepatic hydatidosis had been regarded as the most common primary disease in developing countries for a long time, but only 8 (11.7%, 8/68) cases have been reported in recent 30 years. Other causes were trauma (10.2%, 7/68) and chronic pancreatitis (2.9%, 2/68). One case of hepatic abscess, subphrenic abscess, syphilis gummosa and acute cholecystitis each in a suprahepatic gall bladder as other single primary diseases has been respectively reported. Four cases were complicated with multiple primary diseases in this review (Figure 2).

Symptoms

Biliptysis was presented in all the 68 patients (Figure 3). The volume of scant bile-staining of the sputum and the expectoration of copious volumes of bile ranged from 200 mL to 600 mL and reached a maximum of 1.2 L daily. Respiratory symptoms, such as irritating cough, fever and jaundice, were other clinical features of BBF. More than half of the patients (36/68) presented with fever. The highest body temperature exceeded 39.0 ℃. Abdominal pain was present in 14 patients and most in the right upper quadrant, but chest pain occurred in only eight patients. Respiratory disorders occurred in eight cases, including six cases of dyspneic disease. Symptoms like hepatic decompensation, portal hypertension, anorexia, anemia, nausea, vomiting and diabetes were sporadic. Pneumonia as the most common comorbidity was diagnosed in 10 patients, but bacteriologic results were only provided in two cases.

Therapeutic options

Less-invasive procedures had a tendency to be employed in treatment of BBF. Half of the patients underwent therapeutic endoscopy (49.2%, 33/67), including endoscopic retrograde biliary drainage (ERBD) in 24 cases, sphincterotomy in 16 cases, endoscopic nasobiliary drainage (ENBD) in 3 cases and endoscopic stone extraction in three cases. Percutaneous drainage was conducted in 5 cases. Percutaneous transhepatic cholangial drainage (PTCD) was used as a main treatment only in two patients.

Surgical procedures are invasive, and usually used as a final choice. In this review, 28 patients received open operations (41.7%, 28/67), including pulmonary lobectomy, resection and kposthesis of fistulous tract in diaphragm, hepalobectomy, hepaticoenterostomy and abscess drainage alone or in combination.

With the advance of modern medical techniques, histoacryl embolization under bronchoscopic guidance and n-Butyl cyanoacrylate *via* a bronchial approach brought new insights into this field. However, only one case each by the two techniques was reported by Kim JH and Goldman SY, respectively, and both patients died in the following four months because of hepatic failure or cancer[6,14].

Clinical outcome and follow-up

The outcome at the end of follow-up period (range from 2 wk to 72 mo) was good in most of the 28 cases,

Table 1 Basic data collected from case reports in the literature published over past 30 years

Sex and age	Fistulae orifice in side of lung	Primary diseases	Clinical manifestation				Therapeutic approaches	Outcome	Follow-up duration (mo)
			Cough and biliptysis	Fever	Jaundice	Other manifestation and/or comorbidity			
F 79	Left	Biliary stenosis	Yes	No	No	Pneumonia, diabetes, paroxysmal atrial fibrillation, coronary artery disease	Surgery	Cure	NA
M 19	Right	Hepatic hydatidosis	Yes	Yes	No	NA	ERBD	Cure	15
M 35	Right	Cholangiolithiasis	Yes	NA	NA	Abdominal distension and pain	ENBD	Cure	11
M 47	Right	Hepatic hydatidosis	Yes	Yes	NA	Abdominal pain	ERBD	Cure	7
F 18	Right	Trauma	Yes	NA	NA	Diabetes and asthma	ERBD	Cure	12
F 43	Right	Hepatocellular carcinoma	Yes	Yes	NA	Abdominal pain	Percutaneous drainage	Cure	NA
F 56	Right	Liver abscess and biliary stenosis	Yes	Yes	Yes	Pneumonia	Histoacryl embolization under bronchoscopic guidance	Cure but died 3 mo later	3
M 29	Right	Biliary stenosis	Yes	Yes	NA	Pneumonia	Surgery	Cure	NA
M 22	Right	Trauma	Yes	No	NA	NA	ERBD	Cure	5
M 57	Right	Cholangiolithiasis and chronic pancreatitis	Yes	Yes	Yes	Chest pain, pleuritic, alcoholic cirrhosis, diabetes and COPD	ERBD and bron choalveolar lavage	Cure	NA
M 55	Right	Biliary stenosis	Yes	NA	NA	NA	Surgery	Cure	7
M 41	Right	Hepatic hydatidosis	Yes	Yes	Yes	NA	ERBD	Cure	30
M 35	Right	Hepatic hydatidosis	Yes	Yes	Yes	Dyspnea	Surgery	Cure	12
M 20	Right	Trauma	Yes	NA	NA	NA	Surgery	Cure	NA
M 38	Right	Hepatic hydatidosis	Yes	Yes	Yes	NA	Surgery	Cure	NA
M 66	Right	Hepatocellular carcinoma	Yes	Yes	NA	Pneumonia	Surgery	Cure	NA
F 49	Right	Metastatic liver tumors	Yes	NA	NA	Pneumonia	N-Butyl cyanoacrylate *via* a bronchial approach	Cure	3
M 30	Right	Liver abscess	Yes	NA	NA	NA	Surgery	Cure	NA
F 64	Right	Biliary stenosis	Yes	Yes	Yes	Hepatomegaly	Percutaneous drainage	Failure	NA
M 65	Right	Metastatic liver tumors	Yes	No	No	Shortness of breath, pleuritic and chest pain	ERBD	Cure but died 3 mo later	3
M 12	Right	Undifferentiated sarcoma	Yes	NA	NA	NA	Surgery	Cure	NA
M 76	Right	Hepatocellular carcinoma	Yes	Yes	Yes	Pneumonia	Surgery	Failure	NA
F 68	Right	Biliary stenosis	Yes	No	No	Pneumonia	ERBD	Cure	24
M 71	Right	Cholangiocarcinoma	yes	Yes	NA	Pneumonia	ERBD	Cure	9
F 63	Right	Biliary stenosis	Yes	NA	NA	NA	Surgery	Cure	NA
M 52	Right	Metastatic liver tumors	Yes	NA	NA	Pneumonia and abdominal pain	Percutaneous drainage	Cure	3
F 55	Right	Hepatocellular carcinoma	Yes	Yes	NA	Dyspnea	ENBD	Cure	3
F 40	Right	Biliary stenosis	Yes	NA	NA	NA	ERBD	Cure	3
M 69	NA	Hepatocellular carcinoma	Yes	NA	Yes	Dyspnea and cirrhosis	ERBD	Cure but died 5 mo later	5
M 44	Right	Hepatocellular carcinoma	Yes	Yes	Yes	Abdominal pain	Surgery	Failure	NA
F 71	Right	Cholangiolithiasis	Yes	Yes	No	Chest pain	ERBD	Cure	NA
F 56	Right	Metastatic liver tumors	Yes	NA	NA	NA	ERBD	Failure	NA
M 65	Right	Metastatic liver tumors and cholangiolithiasis	Yes	NA	NA	Pneumonia	ERBD and Surgery	Cure	7
M 34	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	Surgery	Cure	NA
M 67	Right	Cholangiolithiasis	Yes	Yes	NA	Nausea, vomiting	Percutaneous drainage and ERBD	Cure	NA
M 38	Right	Trauma	Yes	Yes	No	Tachypnoea	ERBD	Cure	NA
M 20	Right	Trauma	Yes	Yes	Yes	Shortness of breath	Surgery	Cure	NA
F 70	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	ENBD	Cure	NA
M 40	Right	Biliary stenosis	Yes	NA	NA	NA	Histoacryl injection through the micro-catheter and ERBD	Cure	9

F 47	Right	Cholangiolithiasis	Yes	Yes	NA	Chest pain and abdominal pain	ERBD	Cure	10
M 61	Right	Carcinosarcoma	Yes	Yes	NA	Chest pain	Surgery	Cure	7
F 46	Right	Subphrenic abscess	Yes	Yes	NA	NA	Surgery	Cure	24
F 46	Right	Metastatic liver tumors	Yes	NA	Yes	NA	ERBD	Cure but died 9 mo later	9
M 56	Right	Metastatic liver tumors	Yes	Yes	Yes	Chest pain	ERBD	Cure	NA
M 61	Right	Biliary stenosis	Yes	NA	Yes	NA	ERBD	Cure but died 5 mo later	NA
F 64	Right	Biliary stenosis	Yes	Yes	Yes	Abdominal pain	ERBD	Cure	2
F 53	Right	Hepatic hydatidosis	Yes	Yes	Yes	Abdominal pain, ascites and respiratory distress	Endoscopic sphincterotomy alone	NA	NA
M 26	Right	Hepatic hydatidosis	Yes	Yes	Yes	Ascites and anemia	ERBD	Cure	NA
M 44	Right	Hepatic hydatidosis	Yes	NA	NA	NA	Surgery	Cure	NA
F 73	Right	Hepatocellular carcinoma	Yes	NA	NA	NA	ERBD	Cure but died 5 mo later	5
F 57	Right	Syphilis gummosa	Yes	Yes	NA	NA	Surgery	Failure	NA
M 54	Right	Chronic pancreatitis	Yes	Yes	NA	Chest pain and diabetes	Surgery	Cure	NA
F 58	Right	Uterine leiomyosarcoma with hepatic metastases	Yes	Yes	Yes	Dyspnea	PTCD	Cure	11
M 61	Right	Cholangiolithiasis	Yes	Yes	Yes	Abdominal pain, dyspnea	Endoscopic stone extraction and sphincterotomy	Cure	NA
F 71	NA	Metastatic liver tumors	Yes	NA	No	Abdominal pain	Surgery	Failure	NA
F 56	NA	Cholangiolithiasis	Yes	NA	NA	NA	Endoscopic sphincterotomy and stone extraction	Cure	NA
F 38	Right	Mucinous adenocarcinoma	Yes	NA	NA	NA	PTCD	Cure	NA
F 87	Right	Cholangiolithiasis	Yes	Yes	NA	NA	Endoscopic stone extraction and sphincterotomy	Cure	NA
M 18	Right	Biliary stenosis	Yes	NA	NA	NA	PTCD and balloon dilation cholangio plasty	Cure	NA
M 47	Right	Hepatic abscess and chronic pancreatitis	Yes	Yes	NA	Abdominal pain	Surgery	Cure	NA
M 63	Right	Acute cholecystitis in a suprahepatic gallbladder	Yes	NA	Yes	Abdominal pain, portosystemic encephalopathy and ascites	Surgery	Failure	NA
M 58	Right	Metastatic liver tumors	Yes	Yes	Yes	Hemoptysis	Surgery	Failure	NA
M 44	Right	Chronic pancreatitis	Yes	NA	NA	NA	Surgery	Cure	NA
M 15	Right	Trauma	Yes	Yes	NA	NA	Surgery	Cure	NA
M 14	Right	Cholangiolithiasis	Yes	Yes	Yes	Chest pain	Surgery	Cure	72
M 45	Right	Cholangiolithiasis	Yes	Yes	NA	Abdominal pain	Surgery	NA	72
F 26	NA	Biliary stenosis	Yes	NA	NA	NA	NA	NA	NA
M 21	Right	Trauma	Yes	NA	NA	Anorexia	Percutaneous drainage	Cure	4

M: Male; F: Female; ERBD: Endoscopic retrograde biliary drainage; ENBD: Endoscopic nasobiliary drainage; PTCD: Percutaneous transhepatic cholangial drainage; NA: Not applicable/available. COPD: Chronic obstructive pulmonary diseases.

and the recovery rate was 87.7% (57/65). Therapeutic endoscopy was safer than surgery, as 96.8% (30/31) *vs* 76.9% (20/26). Intraoperative complications as the cause of death occurred in one patient and recurrent BBF was found in four cases. Among the recurrent cases, one was cured by open surgery, one by percutaneous drainage and the other two cases by ERBD.

DISCUSSION

Local infection has been considered as a classic cause of BBF since Peacock's report in 1850[1,59]. According to this review, tumor is the major cause of BBF. It should be emphasized that the disease spectrum is changing. It may be correlated with the development of radical surgery and its complications. Primary tumors were all liver cancers in BBF patients as noticed in this review. Among the seven cases with information of tumor location, only two cases had the tumor near diaphragm surface and one case had diaphragm invasion. Metastatic tumors derived from gastrointestinal tract were reported in nine cases of BBF. The pathogenesis of BBF is various. It may involve the iatrogenic damage, diaphragm invasion, intrahepatic or extrahepatic biliary obstruction and tumor cachexia. Although biliary obstruction is the most common pathogeny in literature[57], its nature is not easy to

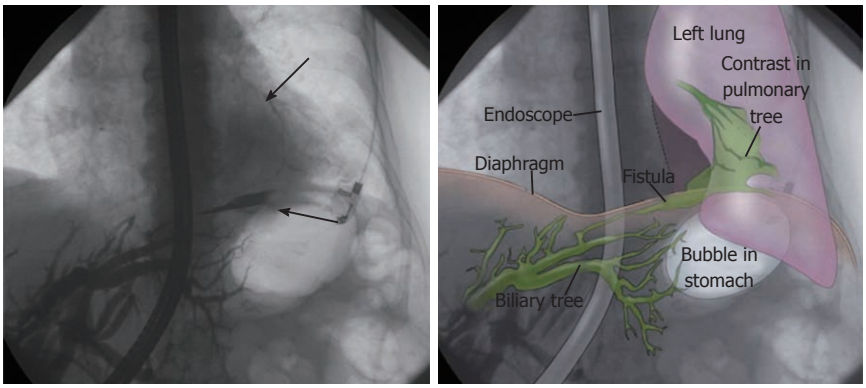

Figure 1 Picture reprint from the article of Weis[2]. The arrows show the bronchobiliary fistula involving the left lung.

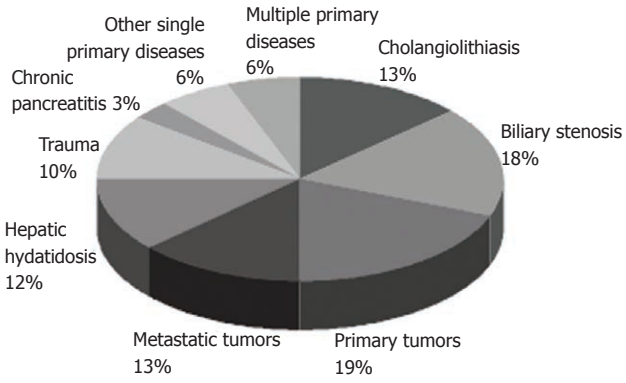

Figure 2 Primary diseases of bronchobiliary fistula. Multiple primary diseases include liver abscess and biliary stenosis, cholangiolithiasis and chronic pancreatitis, metastatic liver tumors and cholangiolithiasis, hepatic abscess and chronic pancreatitis.

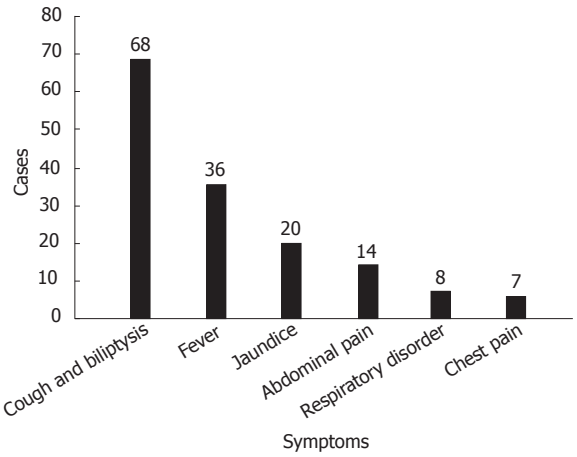

Figure 3 Clinical manifestations of bronchobiliary fistula.

define, and in most cases, it resulted from the presence of severe inflammation close to the primary lesion or in the hilus of the liver. The obstruction of the bile duct is often due to lithiasis, tumor, hydatid cyst, or postoperative stricture.

It should be emphasized that the recent surgery or invasive therapies before biliptysis appearance was observed in 51 cases (75%). And in seven cases, the operation induced the stenosis of bile duct. It is considered that the stress, especially the iatrogenic damage is correlated with bronchobiliary fistula. We also found a congenital case[60]. Therefore, we suspect that congenital fragile structure in diaphragma may be the basic pathogenesis in some cases.

Clinical diagnosis is usually made by bileptysis. In some cases, it is inappropriately diagnosed as acute pneumonitis or chronic irritable cough producing greenish sputum[59]. Patients having a long history of biliary tract disease can be diagnosed quite easily. However, some patients exhibited minimal signs of this disease. Sutherland *et al*[61] described false bile ptyalism in patients with sickle cell diseases and hemolytic crisis. In the patients without these conditions, the presence of bile in the sputum is defined as pathognomonic bronchobiliary fistula. Conventionally, endoscopic retrograde cholangio-pancreatography (ERCP) or percutaneous transhepatic cholangio (PTC) provided direct photographic evidence. This has been the most preferred choice for BBF. But, contrast-enhanced magnetic resonance cholangio (MRC) and hepatobiliary imino-diacetic acid (HIDA) scan not only ensures a definite diagnosis, but also has distinct advantage over other conventional non-invasional techniques[62,63].

In terms of medical treatment, somatostatin and its analogues were often used for treating BBF, because it reduced its secretion in the gastrointestinal tract. But, up till now, not a single case has been completely cured only with medical treatment[28]. Many patients complained that posture had an adverse impact on the volume of biloptysis. But only few doctors considered this factor while treating these patients. Therefore, we suggest that doctors should instruct BBF patients, especially those without biliary obstruction, to refrain from bile postural drainage. In other words, patients should be advised to take orthostatic position and avoid supine position. To alleviate the symptoms and accelerate concrescence of the fistula, bile reflux should be decreased. In addition, electrolyte disturbances and digestive system disturbances occurred when a significant amount of bile is lost. Therefore, supporting therapy should be administered with appropriate prophylactic attention.

Recently, when resolution of a distal biliary obstruction was accomplished, non-surgical interventions *via* ERCP or PTC were successfully conducted. ERBD became much more prevalent. It was considered in more than 60% (24/39) of cases for non-surgical treatment in this review. Based on this review, its single application recovery rate is also much higher than traditional surgery (95.8%:76.9%). Although PTCD and ENBD may lead to severe electrolyte disturbances and disturbances of the digestive system triggered by bile loss, it is convenient to recheck the healing of fistula using the radiographic technique with contrast media injection *via* drainage tube. This is practically more advantageous for managing the disease. In the past few years, histoacryl embolization under bronchoscopic guidance or the n-Butyl Cyanoacrylate *via* a bronchial approach were reported as new therapeutic methods[6,14]. However, these methods should be proved by more clinical cases.

The open surgery should be the first choice when interventional techniques have failed or BBF secondary to tumors, biliary obstruction and trauma occurred. The type of operation depends on the primary tumor type, BBF location and involvement. The following surgical procedures were performed in BBF: drainage of right subphrenic or hepatic abscess, closure of fistula, resection of hydatid cystis or tumor, biliary drainage using T-tube, and bilioenteric anastomoses (e.g., Roux-en-Y hepaticojejunostomy). In case of diaphragmatic, pleural, bronchial, or pulmonary damages, closure of the diaphragm, pleural drainage, decortication or different pulmonary resections have been used. Gugenheim *et al*[59] recommended a two-stage approach for treating BBF: (1) external biliary drainage by percutaneous or surgical drainage of subphrenic abscess and/or direct percutaneous drainage of the intrahepatic biliary tract; and (2) treatment of the underlying cause. In the patients with biliary obstruction, the priority management was to treat the biliary disease.

Early diagnosis and treatment can alleviate the patient' s sufferings. But there has been no evidence as to whether they can improve the prognosis of these patients. We have not obtained any information about the prevention of this disease.

In conclusion, we reviewed limited information of BBF published in the past 30 years. We could not completely understand its etiology and pathogenesis. The published experience suggests that ERCP or PTCD should be a priority. But new diagnostic techniques such as contrast-enhanced MRC and HIDA scan have greater advantages. While considering the medical treatment, quite a few doctors have advocated non-operative therapy as the preferred choice. Individualized and multidisciplinary treatment should be emphasized in patients with primary diseases as the condition of each individual patient is quite different and complex.

COMMENTS

Background

Bronchobiliary fistula (BBF) is an uncommon disorder involving biliary channels and the bronchial tree. Acquired BBF without proper management can induce death. With the improvement of non-invasive approaches, more satisfying outcome can be expected.

Research frontiers

Increasing non-invasive and less-invasive approaches to the management of acquired BBF can offer promising benefits to the patients with this condition. In this literature review, the authors point out that the definite procedure such as surgery should be considered only when the non-invasive methods failed.

Innovations and breakthroughs

Invasive procedure such as surgical excision of the fistula has been used widely, while less-invasive methods like external and internal stenting which can reduce biliary obstruction are of more value, since they are much safer and easier. The authors of this review searched the literature, provided more diagnostic and therapeutic options in management of acquired BBF.

Applications

BBF is rare and easily misdiagnosed as respiratory disease. Patients with this disease usually had poor outcome. However, there has been no guideline in BBF treatment up to date. This study reviewed the management strategy of BBF and summarized the experience from literatures in the past 30 years. It may contribute to the clinical treatment of BBF.

Terminology

Hepatobiliary imino-diacetic acid scan is a nuclear imaging procedure to evaluate the status of the gallbladder. N-Butyl cyanoacrylate is a tissue adhesive that applied as a monomer to moist tissue and polymerizes to form a bond. It is biodegradable slowly and used in all kinds of surgeries.

Peer review

This paper is a review of the world literature regarding BBF. Authors presented a spectrum of case reports concerning BBF. Idea of the summary about the knowledge of BBF is interesting.

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BRIEF ARTICLE

Enhanced CT and CT virtual endoscopy in diagnosis of heterotopic pancreas

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Abstract

AIM: To improve the diagnosis of heterotopic pancreas by the use of contrast-enhanced computed tomography (CT) imaging and CT virtual endoscopy (CTVE).

METHODS: A total of six patients with heterotopic pancreas, as confirmed by clinical pathology and immunohistochemistry in the Sixth Affiliated People's Hospital of Shanghai Jiao Tong University, Shanghai, China, were included. Non-enhanced CT and enhanced CT scanning were performed, and the resulting images were reviewed and analyzed using three-dimensional post-processing software, including CTVE.

RESULTS: Four males and two females were enrolled. Several heterotopic pancreas sites were involved; three occurred in the stomach, including the gastric antrum (n = 2) and lesser curvature (n = 1), and two were in the duodenal bulb. Only one case of heterotopic pancreas lesion occurred in the mesentery. Four cases had a solid yet soft tissue density that had a homogeneous pattern when viewed by enhanced CT. Additionally, their CT values were similar to that of the pancreas. The ducts of the heterotopic pancreas tissue, one of the characteristic CT features of heterotopic pancreas tissue, were detected in the CT images of two patients. CTVE images showed normal mucosa around the tissue, which is also an important indicator of a heterotopic pancreas. However, none of the CTVE images showed the typical signs of central dimpling or umbilication.

CONCLUSION: CT, enhanced CT and CTVE techniques provide useful information about the location, growth pattern, vascularity, and condition of the gastrointestinal wall around heterotopic pancreatic tissue.

Key words: Heterotopic pancreas; Computed tomography; Contrast enhancement; Computed tomography virtual endoscopy

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INTRODUCTION

Heterotopic pancreas is a condition in which pancreatic tissue is found outside the boundaries of the normal pan-

Table 1 Clinical and computed tomography scanning data for the six patients with heterotopic pancreas

Case	Gender	Age	Symptom	Location	Growth pattern and modality
1	M	60 yr	Carcinoid syndrome	Duodenal bulb	Exogenous, superficially lobulated, well-defined border, subserosal outer boundary was rough
2	F	59 yr	Epigastric pain (3 wk)	Gastric antrum	Circumscribed tissue, ill-defined border
3	M	68 yr	Epigastric pain (1 yr)	Gastric antrum	Circumscribed tissue, superficially lobulated, ill-defined border
4	F	3 mo	Identified during choledochal cystectomy	Mesentery	No obvious abnormality detected by computed tomography
5	M	60 yr	Abdominal distension (2 mo)	Lesser curvature aspect of gastric antrum	Superficially lobulated, well-defined border
6	M	38 yr	Cachexia	Duodenal bulb	Exogenous, well-defined border

creas; such tissue is regarded as aberrant pancreas or an accessory pancreatic lesion. This tissue has no anatomical, vascular or neuronal connection with the main pancreas. Such anomalies can become apparent at any age, but they are most commonly found in the fourth, fifth and sixth decades of life, with a slight male predominance. Heterotopic pancreas frequently occurs in association with the gastrointestinal tract (stomach, duodenum, jejunum, ileum) but is also found associated with Meckel's diverticulum, mesentery, omentum, spleen, and gallbladder[1]. In mice, the formation of a heterotopic pancreas may be caused by inactivation of the gene *IPF-1* (also known as *IDX-1*, *STF-1* or *PDX*), which leads to errors in embryological development that can result in the total absence of the pancreas[1]. Symptoms are dependent on the location of the ectopic tissue, although the most common symptoms are epigastric pain, pyloric obstruction, cholecystitis, and intussusception[1,2]. Some cases are identified as a result of such symptoms, but others are identified incidentally, such as during an unrelated surgery or during autopsy[1,3,4].

Cases of heterotopic pancreas have been reported in recent years following descriptions of the general characteristics of the condition observed by endoscopic ultrasonography (EUS). However, recent improvements in multi-slice computed tomography (CT) technology and the wide use of enhanced CT scanning have provided a new approach for identifying heterotopic pancreas. In this study, we present six cases of heterotopic pancreas and highlight the associated CT features[5].

MATERIALS AND METHODS

Patients

We retrospectively analyzed our database of all patients (about 350 000 patients) who underwent CT scanning at the Shanghai 6th People's Hospital Affiliated with Shanghai Jiao Tong University from January 2000 to December 2010. Six cases (four males, two females) were selected who were definitively diagnosed with heterotopic pancreatic tissue postoperatively by pathological examination and immunohistochemistry. With the exception of one patient aged 3 mo and another aged 38 years, the subjects were all aged between 59 and 68 years (Table 1). Three patients complained of epigastric pain or abdominal distension, and two others presented with cachexia or carcinoid syndrome. The other case was identified incidentally (Table 1). This study was reviewed and approved by the Shanghai 6th People's Hospital Affiliated with Shanghai Jiao Tong University.

CT scanning procedure

Multi-slice CT scanning was performed in all of the six cases using the LightSpeed VCT (GE) or Sensation CT (SIEMENS). No abnormality was detected in one of the female patients who therefore did not undergo contrast-enhanced CT scanning. The other five cases underwent enhanced CT scanning after standard CT scanning. With the exception of the 3-mo-old infant, the patients were asked to hold their breath during the scan in order to reduce artifacts. The thickness of the scanning slices was either 5 mm or 7 mm. The CT data of some patients was further refined using reconstruction software, which enabled thinner slice data to be obtained. The reconstruction software was all supplied by multi-slice CT; this was one function of the multi-slice CT. After the reconstruction the thinnest slice was 0.625 mm.

Each patient received the contrast agent iopromide (dose, 50-70 mL) through the median cubital vein. The arterial phase scan was produced approximately 30 s after the start of the injection. Coronal and sagittal images were obtained, and scanning data were analyzed on the GE workstation ADW 4.3. Lesion sizes were measured, and CT values were calculated (Table 1). Images were analyzed by application of three-dimensional post-processing software, including CT virtual endoscopy (CTVE).

RESULTS

Clinical findings

The six patients comprised four males (aged 38, 60, 60 and 69 years) and two females (aged 3 mo and 59 years). All five adult patients had experienced symptoms over a period of time ranging from weeks to years. Three of the patients presented with symptoms that were directly indicative of gastrointestinal disease, which made the lesions

Table 2 Detailed data from non-enhanced and enhanced computed tomography images of five of the six patients with heterotopic pancreas

		CT scanning		Enhanced CT scanning			
		Lesion	Pancreas	Lesion[1]	Pancreas[1]		
Case	Lesion dimensions (cm^3)	CT value (Hu)	CT value (Hu)	CT value (Hu)	CT value (Hu)	Properties	Enhancement pattern
1	1.8 × 1.2 × 2.1	38.2	44.3	93.2	97.4	Solid	Homogeneous, highly enhanced
2	1.2 × 1.0 × 2.1	28.2	44.7	80	106.9	Solid	Homogeneous, slightly enhanced
3	2.0 × 1.3 × 2.0	6.9	41.5	13.5	95.5	Solid	Heterogeneous, slightly enhanced
5	1.7 × 1.9 × 2.0	–47.8	47.6	–35.8	93.4	Cystic with density of fat	Heterogeneous, slightly enhanced
6	1.9 × 1.3 × 1.0	43.3	47.1	29.1	106.6	Solid	Homogeneous, highly enhanced

Hu is the unit value for computed tomography (CT). [1]The CT values of both the lesion and the pancreas were measured during the arterial phase.

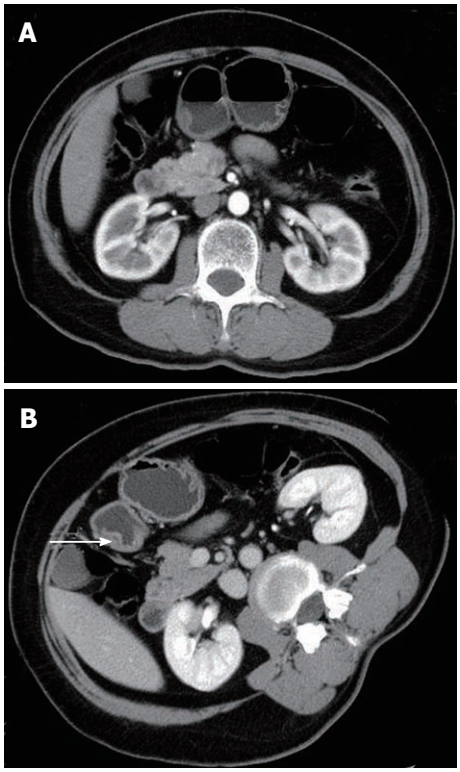

Figure 1 Heterotopic pancreas in the gastric antrum of a 59-year-old woman. A: Arterial phase image showing gastric antrum wall thickening. There is a circumscribed border of the lesion below the mucosal layer. The density at the center of the lesion is slightly lower than at the periphery. B: Each patient was asked to lie on their right side during venous phase scanning. The central area of the low-density region (white arrow) is obvious.

simpler to identify. However, two of the patients had non-specific symptoms, including carcinoid syndrome or cachexia associated with other systemic diseases. As these symptoms did not directly indicate a heterotopic pancreas, the lesions were more difficult to identify. They were discovered after physical examination and other tests. The 3-mo-old girl was jaundiced, and although CT scanning revealed the presence of a choledochal cyst, the heterotopic pancreatic tissue that indeed was later found to be present in the mesentery was not detected (Table 1).

CT techniques

The CT values of the heterotopic pancreatic tissues were calculated, and these ranged from -47.8 to 43.3 Hu (Table 2). The density measured in four cases was indicative of soft tissue, and this density correlated with the fatty tissue in only one case. The lesions were frequently superficially lobulated, with circumscribed borders, and they protruded into the gastrointestinal cavity or peritoneal cavity. After carrying out contrast-enhanced CT, the CT value of each lesion was based on the arterial phase scanning data. The three soft tissue lesions were homogeneously enhanced to varying extents. The other two lesions were heterogeneously enhanced (Table 2).

The scanning data were analyzed in depth using the GE workstation. The lesions could be seen from any angle in addition to the traditional axial, coronal, and sagittal views. Post-processing software was used to reveal further detail. In two cases, there was a strip of low density, which could be seen in both the axial and sagittal images; this may have represented the duct of the heterotopic pancreas (Figures 1 and 2).

CTVE imaging of each entire lesion revealed structural features of the mass, such as a spherical shape and superficial lobulation, amongst others. This also aided in identifying the layer of the gastrointestinal wall in which the lesion was located, and it showed that the mucosa around the lesion was normal (Figure 3).

DISCUSSION

The incidence of heterotopic pancreas is low, with only 40% of patients experiencing symptoms and 60% of cases being found incidentally during surgery for other disorders[6-9]. Although CT scanning is a highly sensitive technique, it can still be difficult to detect this abnormality. In this study, a heterotopic pancreas was detected in five of six patients by means of CT scanning. Moreover, contrast-enhanced CT scanning should always be per-

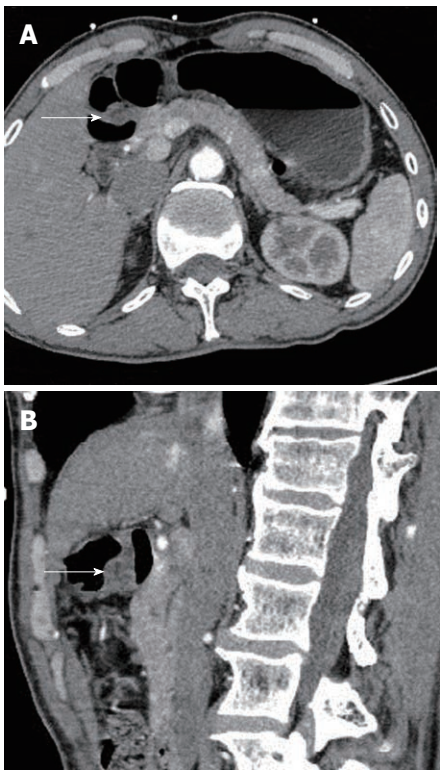

Figure 2 Heterotopic pancreas in the gastric antrum of a 68-year-old woman; the lesion is protruding into the stomach cavity. This heterotopic pancreas is of lower density than the pancreas, is heterogeneous, and slightly enhanced. In both the axial (A) and sagittal images (B), there is a low-density strip (white arrow) in the center of the lesion, which may represent the duct of the heterotopic pancreas. From the sagittal image (B), it can be seen that although the heterotopic pancreas tissue lies in the gastric antrum wall, the wall is still well defined. This suggests that the lesion does not violate the surrounding tissues and organs.

formed, when possible. Heterotopic pancreatic tissue was not observed in only one patient - the infant. For that patient, we postoperatively analyzed the CT images, but the lesion was still undetectable. As the intestines had expanded due to gas and liquid accumulation, as is commonly seen in infants, some of the intestines congregated at the mesenteric root.

In this study, 83.3% (five of six lesions) of the heterotopic pancreas lesions we observed occurred in the stomach, duodenum, and jejunum. In the stomach, 80%-90% of the lesions occurred in the antrum, within 5 to 6 cm of the pylorus[1,10,11]. Two of the three lesions that occurred in the stomach were located in the antrum (Figure 1). Heterotopic pancreas rarely occurs outside the gastrointestinal wall in tissues such as the liver, lung, omentum, mesentery, umbilicus, mediastinum, and fallopian tube[1,11-14].

Histologically, heterotopic pancreas is composed of ductal components, acinar cells, and islet cells. Because of differences in the proportions of the three components, however, the density of heterotopic pancreas tissue can vary[15]. In our study, lesions in four cases were composed of solid soft tissue, which was the commonest type. With the borders being defined to a variable extent, the density was similar to that of the main pancreas in 75% (3/4)

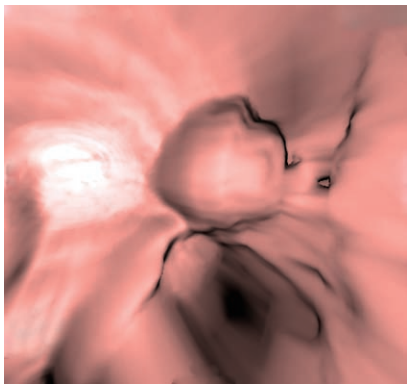

Figure 3 Heterotopic pancreas at the lesser curvature aspect of a 60-year-old man, the lesion is located below the mucosal layer. Computed tomography virtual endoscopy image showing that the lesion is round and superficially lobulated with a depression on one side.

of the cases of this type. Both the non-enhanced and contrast-enhanced CT images were analyzed, and the CT value of each lesion was measured (Table 2). These tissues were also homogeneously enhanced and were similar to the pancreas[16]. Only one case had a heterogeneous enhancement density that was typical of fatty tissue, and this was initially misdiagnosed as a lipoma. Therefore, lesions of the gastrointestinal tract having a density equivalent to fat should be additionally screened for a potential diagnosis of heterotopic pancreas.

Besides the density types discussed above, there is also a less common mixed-density cyst/solid type that was not observed in our study. This has also been considered as a complication of heterotopic pancreas[7,17]. Kim *et al*[14] reported that heterotopic pancreas with predominantly pancreatic acini shows a homogeneous enhancement pattern, whereas lesions with a mixed composition of acini and ducts show a heterogeneous enhancement. In our study, two cases showed a low-density strip of tissue with a well-defined border in the middle of the heterotopic pancreas lesion. In one case, the low-density strip was peripherally enhanced and so may have been the duct of the heterotopic pancreas[13].

Further thin-slice image data were obtained using the multi-slice CT, showing more details of each lesion, such as the interior density, enhancement pattern, location, and the duct. The thin-slice images also yielded high-quality two-dimensional and three-dimensional representations. With selection of the optimal viewing angle for the two-dimensional image, the low-density strip was shown to a greater extent; the presence of this strip is thus one of the characteristics of a heterotopic pancreas. Using EUS, Ryu *et al*[18] also identified the anechoic duct structure in some cases of heterotopic pancreas. Other work has shown that EUS may be more useful than CT in visualizing the pancreatic ducts[19]. In our study, however, the very small, narrow ducts were detectable only upon analysis of thin-slice two-dimensional contrast-enhanced images, and they were not detectable by EUS in the same patients. Therefore, we found that CT images were more sensitive

for detecting the heterotopic pancreas duct.

CTVE is a three-dimensional display technology used in the post-processing of CT scanning data to reconstruct three-dimensional cavity surface images of hollow organs, and it provides images that are similar in detail to those obtained by endoscopy (Figure 3). The mucosa around all lesions we observed was normal. The gastrointestinal mucosa seemed to be undamaged and enhanced[14]. This was an important sign in the diagnosis of heterotopic pancreas.

The features of the heterotopic pancreas have been confirmed by endoscopy[20]. The lesions we observed were superficially lobulated, but none of the cases in our study exhibited typical heterotopic pancreas features, such as central dimpling or umbilication[21]. Notably, Ryu *et al*[18] also did not observe such umbilication. Hazzan *et al*[10] suggested that, although central umbilication of the lesion is one of the characteristic features of a heterotopic pancreas, it is difficult to diagnose because umbilication is often absent in tumors of less than 1.5 cm in diameter. This may explain why umbilication was not observed in our study, i.e., because the lesions were small.

In conclusion, CT scanning, contrast-enhanced CT scanning and CTVE provide useful information about heterotopic pancreas tissue and reveal some of its characteristic features. This combined-technique approach represents a novel way of recognizing and diagnosing the disease. Although these techniques have some limitations, they have been shown to be beneficial for preoperative diagnosis of heterotopic pancreas and therefore may influence the choice of surgical procedure. Resection of heterotopic pancreas tissue is advisable in order to avoid later complications and a second operation[22,23,25].

COMMENTS

Background

Heterotopic pancreas has a low incidence, and most affected individuals are asymptomatic. Previous reports describe the identification of heterotopic pancreas through endoscopic ultrasonography, but few studies have focused on the potential benefits of computed tomography (CT) and contrast-enhanced images. With the improvement in multi-slice CT technology and the widely used post-processing software, this approach provides a new way to detect the features of heterotopic pancreas.

Research frontiers

In recent years, an increasing number of studies have focused on the application of contrast-enhanced CT scanning, post-processing software, and Computed tomography virtual endoscopy (CTVE) technology for the diagnosis of heterotopic pancreas.

Innovations and breakthroughs

CT scanning revealed the location and enhanced features of the lesion. CTVE showed the location, size, and shape of lesions as well as the organs with which they were associated; it also could visualize lumen stenosis and the surrounding mucosa. CTVE is also a reliable way to show the duct of heterotopic pancreas tissue. The advantages of this approach are that it is non-invasive and can reveal the extent of disease.

Applications

The findings of this study will be advantageous for the preoperative diagnosis of heterotopic pancreas. The approach also demonstrates a new use of CTVE.

Peer review

It is an interesting proposition, even though founded on retrospective analysis.

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CASE REPORT

A case of gas gangrene in an immunosuppressed Crohn's patient

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Abstract

Clostridium septicum (*C. septicum*) gas gangrene is well documented in the literature, typically in the setting of trauma or immunosuppression. In this paper, we report a unique case of spontaneous clostridial myonecrosis in a patient with Crohn's disease and sulfasalazine-induced neutropenia. The patient presented with left thigh pain, vomiting and diarrhea. Blood tests demonstrated a profound neutropenia, and magnetic resonance imaging of the thigh confirmed extensive myonecrosis. The patient underwent emergency hip disarticulation, followed by hemicolectomy. *C. septicum* was cultured from the blood. Following completion of antibiotic therapy, the patient developed myonecrosis of the right pectoral muscle necessitating further debridement, and remains on lifelong prophylactic antibiotic therapy.

Key words: Crohn's disease; Inflammatory bowel disease; Sulfasalazine; Neutropenia; *Clostridium septicum*

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INTRODUCTION

Clostridium septicum (*C. septicum*) gas gangrene is not uncommonly described in the literature, invariably in the immunosuppressed patient. Though historically described as a complication of war wounds, in the present time it is usually associated with postoperative infections[1]. Crohn's disease is an inflammatory bowel disease with variable luminal and extra-luminal manifestations. Because of the autoimmune nature of the disease, treatment is immunomodulatory in nature and common side effects include marrow suppression. We describe the development of *C. septicum* gas gangrene in a patient with Crohn's disease, who was neutropenic as a result of treatment with sulfasalazine.

CASE REPORT

A 26-year-old man presented to the emergency department with the complaint of a few hours of feeling unwell with vomiting, diarrhea and pain in the left anterolateral thigh. His background history included longstanding diarrhea secondary to Crohn's colitis diagnosed by colonoscopy 2 mo previously. The colitis was predominantly cecal, and the patient had been commenced on sulfasalazine treatment. Sulfasalazine had however been stopped 2 wk prior to presentation due to lack of efficacy, and prednisone was substituted.

On initial examination the patient looked unwell and was tachycardic (150 bpm), though afebrile and normotensive. Respiratory and abdominal examinations were unremarkable, and the left anterolateral thigh was tender

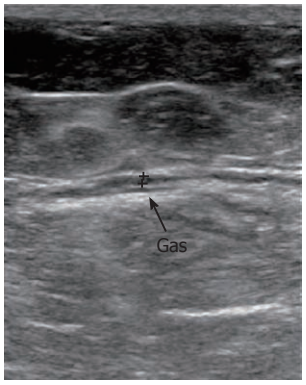

Figure 1 Ultrasound scan of left anterior thigh demonstrating gas. Princess Alexandra Radiology Department, 2007.

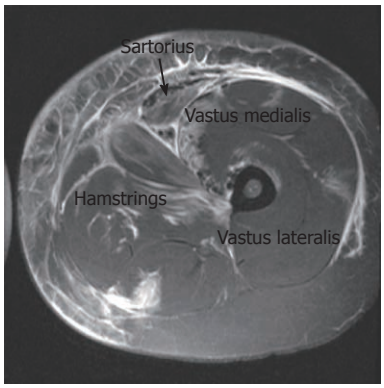

Figure 2 T2 fat suppressed magnetic resonance imaging of the left thigh, demonstrating changes of necrotizing myositis and gas formation. Princess Alexandra Hospital Radiology Department, 2007.

to palpation but otherwise normal in appearance. An initial ultrasound scan of the thigh also excluded any abnormality. Within 2 h the patient developed a fever to 38.5 ℃ and marked erythema of the left thigh with pustular formation. Initial laboratory investigations demonstrated a profound neutropenia (neutrophil count 0.2 × 10^9/L, white blood cell count 1.6 × 10^9/L) and myoglobinuria. An urgent repeat ultrasound scan of the anteromedial aspect of the left thigh demonstrated several small collections corresponding to pustule sites on the skin and visualized free gas near the obturator foramen (Figure 1). Magnetic resonance imaging confirmed extensive necrotic myositis and extensive gas formation in the left vastus medialis down to the extensor tendon and neurovascular bundle and in the rectus femoris (Figure 2). A presumptive diagnosis of infective myonecrosis was made. The patient underwent emergency left hip disarticulation and exploratory laparotomy. Two days later an abdominal computed tomography (CT) scan was performed for investigation of induration of the abdominal wall, and demonstrated cecal edema with free gas in the cecal wall. Right hemicolectomy was then performed for colitis and gangrene. *C. septicum* was cultured from blood samples taken at initial presentation. Antibiotic treatment of intravenous lincomycin, meropenem and penicillin was given.

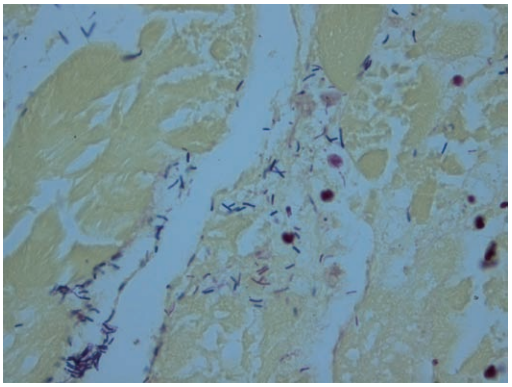

Figure 3 Necrotic chest wall muscle, with Clostridium septicum species. Pathology Queensland, Queensland Health-Princess Alexandra Hospital, 2007.

Routine hip stump washouts were performed and excluded progression of the necrotizing myositis. His recovery was complicated by a right pleural effusion requiring drainage. The patient was discharged from hospital on oral amoxicillin after a 3-wk stay. Two weeks after completion of the amoxycillin treatment the patient again presented with pain and erythema over the right pectoral muscle. A CT scan demonstrated an enlarged right pectoralis major muscle with inflammation of the overlying subcutaneous fat. The patient was taken to theatre where the necrotic pectoralis major muscle was debrided. *C. septicum* was cultured from the interoperative specimen (Figure 3). Antibiotic treatment during the perioperative period was intravenous benzylpenicillin, and the patient was also treated with hyperbaric oxygen therapy postoperatively. He had an uneventful recovery, and now remains on lifelong oral ampicillin prophylaxis.

DISCUSSION

C. septicum is a large, spore-forming, gram-positive anerobic bacillus[2-4] found in the gastrointestinal tract of approximately 2% of the healthy population[5]. *C. septicum* is typically found in the cecum and ileocecal area, where factors including poor vascular supply, local redox potential, pH and the osmotic and electrolyte environment provide an environment conducive to proliferation[5-7]. The rapid proliferation and systemic toxicity that *C. septicum* causes is thought to be attributed to the production of four exotoxins[4]. The alpha toxin causes intravascular hemolysis, necrosis of host tissue, and increases capillary permeability, thus producing tachycardia and hypotension[2]. A hallmark of clostridial myonecrosis is a paucity of inflammatory cells in the affected tissue[2,5,8]. The necrotic process spreads rapidly to adjacent healthy tissue, causing massive necrotizing gangrene within hours[2].

C. septicum gas gangrene can be traumatic or non-traumatic. Traumatic gas gangrene historically complicated war wounds, and is now usually associated with postoperative infections[1]. Non-traumatic clostridial infections nearly always occur in the immunosuppressed patient,

commonly in the setting of hematological or gastrointestinal cancer[5,7]. Diagnosis in a patient with spontaneous myonecrosis is often difficult as systemic symptoms are vague, and early localized findings can be mistaken for cellulitis. However, in a novel presentation, the triad of pain, tachycardia out of proportion to fever and crepitus is highly suggestive of clostridial myonecrosis[2,5].

We propose that in our patient, the ulcerated cecum allowed entry of *C. septicum* into the abdominal cavity, and this in concert with neutropenia secondary to sulfasalazine provided an environment predisposing to rapid proliferation of the bacilli.

Sulfasalazine is a medication commonly used in the treatment of inflammatory bowel disease, and a not uncommon complication is neutropenia. Mortality is high in *Clostridium* sepsis in the neutropenic milieu, and death usually occurs within 24 to 48 h[4,8]. For this reason, early diagnosis followed by immediate, aggressive surgical debridement is critical to survival[2,9]. It is therefore advisable that blood counts be monitored in patients who are commenced on this treatment, and that neutropenic patients receive emergency management should symptoms of sepsis arise.

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Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
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42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

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June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

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World Journal of Gastroenterology

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Acknowledgments

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Chinese journal article (list all authors and include the PMID where applicable)

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

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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