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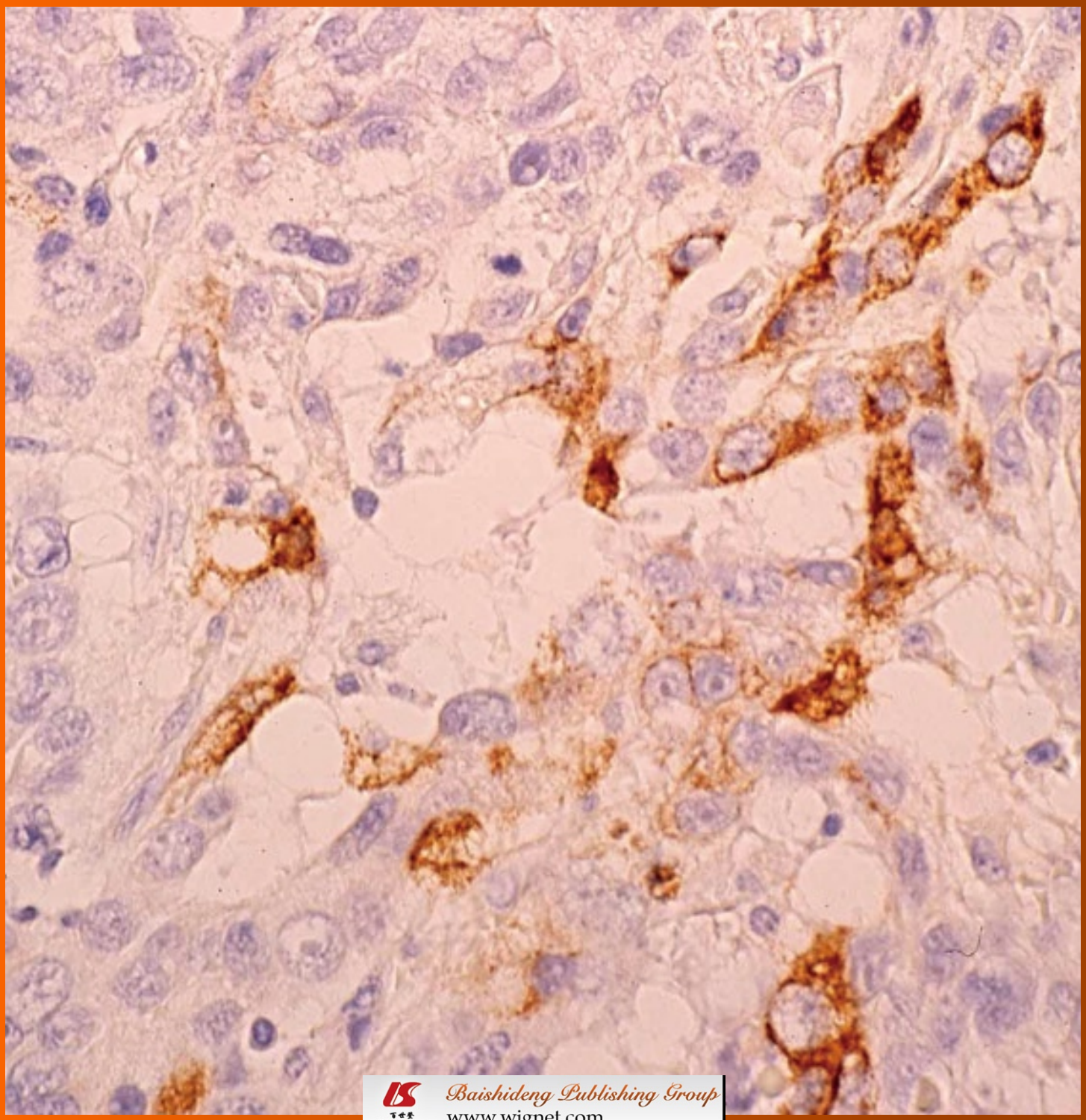

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EDITORIAL

Emerging roles of connexin hemichannels in gastrointestinal and liver pathophysiology

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Abstract

Connexin hemichannels have long been considered as mere structural precursors for gap junctions. In the last decade, it has become clear that they also act as individual channels, connecting the intracellular compartment and the extracellular environment. Impairement of connexin hemichannel functionality may result in disturbance of homeostasis, as exemplified in the current paper for the intestine and the liver. Research in this field still has a number of shortcomings, of which some are also discussed here.

Key words: Hemichannel; Connexin; Pathophysiology

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CONNEXIN CHANNELS: STRUCTURAL ASPECTS

The maintenance of tissue homeostasis is governed by the well-orchestrated interplay between three major communicative networks located at the extracellular, intracellular and intercellular level. Direct intercellular communication is mediated by gap junctions, which arise from the interaction of two hemichannels of neighbouring cells. Hemichannels, in turn, are composed of six connexin (Cx) units (Figure 1). The connexin family comprises as many as twenty isoforms in mammals. They are named according to their molecular weight and are expressed in a cell-specific way[1-5]. In the gastrointestinal tract, Cx40, Cx45 and particularly Cx43 are expressed by the intestinal smooth muscle cells and the interstitial cells of Cajal[6,7]. In the liver, hepatocytes abundantly produce Cx32 and small quantities of Cx26, while non-parenchymal hepatic cells mainly harbour Cx43[3,4].

CONNEXIN CHANNELS: FUNCTIONAL ASPECTS

Gap junctions provide a pathway for the intercellular exchange of small and hydrophilic substances, including nucleotides (e.g. ATP) and ions (e.g. Ca^{2+}). As numerous physiological processes are driven by these substances, gap junctional intercellular communication is considered as a key mechanism in the maintenance of tissue homeostasis[1-5]. Gastrointestinal gap junctions play a specific role in pacemaking and neurotransmission, and thus in

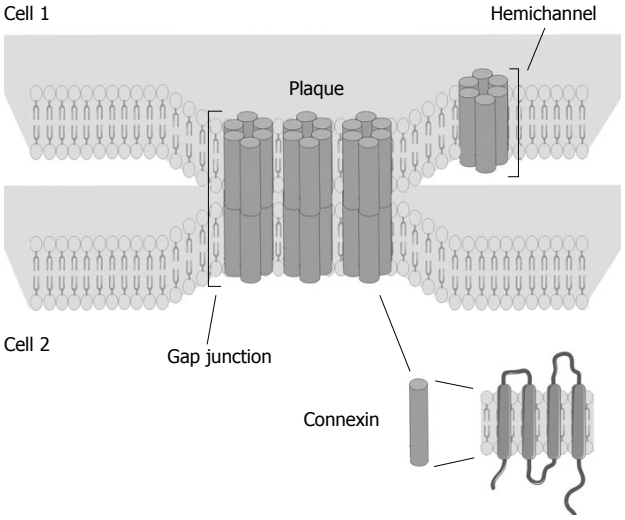

Figure 1 Molecular architecture of gap junctions. Gap junctions are grouped in plaques at the cell membrane surface and are composed of twelve connexin proteins organized as two hexameric hemichannels. The connexin structure consists of four membrane-spanning domains, two extracellular loops, one cytoplasmic loop, one cytoplasmic amino tail and one cytoplasmic carboxy tail.

the regulation of motility[7,8]. Hepatocellular gap junctions, on the other hand, essentially underlie most liver-specific functions, such as xenobiotic biotransformation and albu min secretion[3,4]. Gap junctions are also master regulators of cell growth and cell death[1,2]. In fact, a growing number of reports point to non-gap junctional functions for connexins in these events. Indeed, connexin proteins themselves can alter the production of critical homeostasis regulators, such as caspases and cyclins, irrespective of their channel properties. The mechanisms that govern these atypical connexin functions remain elusive, but may involve direct interaction with these regulatory molecules or the modulation of their gene transcription[1,2,9-11]. Another gap junction-independent cell signalling platform for connexins relates to hemichannels. For a long time, hemichannels have been considered as merely structural precursors for gap junctions, remaining closed until docking with counterparts from adjacent cells prior to gap junction formation. It has now become clear that connexin hemichannels also provide a pathway for communication, albeit between the intracellular compartment and the extracellular environment. The substances that travel thr ough hemichannels are very similar to those that are intercellularly exchanged via gap junctions[1,2,9-12]. To make the picture even more complicated, a second set of gap junction-related proteins has been described in recent years, the pannexins, which are structurally similar but phylogenetically unrelated to the connexin family. At present, three pannexins, namely Panx1, Panx2 and Panx3, have been identified in humans and rodents, and they preferentially occur in a hemichannel configuration[1,13,14].

CONNEXIN HEMICHANNELS IN THE INTESTINE AND THE LIVER

Thus far, most attention has been paid to connexin hemichannels in nervous tissue as well as their involvement in cerebral ischemia, in which their opening results in cell death[1,15,16]. Nevertheless, a limited number of studies have addressed hemichannels in the gastrointestinal tract and the liver. In an attempt to characterize gap junctions in a human intestinal epithelial cell line, Clair and colleagues found that functional hemichannels composed of Cx26, Cx32 and Cx43 are abundantly present at the basal side of these polarized cells[17]. Research from the same group showed that the Cx26 hemichannels facilitate the pathogenesis induced by *Shigella flexneri*, the causative agent of bacillary dysentery that invades the colonic mucosa where it elicits an intense inflammatory reaction responsible for destruction of the mucosa. Thus, *Shigella* invasion induces the opening of Cx26 hemichannels, allowing extracellular release of ATP, which in turn favours bacterial dissemination[18]. In a recent *in vivo* study conducted by Guttman *et al*, increasing levels of Cx43 were observed in mouse colon infected with the diarrhea-causing *Citrobacter rodentium*, whereby unpaired hemichannels were formed at both the apical and the lateral membrane surface of the colonocytes. Using animals genetically deficient in Cx43, it was subsequently demonstrated that Cx43 hemichannel opening triggers water release during infectious diarrhea[19]. Our group was the first to report the occurrence of Cx32 hemichannels in hepatocytes. Using an *in vitro* model of Fas-mediated apoptosis, a cell death mode involved in most liver pathologies, we found that *de novo* synthesized Cx32 gathers in hemichannels during apoptosis, and that hemichannel opening is critical for the termination of the cell death response[20].

CONCLUSION

Although a large body of evidence is nowadays available, the concept of functional connexin hemichannels still remains controversial[1,21]. A major point of debate concerns the identity of the molecular constituents of hemichannels. Critics claim that most, if not all, of the functions that have been attributed to connexin hemichannels, can actually be ascribed to other channel types, consisting for example of pannexins[21,22]. In addition, it seems challenging to discriminate between the functionality of connexin hemichannels and that of gap junctions, especially in an *in vivo* environment[1,21]. These constraints mainly arise from the ubiquitous lack of appropriate testing approaches to unequivocally study connexin hemichannels[21,23], including pannexin hemichannel- and connexin hemichannel-specific inhibitors. Undoubtedly, this research field will become more appealing when such experimental tools are avaliable. Moreover, it can be anticipated that the basic knowledge that will be gained by doing so, will also open new perspectives for the development of new clinical (gastrointestinal and liver) therapies.

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TOPIC HIGHLIGHT

Sharon DeMorrow, PhD, *Series Editor*

Immune-mediated bile duct injury: The case of primary biliary cirrhosis

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Abstract

Autoimmune cholangitis would be the appropriate name to define the immune-mediated bile duct injury following the breakdown of tolerance to mitochondrial proteins and the appearance of serum autoantibodies and autoreactive T cells. Nevertheless, the condition is universally named primary biliary cirrhosis (PBC). The disease etiology and pathogenesis remain largely unknown despite the proposed lines of evidence. One twin study and numerous epidemiology reports suggest that both a susceptible genetic background and environmental factors determine disease onset while a recent genome-wide association study proposed highly significant associations with several common genetic polymorphisms in subgroups of patients. Specific infectious agents and chemicals may contribute to the disease onset and perpetuation in a genetically susceptible host, possibly through molecular mimicry. Importantly, several murine models have been proposed and include strains in which PBC is genetically determined or induced by immunization with chemicals and bacteria. From a pathogenetic standpoint, new exciting data have demonstrated the unique apoptotic features of bile duct cells that allow the mitochondrial autoantigens to be taken up in their intact form within apoptotic blebs. We are convinced that the application of the most recent molecular techniques will soon provide developments in PBC etiology and pathogenesis with likely implications in diagnostics and therapeutics.

Key words: Autoimmune cholangitis; Anti-mitochondrial antibody; Epithelial cell apoptosis; Innate immunity

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INTRODUCTION

The intrahepatic biliary tree is the only target of the immune-mediated injury associated with primary biliary cirrhosis (PBC). In fact, PBC is an autoimmune cholangitis characterized at histology by the non suppurative inflammation and destruction of intrahepatic bile ducts while serology is characterized by the highly specific antimitochondrial autoantibodies (AMA). From a clinical standpoint, PBC is considered a peculiar, yet representative, autoimmune disease[1]. It affects women more frequently than men with a female to male ratio of 10 to 1, mostly at post-menopausal age with anecdotal

cases described in younger subjects[2]. The diagnosis of PBC is made when 2 out of 3 criteria (i.e. presence of serum AMA, increased serum enzymes indicating cholestasis (i.e. alkaline phosphatase) for longer than 6 mo and a compatible or diagnostic liver histology) are fulfilled[1,3]. Clinical symptoms commonly found in reference textbooks include fatigue, pruritus and jaundice; yet the changing disease scenario has now led jaundice to be a very rare sign at presentation[4] while the impact and specificity of fatigue remains a hot topic for debate[5]. The increasing availability of serological tests for routine AMA has significantly changed the spectrum of disease presentation[6]. The need for a liver biopsy at the time of PBC diagnosis remains debatable and it is currently indicated only in patients lacking one of the other criteria, patients requiring accurate staging (although the possibility of sampling errors should be accounted for) or in patients enrolled in clinical trials. Discriminating PBC from other autoimmune or inflammatory liver diseases is usually easy, mostly based on serum autoantibody profiles. Liver histology can be classified into four stages[7]. At earlier stages, bile duct obliteration and granulomas (possibly found at all stages) are strongly suggestive for PBC. Stage Ⅲ demonstrates septal or bridging fibrosis with ductopenia (over half of the visible interlobular bile ducts having vanished) while stage Ⅳ corresponds to frank cirrhosis virtually undistinguishable from end stage liver diseases of different etiologies.

The complete pathways of PBC immunopathogenesis remain unknown yet several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC with the disease onset recognizing two necessary components in a permissive genetic background and an environmental trigger[8].

ETIOLOGY

As in most complex diseases, it is now widely accepted that PBC results from an environmental stimulus intervening on a genetically susceptible background. We will first discuss the somehow overlooked role of female predominance in autoimmunity in general and in PBC. We will then review what is known of the genetic bases of PBC susceptibility and the environmental causes of its development.

Female preponderance

Among autoimmune diseases, PBC, Sjogren's syndrome, SLE, autoimmune thyroid disease and scleroderma manifest the highest female predominance with 80% of patients being women. Based on this long-established observation, three main research directions have been addressed and will be now discussed.

Sex hormones (i.e. estrogens, androgens and prolactin) have been the first proposed candidates in the sex bias observed in autoimmunity due to their modulatory functions within the immune response, particularly acting on the development of immune cells. Sex hormones may also directly influence the homing of lymphocytes to a target organ and the process of antigen presentation, thus influencing the organ specificity of AID as well as the breakdown of tolerance. The effect of estrogens is different in normal conditions and in autoimmunity with a biphasic effect; lower levels facilitate the immune response while higher levels suppress it. These data indicate that estrogen is capable of modulating both pro- and anti-inflammatory activities of CD4+ T cells and thus has the potential to influence the outcome of CD4+ T cell-mediated immune responsiveness. Estrogens may thus be central to the regulation of the balance of Th1/Th2 cytokines within sites of inflammation and to the appropriate or inappropriate termination of the inflammatory response in infections, tolerance development or autoimmunity. Several authors have attempted to study sex hormone changes in women with PBC. These have included epidemiological studies in which a negative association with parity was first denied and ultimately confirmed[9]. Of interest, taking hormonal replacement therapies following menopause was found in this latter study to be significantly associated with PBC although this may be secondary to the proposed enhanced rate of bone loss in chronic cholestasis. Furthermore, the differences in plasma estrogen levels between women with PBC and controls observed in earlier studies may be secondary to long-standing cholestasis or may account for the wide variability of their measurements during the reproductive cycle and should be considered as non conclusive.

A second hypothesis on female predominance is the persistence of fetal genome parts in women. It has been hypothesized that the pathogenesis and female predominance of autoimmunity may be secondary to the presence in affected women of allogenic male fetal cells se veral years after pregnancy (i.e. fetal microchimerism). Microchimeric cells were first found in peripheral blood mononuclear cells from patients with scleroderma and it was suggested that non-autologous cells may be mediating a graft-versus host disease-like reaction in these patients but other studies have failed to recapitulate these findings. Several studies found no significant difference in frequency of male microchimerism in female PBC and controls[10]. We are convinced that available data on the role of fetal microchimerism in autoimmunity in general are still controversial while these should be considered as negative in PBC.

A new fascinating hypothesis on the female predominance of autoimmunity is based on major defects of sex chromosomes[11] and supported by data in other fields[12-15]. X chromosome inheritance displays a peculiar pattern compared to autosomal chromosomes since women are functional mosaics for X-linked genes. In females, most genes on one X chromosome are silenced as a result of X-chromosome inactivation (XCI). The result of XCI is to achieve equivalent levels of X-linked gene products between males and females. More recent data have undermined this dogmatic view by de monstrating that at least 15% of X-linked genes are capa

ble of escaping XCI in healthy women and are thus expressed from both X chromosomes. Up to 10% of total X-linked genes manifest variable XCI patterns in different individuals[16]. We recently proposed a role for X chromosome based on experimental evidence that women with autoimmune diseases have a significantly higher frequency of peripheral blood cells with a single X chromosome (i.e. X monosomy) compared to healthy women (Lancet). Importantly, this was observed in diseases with different organ specificities including PBC[17], scleroderma and autoimmune thyroid disease[18]. X chromosome loss is indeed preferential and involves more frequently a parentally inherited one[19], suggesting a possible critical involvement of X chromosome gene products defects in female preponderance of PBC and other autoimmune diseases while new factors such as microRNAs[20] or epigenetics[21,22] should not be overlooked. Other authors have suggested that women affected with specific female-preponderant autoimmune diseases, i.e. scleroderma, manifest a skewed XCI pattern in their peripheral white blood cells[23]. In PBC, however, we failed to demonstrate such preferential inactivation[19].

Genomics

The genetic bases of PBC are not related to a single gene or Mendelian compatible with the complex etiology previously discussed. Variable rates of familial PBC are seen in different geographical areas, possibly due to different methods of case definition, but generally 1%-6% of PBC cases have at least one family member manifesting the disease while our most recent data obtained interviewing 1032 patients throughout the US indicate 6% of cases with a first-degree relative also affected[9]. Such familial prevalence rates are significantly higher than general population prevalence estimates, thus indicating a genetic predisposition to the disease. However, the difficulty in evaluating these data is that prevalence rates in the general population are still uncertain and control groups are not always included in the family studies. On the other hand, concordance rates in monozygotic twins for late-onset female-predominant autoimmune diseases range on average well below 50%. We first reported that concordance rates for PBC are 63% in 8 monozygotic sets and null in dizygotic twins[24]. The phenotypical discordance observed in some twin pairs could be caused by epigenetic factors, differences in exposure to environmental factors or mere serendipity.

Studies on polymorphisms associated with PBC are based on case-control designs[25] and these approaches are limited by poor control matching criteria and sample size or selection while very few proposed associations have been independently confirmed in other populations. Of interest, a recent multi-center study reported the first genome-wide association study and identified interleukin 12 and its relative receptor as susceptibility genes for PBC as well as STAT4 and HLA genotypes[26]. These data were significantly strengthened by our most recent independent study and meta-analysis[27].

A representation of smaller studies on candidate genes is to be subdivided into two separate groups of genes. Significant associations with specific MHC alleles have been reported in most autoimmune diseases[28-30], in some cases constituting a clinical marker[31]. The scenario in PBC is quite different with limited evidence provided thus far. We contributed in 2003 and 2008 to this issue and reported that PBC is significantly associated with various HLA-B alleles in a small proportion of the patients studied[32,33]. The association between PBC and HLA genes should be ultimately seen as weak, if any. The challenge to identify susceptibility gene(s) that predispose for the development of PBC is still open. The majority of such studies not only have been derived solely from case-control designs[25] but were also limited by poor control matching criteria and sample size or selection. A plethora of association studies have been conducted, mainly focused on immune genes that affect the immune system belonging to both the HLA family and non-HLA immune modulators genes, including CTLA-4, IL-1, IL-10 and vitamin D-receptor. The discussion of these data goes beyond the aims of this article and details have been reviewed in dedicated articles[25,34,35].

Environmental factors

An environmental insult (more likely not harmful in the general population) is believed to result in tolerance breakdown and PBC onset in the presence of a susceptible genetic background. Epidemiological data combined with experimental evidence on infectious agents and xenobiotics strongly support this view.

Our 2005 epidemiological study on 1032 patients with PBC and 1041 rigorously matched controls[9] demonstrated that a high risk of developing PBC is associated with a positive family history for PBC, a history of urinary or vaginal infections, co-morbidity with other autoimmune diseases, lifestyle factors such as smoking and previous pregnancies. We also observed that the frequent use of nail polish also slightly increased the risk of having PBC. In most cases, these factors had been previously suggested in smaller studies. Based on these observations, two main classes of environmental factors have been suggested in PBC and include infectious (bacteria, viruses) and chemical (xenobiotics) factors. The ability of infectious agents, particularly bacteria, to induce autoimmune responses has been supported by animal models and molecular mimicry in several autoimmune diseases and remains the most widely studied mechanism. The molecular mimicry hypothesis states that microbes contain peptides sharing different degrees of similarity with self-proteins, thus leading to a promiscuous immune response (antibody- and cell-mediated) in turn capable to recognize both microbial and self-epitopes. This cross-reactivity is not particularly surprising given the conserved sequence of mitochondrial enzymes across all species, from eubacteria to mammals[36]. Mitochondria originated following uptake of bacteria into the precursors of eukaryotic cells and maintenance as intracellular

symbionts and this makes it difficult to determine a causal role for microbial proteins in pathogenesis given their phylogenetic relationship to the human autoantigen. One line of argument that we have taken is that the breaking of tolerance and induction of autoimmunity would be more likely to occur when the microbial protein is extremely similar in sequence while it would not be necessary for tolerance breakdown to take place in the disease target organ[37]. In this scenario, T-cell activation produces cross-reacting T-cells leading to self-tissue destruction and this ultimately perpetuates the autoimmune injury, possibly through the degeneracy of the T-cell receptor and cross-priming. Of the bacterial strains suggested to lead to PBC through molecular mimicry[38], most evidence has been gathered for *Escherichia coli*(*E. coli*), primarily based on the reports of the increased incidence of recurrent urinary tract infections in patients with PBC[9]. Conflicting evidence has been obtained on the role of *Chlamydia pneumoniae* in the pathogenesis of PBC but original data were not independently recapitulated. Finally, our group has provided serological and molecular data suggesting that a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, *Novosphingobium aromaticivorans*(*N. aromaticivorans*), is the best candidate yet for the induction of PBC as it elicits a specific antibody-reaction (up to 1 000-fold higher than against *E. coli*) and its 16S rRNA specific sequences can detected in 25% of human fecal samples[39]. Most recently, Mattner *et al*[40] were able to induce serum autoantibodies and PBC-like liver lesions following immunization with *N. aromaticivorans*. Whether the bacterial impact should be regarded as based solely on cross-reactivity or on the presentation of mimicry antigens remains to be largely investigated. Similarly, we cannot but hypothesize a connection between the bacterial infection and the xenobiotic theory at the present status of knowledge.

Xenobiotics can be defined as foreign compounds that are believed to alter or complex to defined self or non-self proteins and induce a change in the molecular structure of the native protein sufficient to induce an immune response[41]. Similar to molecular mimicry, therefore, such immune response may lead to the cross-recognition of the self form and ultimately chronic autoimmunity. The main detoxifying organ is the liver, thus potentially exposing hepatocytes and biliary epithelial cells to chemical byproducts. Earlier serological data obtained in our laboratory by Long *et al*[42] in 2001 demonstrated that specific halogenated organic compounds attached to the major mitochondrial epitope backbone were recognized by sera from PBC patients with a higher affinity than the native forms. These results were of critical importance supporting for the first time an organic compound serving as mimic for an autoantigen. Afterwards, the same halogenated compound was found capable to induce autoantibody production in animal models, albeit no liver lesions were observed in the short follow-up[43]. More recently, Leung *et al* reported the induction of PBC-like liver lesions following longer follow-ups in guinea pigs exposed to a specific halogenated compound which was also capable to induce liver lesion in a specific strain of non obese diabetic (NOD) mouse[44,45]. Finally, the use of a multiplex approach led to determine that 2-nonynoic acid is recognized by PBC sera with high affinity[46]. This is particularly interesting since this compound does not occur naturally and is found in several cosmetic products, including nail polish[9]. One should note that data on molecular mimicry in PBC were mainly obtained from the study of autoantibodies in sera from patient or animal models while the study of cellular autoimmunity is limited.

PATHOGENESIS

The etiology and pathogenesis of PBC remain largely enigmatic. The disease should be regarded as multifactorial and we are convinced that the numerous lines of evidence provided by clinical and experimental research will ultimately provide an answer to the several questions remaining on the table. Prior to illustrating the available evidence on the immune mechanisms involved in PBC pathogenesis, it should be clear that the etiology of the bile duct injury and in particular the strict organ-specificity of the immune-mediated tissue damage remains to be elucidated and current hypotheses will be discussed. The lines of evidence should not be regarded as mutually exclusive from any causative factor such as susceptibility genes but rather as terminal mechanisms of the pathway leading to the clinical manifestations[47] ultimately orchestrated by a specific cytokine pattern[48-50].

First, the pathogenetic role of AMA is not yet clear, as discussed in further details below. AMA belong mainly to the IgG isotype, in particular IgG3 subclasses, and thus are potentially pathogenic through different mechanisms, e.g. complement activation, antibody-dependent cytotoxicity. There are no direct experimental evidence, however, supporting the involvement of these mechanisms in the pathogenesis of PBC as serum AMA are elicited in animal models following several types of immunization; yet PBC-like liver damage is caused only in selected cases[51]. More importantly, AMA can be also of IgA isotype. The role of such AMA-IgA has been ignored for long time but may be critical in the pathogenesis of PBC. It is now well established that AMA-IgA, indeed, can be detected not only in sera but also in bile, saliva and urine of patients with PBC, in some cases correlating with disease severity[52]. Moreover, IgA represents the principal Ig isotype in epithelial surfaces, including biliary epithelia. AMA-IgA has been reported to co-localize with the major AMA autoantigen (i.e. the E2 subunit of pyruvate dehydrogenase, PDC-E2) both inside the cell cytoplasm as well as the apical membrane of cholangiocytes in PBC but not in controls. Thus, AMA-IgA and in particular AMA-IgA bound to mitochondrial antigen could be able to disrupt cell metabolism and may also induce cellular dysfunction and damage thus leading to a tissue specific injury. We cannot preclude the possibility that the apical staining obtained with anti-PDC-E2 monoclonal

antibodies may be secondary to the presence of immune complexes formed by secreted IgA and AMA antigens, as some line of evidence seems to suggest[53].

Over the past decade one hypothesis for the selective destruction of biliary epithelial cells was proposed implying that the immunodominant autoantigen PDC-E2 should be aberrantly exposed on cholangiocytes cell surface where it may be recognized by AMA and/or antigen-specific T cells[52-54]. Studies based on in situ hybridization of PDC-E2 mRNA failed to demonstrate significant differences in its amount in PBC liver compared with other liver diseases. PDC-E2 may be selectively over-expressed in small bile duct cholangiocytes as indirectly suggested by early experimental evidence showing a positive staining of a murine anti-PDC-E2 monoclonal antibody selectively on the surface of biliary epithelial cells in the liver of patients with PBC but not in normal controls. On the other hand, co- or post-translational modifications of PDC-E2 may cause its abnormal turnover leading to its accumulation. Chemicals (i.e. xenobiotics) disposed by the liver may have a role in this scenario by accumulating in the biliary epithelial cells and modifying PDC-E2 locally. Solid data to support these fascinating mechanisms are lacking or weak and we cannot rule out that the molecules expressed and identified on the ductular surface and recognized by AMA may not be PDC-E2 itself but possibly unrelated PDC-E2 mimics cross-reacting with human PDC-E2.

Does apoptosis hold the key to organ specificity?

Apoptosis of biliary epithelial cells in PBC warrants further discussion and may prove to be crucial for immune tolerance breakdown[55,56] as illustrated in other experimental settings[57]. It was first reported that PDC-E2 remains intact and retains its immunogenicity during cholangiocyte apoptosis due to a cell-specific lack of glutathionylation of biliary epithelial cells[58]. The intact PDC-E2 in apoptotic fragments could be taken up by local antigen presenting cells and transferred to regional lymph nodes for priming of cognate T cells thus initiating PBC. This is indeed an attractive possibility, however, solid data of such antigen presentation are awaited and it cannot be excluded that the reported mechanisms are not PBC specific. A major contribution came from Lleo *et al* who most recently demonstrated that PDC-E2 is found in the blebs of human intrahepatic bile duct cells undergoing apoptosis[56] and that they could be presented to local dendritic cells to initiate the immune response[59]. More importantly, this phenomenon was not observed in other epithelial cell lines and appears to confirm the importance of apoptosis in the perpetuation of the autoimmune injury[55] as well as the view that PBC bile duct cells are not unique[60].

Is PBC an autoimmune disease?

Whether PBC is indeed an autoimmune disease remains to be clearly determined, based on somehow conflicting clinical and experimental data. An autoimmune pathogenesis for PBC is accepted based on clinical and experimental findings that make this condition somehow a model and a paradox for autoimmunity. The former is represented by the PBC features that are common to the autoimmune spectrum such as the female predominance, the genetic predisposition or the presence of specific autoantibodies in the vast majority of cases as well as the frequent comorbidities[61]. Serum autoantibodies, however, in the case of PBC also represent the basis for the disease being a paradox as their direct pathogenetic role is still poorly defined[62]. In fact, serum autoantibodies are detected in approximately 90% of patients; yet seronegative cases manifest a similar disease progression[63]. Furthermore, in the autoimmunity paradigm, the passive transfer of autoantibodies should reproduce the clinical features and experimental immunization with the antigen should produce a model disease. This has been reproduced only in part for PBC, as illustrated in details below. In autoimmune diseases, the reduction of autoantibody titers will correlate with disease amelioration; this criterion is also poorly met in PBC where there is no correlation between the pattern or titer of AMA and progression or severity of the disease. Finally, it is well-established that most autoimmune diseases are responsive to immunosuppressive therapy while no such agent has proven effective for PBC. We will now discuss the major characteristics of PBC in terms of adaptive (humoral and T cell) and innate immunity as well as the emerging role of T regulatory cells. Ultimately, the putatively comprehensive animal models will be discussed.

Autoantibodies

Serum AMA are highly specific for PBC and detected in nearly 100% of patients when tested using techniques based on recombinant mitochondrial antigens (immunoblotting or ELISA) which allow higher sensitivity and specificity. In most clinical settings, however, indirect immunofluorescence remains the test used for initial screening of cases and might lead to falsely positive or negative results. AMA autoantigens have been known since 1987[64]. Antibodies react with lipoilated domains within components of the 2-oxoacid dehydrogenase (2-OADC) family of enzymes within the mitochondrial respiratory chain, most frequently the E2 and E3 binding protein (E3BP) components of the pyruvate dehydrogenase complex (PDC-E2) and the E2 components of the 2-oxo glutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes. The immunodominant epitopes in all major antigens contain the motif DKA, with lipoic acid attached to lysine (K). The necessary/sufficient role of lipoic acid in the epitope recognition by AMA is unclear based on serum reactivity studies, as briefly mentioned above. Serum antinuclear antibodies (ANA) have been detected in as many as 30% of patients with PBC and indirect immunofluorescence more specifically produce a 'nuclear rim' or 'multiple nuclear dots' pattern, based on the recognition by the autoantibodies of gp210 and nucleoporin 62 (within

Table 1 Hints towards an innate immunity involvement in primary biliary cirrhosis

	Peculiarity
Monocytes	Increase in absolute number
	Increase in pro-inflammatory cytokines upon infectious challenge
Elevated IgM	B cell response to bacterial stimuli
NKT cells	Increased NKT cells in PBC peripheral blood and liver
	Increased NKT cytotoxic activity
Liver histology	Focal duct obliteration with granuloma formation

PBC: primary biliary cirrhosis models.

the nuclear pore complex) and Sp100 and PML (possibly also cross-reacting with small ubiquitin-like modifiers, SUMO) respectively[65-67]. Rim-like ANA, in fact, react against proteins of the nuclear pore complexes (NPC), supramolecular structures that include gp210 (a 210-kDa transmembrane glycoprotein involved in the attachment of NPC constituents within the nuclear membrane), p62 (a nuclear pore glycoprotein), and the inner nuclear membrane protein lamin B receptor (LBR). Serum anti-gp210 ANA are detected in about 25% (10%-40%) of AMA-positive and up to 50% of AMA negative patients (in both cases with high specificity). Autoantibodies reacting with p62 or LBR are found in about 13% and 1% of patients with PBC respectively. Interestingly, the presence of anti-gp210 and anti-p62 ANA in the same serum is rare. It was first supposed that ANA-positive patients are more frequently AMA-negative, possibly because of the lack of a masking effect of these latter antibodies in such sera, yet this remains to be determined. Interestingly, these PBC-specific ANA have been consistently found associated with more severe[68] and rapidly progressing disease[65,66]. Similar to AMA, the pathogenic role of ANA in PBC remains enigmatic although cross-sectional and longitudinal data demonstrate an association between ANA positivity and a poorer prognosis.

T cell autoimmunity

T-helper (CD4+) TCR+ and CD8+ T cells are most commonly seen around injured bile ducts in PBC. Autoreactive T cells have been well characterized in PBC from both the liver and in peripheral blood of affected individuals. Autoreactive cytotoxic T lymphocytes (CTL) have been well characterized in PBC and currently considered major effectors in the tissue injury encountered in PBC. The MHC class I restricted epitope for CTLs, namely amino acid 159-167, also maps in close vicinity to the epitopes recognized by CD4+ cells and by AMA. Moreover, the use of tetramer technology has shown a 10-fold higher prevalence of PDC-E2159-167 specific CTL in the liver as compared to peripheral blood of patients with PBC. PDC-E2 specific autoreactive CD4+ T cell (T-helper) clones were first isolated by in vitro stimulation of intrahepatic or peripheral lymphocytes to PDC-E2. It is of note that the autoepitope for T cells overlaps with the B cell (AMA) counterpart and includes the lipoilated amino acid of the inner lipoilated domain. Similar to CTL, there is also a specific 100-150 fold increase in the number of autoreactive CD4+ T cells in the PBC hilar lymph nodes and liver when compared with peripheral blood, regardless of the AMA status[69].

Innate immunity

Following decades in which adaptive immunity was considered self-sufficient to explain autoimmunity, the study of innate immunity has received a significant impetus over the past few years and is no longer overlooked by clinical immunologists. This interest has been growing[70] since evidence has been provided that the cellular components of the innate immune system such as monocytes, dendritic cells (DC), natural killer (NK) and NK T cells modulate the function of both the humoral and cellular adaptive immune responses. Immunological features such as elevated levels of polyclonal IgM and hyper-responsiveness to CpG, increased levels of NK cells and cytokine responses in patients with PBC compared to controls ultimately suggest a determinant role of innate immunity in the onset and perpetuation of PBC (Table 1). The liver express effective immune responses against a wide range of pathogens from viruses to multicellular parasites. In that sense, the environment of an inflammatory milieu seems to be critical for effective immunogenic signals to be delivered to intrahepatic T cells. The tolerogenic potential of intrahepatic DC and sinusoidal endothelial cells is, on the other hand, based on their hyporesponsiveness to lipopolysaccharide as a "pathogen associated molecular pattern" (PAMP) by reason of an altered expression of toll-like receptors (TLR), with deprivation of crucial upregulating signals for antigen presentation and co-stimulatory activity. Some of the innate immunity cells are known for their regulatory function in detailing the quality and quantity of subsequent adaptive immune responses including antigen-specific antibody and T cell responses. Innate immunity is involved in several aspects of autoimmunity[71,72] and in PBC these may include the presence of epithelioid granulomas. Recent data have supported a role for memory B cells, monocytes and NKT cells. It is common to observe elevated levels of IgM in PBC sera, independent of the AMA or ANA status, while their reduction during medical treatment[73] support a direct link. It has been reported that hyper-IgM is secondary to a chronic polyclonal innate immune response of memory B cells to bacterial stimuli represented by unmethylated CpG motifs that share immunostimulatory effects on human cells[74]. Following stimulation with synthetic oligodeoxynucleotides containing such motifs, cultured peripheral blood mononuclear cells from patients with PBC also secreted higher amounts of IgM compared to controls and this is reduced by ursodeoxycholic acid[75]. Alternatively, one may hypothesize that the hyper-IgM in PBC patients is the result of a failed attempt at preservation of the state of tolerance. This could follow the exposure to chemical-metabolizing bacteria as indicated in recent reports of the presence of IgG antibodies against xenobiotic modified PDC-E2 and of a

Table 2 Features of spontaneous and induced murine primary biliary cirrhosis models resembling data in the human condition

	Mouse model	Adaptive immunity	Innate immunity
Spontaneous	Ae2(a.b)-deficient	AMA Lymphocytic CD8+ infiltrates Decreased T regulatory cells PBC-like liver lesions	--
	dnTGFbRII	AMA Deficient T reg function	NKT cells worsen liver injury
	$IL2Ra^{-/-}$	AMA Portal tract CD4+ and CD8+ cells	--
	NOD.c3c4	Lymphocytic infiltrate AMA, ANA	--
Induced	*N. aromaticivorans* on NOD 1101	AMA PBC-like liver lesions Disease transfer by T cells	NKT cells are required
	Xenobiotic on C57BL/6	Lymphocytic CD8+ infiltrate AMA PBC-like liver lesions	--

AMA: antimitochondrial autoantibodies; ANA: antinuclear antibodies; NOD: non obese diabetic; PBC: primary biliary cirrhosis models.

possible association of *N. aromaticivorans*. The monocyte activation by PAMP through TLR induces the release of pro-inflammatory cytokines by monocytes, characteristic of the innate immune response, including IL-1, IL-6, IL-18, IL-12 and TNF-α which mediate the amplification of T-cell mediated immune response against pathogens. Peripheral monocytes from patients with PBC and controls challenged with different ligands for TLR2, TLR3, TLR4, TLR5 and TLR9 produce a significantly increased level of all pro-inflammatory cytokines compared to healthy controls[76]. From the innate immunity perspective, these findings suggest that peripheral blood monocytes from patients with PBC are more sensitive to infectious stimuli resulting in the secretion of pro-inflammatory cytokines. The mechanisms for such increased sensitivity are currently unknown but might reflect or be secondary to the higher frequency of recurrent Gram-negative bacterial infections (e.g. urinary tract infections) in PBC. This effect is seen as a consequence of the constant exposure of monocytes and B cells to bacterially derived products from the portal blood but with inflammation compensating for such hyporesponsiveness and inducing efficient intrahepatic T-cell priming and subsequent effective cellular immune responses.

The role of NKT cells in autoimmunity is also attracting growing attention. The presence of NKT cells restricted for the α-galactosylceramide (α GalCer) in a CD1 context has been evaluated and reported a higher prevalence of these cells in the affected tissue than in peripheral blood[77]. This behavior resembled that of NKT cells in other liver diseases and healthy controls while Chuang *et al* recently demonstrated a marked increase in the frequency and absolute number of blood and liver NKT cells in PBC patients. We are well aware that the innate immune system hyper-responsiveness is likely not sufficient for the breakdown of tolerance; we can hypothesize that these alterations might play a role in the initiation and/or perpetuation of the autoimmune injury. This is particularly intriguing considering the study by Mattner *et al* demonstrating that *N. aromaticivorans* is capable of inducing autoreactive AMA and chronic T cell-mediated autoimmunity against small bile ducts in a murine model of PBC in an NKT-dependent fashion[40].

T regulatory cells

T regulatory cells (Tregs) are $CD4^+$ $CD25^{high}$ cells and play a role in the prevention of autoimmune disease as demonstrated in several clinical settings[78]. As an example, in chronic autoimmune hepatitis therapeutic attempts are ongoing in animal models based on cell expansion[79]. In fact, some studies have demonstrated that the transfer of T cells lacking the $CD4^+$ $CD25^{high}$ Tregs subset into athymic nude mice results in the development of various T cell-mediated autoimmune diseases[80]. Experimental data demonstrate that PBC patients displayed significantly lower frequencies of $CD4^+$ $CD25^{high}$ Tregs as percentages of total TCR-$\alpha\beta^+$/$CD4^+$ T cells which may contribute to the breakdown in tolerance in PBC[81], possibly through interleukin 2[82]. The recently defined field of CD8+ FoxP3+ regulatory cells has not been investigated in PBC.

ANIMAL MODELS

The development of an animal model is of obvious importance in elucidating the mechanism(s) responsible for the initiation and progression of PBC and to investigate new medical treatments. Several models, mostly murine[83], have been proposed for PBC and are illustrated in Table 2. These include both spontaneous and induced models and have subsequently been utilized to prove specific mechanistic hypotheses in PBC pathogenesis and data have demonstrated that CD8+ T cells are required to transfer disease[84] while B cell depletion has unexpected consequences on biliary disease[85].

Two animal models, i.e. dnTGFβRⅡ and IL-2Rα-knockout mouse, point out the possible crucial role of Tregs deficiency in the loss of immune tolerance with consequent development of autoimmune response against PDC-E2 in PBC. In particular, a mouse with dominant negative form of transforming grown factor β (TGFβ) receptor Ⅱ, (dnTGFβRⅡ) showed PBC-like liver disease, e.g. 100% AMA positivity against PDC-E2[86]. TGFβ receptor Ⅱ is essential for signal transduction of TGFβ that is a key regulator of lymphocytes activation[85]. A mouse deficient for IL2 receptor IL-2R which is highly expressed on Tregs developed 100% AMA positivity

against PDC-E2, 80% ANA positivity and lymphocyte infiltration around the portal tracts associated with cholangiocyte injury[87]. As previously mentioned, animal models support our hypothesis that xenobiotics can induce tolerance breakdown. Firstly, loss of tolerance has been demonstrated in rabbits immunized with 6-bromohexonate, a xenobiotically modified hapten mimicking lipoic acid, coupled with bovine serum albumin[43]. The immunized rabbits were able to produce not only antibodies against the xenobiotic but also high titer of anti-PDC-E2 antibodies. Anti-PDC-E2 antibodies in this model were not, however, sufficient to induce specific hepatic lesions, at least in the short follow-up[43]. More recently, induction of PBC-like lesions was obtained in a NOD background by Wakabayashi *et al* and in guinea pigs by Leung *et al* exposed to xenobiotic immunization[88,89]. Furthermore, an animal model is derived from a variant of the NOD mouse model (NOD.c3c4). It has been described that NOD.c3c4 manifests autoimmune cholestasis and PBC-specific serology, showing AMA positivity of 50%-60% and ANA positivity of 80%-90%. Histologically, it presents lymphocyte infiltration around portal tracts with chronic nonsuppurative destructive cholangitis and epithelioid granuloma formations; nevertheless, the morphological features of bile ducts differ somewhat from those in human PBC[90]. Finally, we submit that the PBC-like model induced by *N. aromaticivorans* immunization[40] awaits further recapitulation.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, we recapitulate our current working hypothesis[8]. Three major events in PBC are represented, i.e. bile duct cell apoptosis, female predominance and genetic susceptibility. A mimicking microorganism (possibly *N. aromaticivorans* as discussed below) enters the human system through the digestive mucosa and its PDC-E2-like proteins are modified within the liver by xenobiotics to form immunoreactive adducts. These modifications could be then sufficient to trigger the innate immune system to initiate a cascade of local inflammatory events resulting in local dendritic cell activation and antigen processing. Mucosal antigen-presenting cells in turn could activate autoreactive T and B cells that are directed to the liver through the portal system. T cells, therefore, could participate directly, not only to the autoimmune injury, but also to its amplification and perpetuation. B cells, on the other hand, could secrete AMA, particularly of the IgA type. AMA-IgA could be then transported to the vascular side of biliary epithelial cells where they could recognize PDC-E2-like molecules located on the luminal surface cell membrane. AMA-IgA/PDC-E2-like molecules engagement could initiate apoptotic signaling cascade. Ultimately, the immune complexes of post-apoptotic PDC-E2 and IgG-AMA and the direct cytopathic effects of autoreactive T cells (and possibly AMA) lead to the selective bile duct destruction.

Recent years have had a tremendous impact on our knowledge of PBC causes and consequences, as well represented by data on apoptosis or genomic associations. We are convinced that future studies should be dedicated to overcoming conceptual and logistical obstacles. Firstly, the role of xenobiotics and bacteria in the onset of PBC should be further studied by means of new mo lecular multiplex tools (including but not limited to proteomics) and available animal models. Secondly, only the collection of large series of patients and, quite crucially, representative families and the use of genome-wide analysis on larger numbers of polymorphisms will allow the determination of the genetic bases of PBC, similar to what was recently observed in other autoimmune diseases. Thirdly and most importantly, it is time to prove the AMA pathogenic role in PBC and the proposed animal models may be a good starting point to achieve this goal. Ultimately, we are convinced that newer discoveries in the field of molecular biology will provide exciting data in PBC pathogenesis, as in the case of new cytokine paradigms[49], microRNA[20], DNA methylation[91] or copy number variations[92], with putative therapeutic implications. Among these, the use of epigenetic drugs[22], antisense miRNA[93] as well as new biologics[94] may be of seminal importance, similar to what was recently observed for new compounds targeting nuclear receptors[95].

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Protein induced by vitamin K absence or antagonist Ⅱ-producing gastric cancer

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Abstract

Protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ) is a putative specific marker of hepatocellular carcinoma (HCC), but it may also be produced by a small number of gastric cancers. To date, 16 cases of PIVKA-Ⅱ-producing gastric cancer have been reported, 2 of which were reported by us and all of which were identified in Japan. There are no symptoms specific to PIVKA-Ⅱ-producing gastric cancer, and the represen tative clinical symptoms are general fatigue, appetite loss, and upper abdominal pain. Serum alpha-fetoprotein (AFP) levels are also increased in almost all cases. Liver metastasis is observed in approximately 80% of cases and portal vein tumor thrombus is observed in approximately 20% of cases. Differential diagnosis between metastatic liver tumor and HCC is often difficult. Grossly, almost all cases appear as advanced gastric cancer. Histologically, a hepatoid pattern is observed in many cases, in addition to a moderately to poorly differentiated adenocarcinoma component. The production of PIVKA-Ⅱ and AFP is usually confirmed using immunohistochemical staining. Treatment and prognosis largely depends on the existence of liver metastasis, and the prognosis of patients with liver metastasis is very poor. PIVKA-Ⅱ may be produced during the hepatocellular metaplasia of the tumor cells.

Key words: Protein induced by vitamin K absence or antagonist Ⅱ; Gastric cancer; Alpha-fetoprotein; Hepatocellular carcinoma; Hepatoid carcinoma

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INTRODUCTION

Since Liebman *et al*[1] reported the increase of protein induced by vitamin K absence or antagonist Ⅱ (PIVKA-Ⅱ) in the serum of hepatocellular carcinoma (HCC) patients, PIVKA-Ⅱ has been regarded as a tumor marker of HCC. PIVKA-Ⅱ has been reported to be a more specific marker than alpha-fetoprotein (AFP), another tumor marker of HCC[2,3]. The production of AFP and PIVKA-Ⅱ by HCC cells has been elucidated morphologically[4]. However, serum AFP or PIVKA-Ⅱ levels may increase in patients with tumors other than HCC, and if such tumors metastasize to the liver, it may be difficult to clinically differentiate them from HCC. Although the number of reported cases of AFP-producing gastric cancer has been gradually increasing[5-9], only 16 cases of PIVKA-Ⅱ-producing gastric cancer have been reported

Table 1 Clinicopathological data of protein induced by vitamin K absence or antagonist Ⅱ producing gastric cancer

Case	Age	Sex	Serum AFP (ng/mL)	Serum PIVKA-Ⅱ (AU/mL)	Macroscopic type	Tumor size (cm)	Histological type	Liver metastasis	Treatment	Prognosis	Ref.
1	56	M	2810	2.45	0-Ⅱc	6.5 × 4.5	por + tub1 + hepatoid	(-)	subtotal gastrectomy	Alive without tumor (17 mo)	[10]
2	43	M	483380	134	Borrmann 3	7.5 × 7.0	por	(+)	best supportive care	Died of disease (3 mo)	[11]
3	42	F	190	2.9	Borrmann 3	ND	por	(+)	TACE + systemic chemotherapy	Died of disease (3 mo)	[12]
4	63	M	120000	> 8	Borrmann 1	ND	por + hepatoid	(+)	systemic chemotherapy	Died of disease (2 mo)	[13]
5	72	M	14500	32.8	Borrmann 1	ND	por	(+)	best supportive care	Died of disease (1mo)	[14]
6	71	M	73	5.15	Borrmann 2	About 2	tub2	(+)	subtotal gastrectomy + enucleation of liver metastasis + HAIC	Alive (2 mo)	[15]
7	71	M	1230	28.5	Borrmann 2	About 4	por	(+)	best supportive care	Died of disease (3 mo)	[16]
8	55	M	247000	320	Borrmann 1	ND	por	(+)	systemic chemotherapy	Died of disease (4 mo)	[17]
9	71	M	18551.7	1.08	Borrmann 3	ND	tub2	(+)	systemic chemotherapy	Died of disease (5 mo)	[18]
10	87	F	490200	2.284	Borrmann 2	3.8 × 2.3/4.5 × 3.2	por + tub2 + hepatoid	(+)	best supportive care	Died of disease (1 mo)	[19]
11	49	M	21552.9	3.7	Borrmann 2	5 × 5	tub2 + hepatoid	(+)	HAIC	Died of disease (5 mo)	[20]
12	68	M	4	15.6	Borrmann 3	about 15	por + tub2	(-)	preoperative chemotherapy + pancreatico-spleno total gastrectomy	Alive without tumor (15 mo)	[21]
13	45	M	13827	0.405	Borrmann 2	ND	tub2	(+)	systemic chemotherapy	Died of disease (6 mo)	[22]
14	61	M	495.2	0.635	Borrmann 3	8.0 × 7.0/9.5 × 8.5	tub2 + pap + por + hepatoid	(+)	total gastrectomy + postoperative adjuvant chemotherapy	Died of disease (6 mo)	[23]
15	61	M	9630	0.091	Borrmann 2	4.5 × 4.0	por + hepatoid	(-)	distal gastrectomy with resection of the extra-gastric tumor	Alive without tumor (9 mo)	[24]
16	71	M	296838	56.387	Borrmann 1	ND	por + hepatoid	(+)	systemic chemotherapy	Died of disease (6 mo)	[25]

HAIC: hepatic arterial infusion chemotherapy; ND: no data; pap: papillary adenocarcinoma; por: poorly differentiated adenocarcinoma; TACE: transcatheter arterial chemoembolization; tub1: well differentiated tubular adenocarcinoma; tub2: moderately differentiated tubular adenocarcinoma; Ref: references; PIVKA-Ⅱ: Protein induced by vitamin K absence or antagonist Ⅱ.

to date[10-25], 2 of which were reported by us[19,23]. The exact definition of PIVKA-Ⅱ-producing gastric cancer has not been established yet. However, we propose to make a diagnosis of PIVKA-Ⅱ-producing gastric cancer when a patient with gastric cancer has high serum level of PIVKA-Ⅱ and PIVKA-Ⅱ production by gastric cancer cells is confirmed by immunohistochemistry, or when in a patient with gastric cancer and high serum level of PIVKA-Ⅱ, the other sources of PIVKA-Ⅱ production, such as HCC and vitamin K deficiency, are ruled out. In this paper, we review the clinicopathological features of PIVKA-Ⅱ-producing gastric cancer; furthermore, we discuss the differential diagnosis between HCC and liver metastasis of PIVKA-Ⅱ-producing gastric cancer, and the mechanism of PIVKA-Ⅱ production by gastric cancer cells. In addition, we compare PIVKA-Ⅱ- and AFP-producing gastric cancers.

CLINICAL FEATURES

Epidemiology

PIVKA-Ⅱ-producing gastric cancer is very rare, with only 16 cases reported to date[10-25], and its precise prevalence has not been elucidated. Table 1 summarizes the clinicopathological features of the reported cases of PIVKA-Ⅱ-producing gastric cancer. Interestingly, all of the reported cases were identified in Japan. The reason for this is unclear, but the following possibilities are conceivable: (1) PIVKA-Ⅱ-producing gastric cancer is especially prevalent in the Japanese; and (2) in Japan, PIVKA-Ⅱ-producing gastric cancer is correctly diagnosed because of sufficient endoscopic, laboratory, radiological, and pathological examinations. The serum AFP levels were increased in all but one case and liver metastasis was detected in all but three of the reported PIVKA-Ⅱ-producing gastric cancer

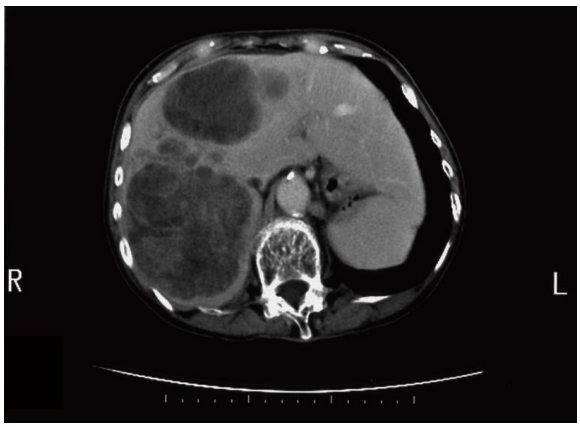

Figure 1 Contrast-enhanced computed tomography image of liver metastasis of protein induced by vitamin K absence or antagonist Ⅱ-producing gastric cancer. Multiple low density nodules, which are poorly enhanced, are observed in the liver.

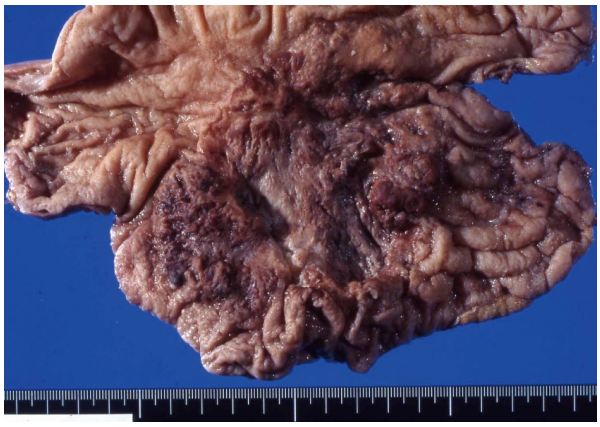

Figure 2 Gross appearance of a case of protein induced by vitamin K absence or antagonist Ⅱ-producing gastric cancer. A large Borrmann type 3 tumor occupies the gastric body.

cases. PIVKA-Ⅱ-producing gastric cancer predominantly occurs in elderly men. The patients' ages ranged from 42-87 years (mean, 62 years). Fourteen cases were male and 2 cases were female; thus, the male-to-female ratio is 7:1.

Symptoms and signs

There are no symptoms specific to PIVKA-Ⅱ-producing gastric cancer, and the representative clinical symptoms are general fatigue, appetite loss, and upper abdominal pain. A mass in the abdomen or right hypochondrium and abdominal distention or discomfort are also occasionally seen. These symptoms are considered to be related to the gastric tumor or metastatic liver tumor. Tarry stool, back pain, edema in the lower leg, and pyrexia were observed in a small number of cases.

Laboratory data

Serum PIVKA-II levels (normal value < 0.04 AU/mL) in the reported cases ranged from 0.091-320 AU/mL (median, 4.4 AU/mL). Serum AFP levels (normal value < 20 ng/mL) were abnormally high in all but one case (range, 4-490200 ng/mL; median, 14164 ng/mL). AFP can be fractionated into 3 isoforms: L1 is produced in non-neoplastic liver disease, L2 is produced in yolk sac tumors, and L3 is produced in HCC and hepatoblastoma[26]. The AFP fractions were examined in 3 PIVKA-Ⅱ-producing gastric cancer cases, and the L3 fraction was increased in all of those cases[18,23,25]. With regard to other tumor markers, serum carcinoembryonic antigen (CEA) levels are often increased.

Anemia is often observed because of malnutrition and/or bleeding from the tumor. Thrombocytosis and hypoproteinemia are occasionally observed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (gamma-GTP) levels are often increased because of frequent liver metastasis, and total and direct bilirubin levels are also occasionally increased. Serum C-reactive protein (CRP), white blood cell count, and erythrocyte sedimentation rate (ESR) are increased if the tumor is complicated by inflammation.

Endoscopy

The most important test in the clinical diagnosis of gastric cancer is upper digestive tract endoscopy. The features describing the gross appearance of PIVKA-Ⅱ-producing gastric cancer are presented in the PATHOLOGICAL FEATURES section.

Clinical imaging findings

Liver metastasis was observed in 13 (81%) of the 16 reported cases of PIVKA-Ⅱ-producing gastric cancer, and there were multiple metastatic foci in the majority of those cases. Metastatic tumors were commonly observed in most parts of the liver. Portal vein tumor thrombus was observed in 3 (19%) of the 16 cases; furthermore, lymph node metastasis was often observed.

By abdominal ultrasonography, metastatic liver foci usually present as an intermingled pattern of hyperechoic and hypoechoic areas. By abdominal computed tomography (CT), metastatic liver tumors are generally observed as low density areas. By contrast-enhanced CT, the marginal zone of metastatic liver tumors may be enhanced, but the internal portion is poorly enhanced (Figure 1). By angiography, arterial blood flow in the metastatic liver tumors is scarce. In patients with portal vein tumor thrombus, a filling defect is observed on portography.

PATHOLOGICAL FEATURES

Gross appearance

PIVKA-Ⅱ-producing gastric cancer occupies the gastric body and the pyloric antrum with almost the same frequency. One reported case presented with a 0-Ⅱc-type early cancer, and the remaining 15 cases presented with advanced cancer (Borrmann type 1, 4 cases; Borrmann type 2, 6 cases; and Borrmann type 3, 5 cases) (Figure 2). There were no Borrmann type 4 cases. The diameter of

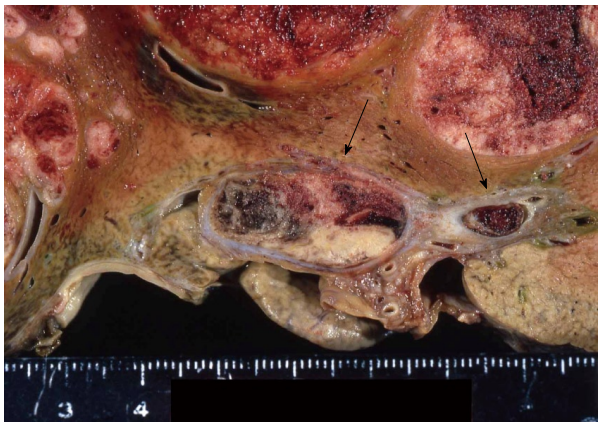

Figure 3 Gross appearance of liver metastasis of protein induced by vitamin K absence or antagonist Ⅱ-producing gastric cancer. Multiple nodular tumors and portal vein tumor thrombi (arrows) are observed.

the tumor ranged from 2-15 cm (mean, 6.5 cm). As mentioned above, multiple liver metastases are frequently detected in PIVKA-Ⅱ-producing gastric cancer, and the presence of portal vein tumor thrombus is not rare (Figure 3).

Microscopic findings

In many cases, gastric biopsy specimens have the histological appearance of poorly or moderately differentiated adenocarcinoma; however, a hepatoid pattern is also fre quently detected by the examination of operation or autopsy materials. Careful examination is required because it may be difficult to differentiate between the hepatoid pattern and poorly differentiated solid-type adeno carcinoma. The tumor generally shows a medullary growth pattern, and there have been no reports of the scirrhous type. Lymphatic and venous invasion is frequently observed, and venous invasion may be very conspicuous. The histological appearance of liver metastasis basically resembles that of a primary gastric tumor, but the hepatoid pattern may be more conspicuous. In a case that we reported[19], a gastric tumor in an area other than the venous invasion primarily demonstrated the histological appearance of a moderately to poorly differentiated adenocarcinoma (Figure 4A), while almost the entire intravenous part of the tumor and liver metastases showed the hepatoid pattern (Figure 4B, C). This finding may suggest that the hepatoid component is especially prone to venous invasion and metastasis to the liver.

Immunohistochemical staining

The primary gastric tumor and metastatic liver tumor show positive immunohistochemical staining for PIVKA-Ⅱ in the majority of the cases, and positive staining is most frequently observed in the area of the hepatoid structure. However, in a case that we reported, positive staining for PIVKA-Ⅱ was primarily observed in the area of the tubular structure[23] (Figure 5A). Cases with very few[19] or no[13,14] PIVKA-Ⅱ-positive tumor cells have also been reported. In both cases with no PIVKA-Ⅱ-positive tumor cells, a biopsy specimen was used for immunohistochemical staining, and the size of the sample may be the cause for the negative staining.

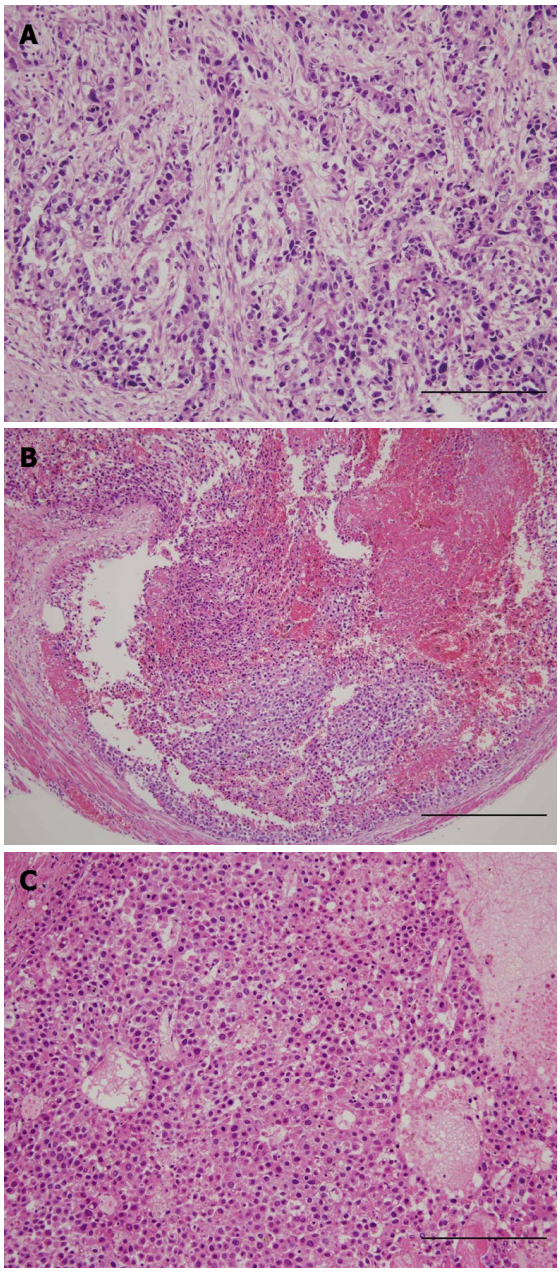

Figure 4 Microscopic appearance of protein induced by vitamin K absence or antagonist Ⅱ-producing gastric cancer. A: Gastric tumor with the appearance of a moderately to poorly differentiated adenocarcinoma (HE stain; the scale bar indicates 50 μm); B: Intravenous tumor with a hepatoid pattern (HE stain; the scale bar indicates 100 μm); C: Metastatic liver tumor with a hepatoid pattern (HE stain; the scale bar indicates 50 μm).

Immunohistochemical staining for AFP is also positive in the primary gastric tumor and metastatic liver tumor in the majority of cases (Figure 5B), and positive staining is most frequently observed in the area of the hepatoid structure. However, in a case in which a biopsy specimen was used for immunohistochemical staining, no AFP-positive tumor cells were detected[12].

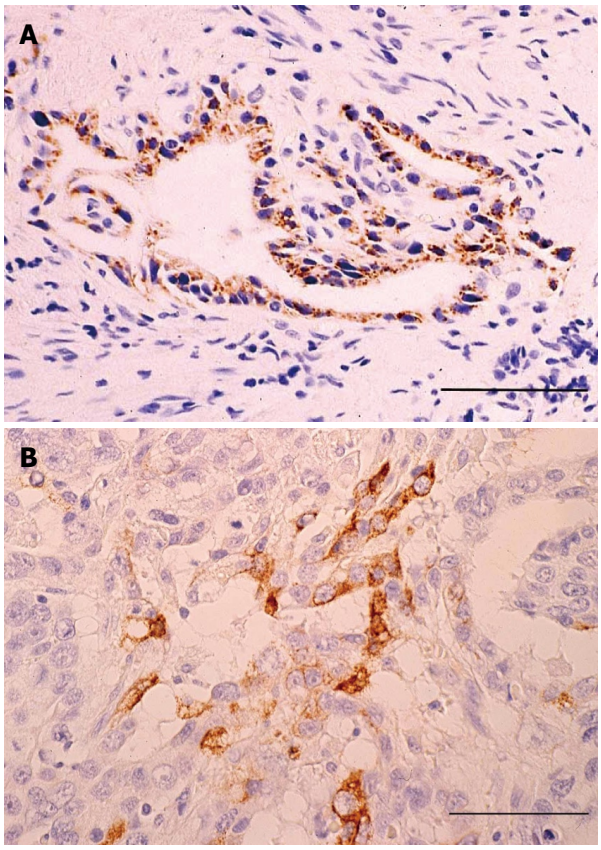

Figure 5 The results of immunohistochemical staining. A: The tumor cells forming glandular structure are positive for protein induced by vitamin K absence or antagonist Ⅱ; B: The tumor cells are also positive for alpha-fetoprotein. All scale bars indicate 25 μm.

DIFFERENTIAL DIAGNOSIS BETWEEN HCC AND LIVER METASTASIS OF PIVKA-Ⅱ-PRODUCING GASTRIC CANCER

It is often difficult to differentiate between HCC and liver metastasis of PIVKA-Ⅱ-producing gastric cancer because PIVKA-Ⅱ is a putative specific marker of HCC and PIVKA-Ⅱ-producing gastric cancer frequently metastasizes to the liver. HCC usually occurs in patients who are infected with the hepatitis virus and have liver cirrhosis or chronic hepatitis. Accordingly, when multiple liver tumors are found in a patient without an underlying liver disease, the possibility of metastatic liver tumor should be considered even if the serum PIVKA-Ⅱ levels are abnormally high, and systemic examination, including upper digestive tract endoscopy, should be performed. If gastric cancer is found in such a patient, histological examination by biopsy and immunohistochemical staining for AFP and PIVKA-Ⅱ is necessary. If a hepatoid pattern is found in the gastric tumor and the tumor cells are positive for AFP and PIVKA-Ⅱ, it is probable that the liver tumor is a result of the metastasis of PIVKA-Ⅱ-producing gastric cancer. However, it is noteworthy that a hepatoid pattern and positive staining for AFP and PIVKA-Ⅱ may not be confirmed in a biopsy specimen because of the limited size of the sample. It was recently reported that immunohistochemical staining for SALL4, a stem cell marker, was positive in all cases of AFP-producing gastric carcinoma and completely negative in HCC[27]. This marker may also be useful to differentiate between PIVKA-Ⅱ-producing gastric cancer and HCC, although further examination is required to confirm this. In addition to the above-mentioned histopathological examination, radiographic examination such as contrast-enhanced CT is also useful for differential diagnosis.

TREATMENT AND PROGNOSIS

Operations were performed on 3 cases without liver metastasis[10,21,24]. In one of those 3 cases, preoperative chemotherapy [low-dose cisplatin (CDDP) and continuous 5-fluorouracil (5-FU) i.v.] was administered[21]. The operative surgical procedures used were subtotal gastrectomy, pancreatico-spleno total gastrectomy, and distal gastrectomy with resection of the extra-gastric tumor according to the spread of the tumor.

Chemotherapy is the main treatment option for patients with liver metastasis, although gastrectomy was also performed in two cases[15,23]. Only best supportive care was performed in four cases with liver metastasis[11,14,16,19]. Systemic chemotherapy or hepatic arterial infusion chemotherapy, and usually, several anti-cancer drugs such as CDDP, 5-FU, and mitomycin C are used in combination; however, the effect is generally poor. Although the efficacy of TS-1, a novel oral derivative of 5-FU, has been reported for several cases of AFP-producing gastric cancer[28,29], long-term survival with TS-1 therapy has not been reported for PIVKA-Ⅱ-producing gastric cancer[22,25].

The prognosis largely depends on the existence of liver metastasis. All 3 patients without liver metastasis are alive without tumor at 9, 15, and 17 mo after surgery. Twelve of the 13 patients with liver metastasis died from their disease and the longest survival period of those patients was 6 mo. The cause of death in the majority of those cases was hepatic failure. The follow-up period for the only patient who was still alive with liver metastasis was only 2 mo[15].

MECHANISM OF PIVKA-Ⅱ PRODUCTION IN GASTRIC CANCER

Glutamic acid, located near the N-terminal of the prothrombin precursor is converted to gamma-carboxyl glutamic acid by vitamin K-dependent carboxylase, and PIVKA-Ⅱ is produced when this mechanism is disturbed. PIVKA-Ⅱ had been known to be produced in the absence of vitamin K or when a vitamin K antagonist is used, and PIVKA-Ⅱ has been regarded as a specific tumor marker of HCC since 1984 when Liebman *et al*[1] reported that it was increased in HCC patients. The increased production of the prothrombin precursor in tumor cells, abnormalities in vitamin K-dependent carboxylation, and vitamin K deficiency in tumor tissue have been spe-

culated to be the underlying mechanisms of PIVKA-Ⅱ production in HCC[30]. We speculate that gastric cancer produces PIVKA-Ⅱ *via* hepatocellular metaplasia because an HCC-like histological pattern is observed in many PIVKA-Ⅱ-producing gastric cancer cases, almost all PIVKA-Ⅱ-producing gastric cancer cases have increased serum AFP levels, and the L3 fraction of AFP was increased (HCC-like pattern) in all cases in which the AFP fractions were examined. The liver is derived from an outpouching of the foregut, as is the stomach, so it is not particularly surprising that the neoplastic gastric mucosa may sometimes differentiate into hepatic-type cells.

In addition, we speculate that many PIVKA-Ⅱ-producing gastric cancers occur initially as common gastric adenocarcinomas and that the hepatoid component arises during tumor progression, given that almost all cases are advanced cancers and the hepatoid pattern is most frequently observed in the deep invasive portion. Fujii *et al*[31] performed loss of heterozygosity analysis on AFP-producing gastric cancer using a panel of microsatellite markers, and concluded that AFP-producing carcinoma foci may evolve through genetic progression and/or genetic divergence. They also described that the silencing of a crucial gene on 13q may be involved in the acquisition of the AFP-producing phenotype. Further examination is required with regard to the genetic evolution of PIVKA-Ⅱ-producing gastric cancer.

COMPARISON BETWEEN AFP- AND PIVKA-Ⅱ-PRODUCING GASTRIC CANCERS

It has been reported that AFP-producing gastric cancer comprises 2.7%-5.4% of all gastric cancer[7,32]. According to a review of 270 cases of AFP-producing gastric cancer by Adachi *et al*[33], AFP-producing gastric cancer shows a male predominance (73%), predominant tumor location in the gastric antrum (57%), lymph node metastasis (83%), and liver metastasis (33%). Histologically, AFP-producing gastric cancer is classified as hepatoid adenocarcinoma-type or non-hepatoid adenocarcinoma-type, with the latter including fetal gastrointestinal tube-type and yolk sac-type[24]. Hepatoid adenocarcinomas are more invasive, especially to the small veins, than the non-hepatoid adenocarcinoma-type[24]. The prognosis for AFP-producing gastric cancer is worse than for conventional gastric cancer, and the 5-year survival rates for hepatoid and non-hepatoid AFP-producing carcinomas are 21.4% and 38.2%, respectively[8]. Thus, it seems that the clinicopathological features of PIVKA-Ⅱ-producing gastric cancer resemble those of AFP-producing gastric cancer, especially AFP-producing hepatoid adenocarcinoma, although the frequency of liver metastasis and portal vein tumor thrombus is higher and the prognosis is worse in PIVKA-Ⅱ-producing gastric cancer than in AFP-producing gastric cancer. The possibility that PIVKA-Ⅱ production is related to a worse prognosis has also been observed in HCC[34].

The mechanisms responsible for the production of AFP and PIVKA-Ⅱ in HCC appear to be independent[30,35], and HCCs produce either or both of AFP and PIVKA-Ⅱ. However, as mentioned above, PIVKA-Ⅱ-producing gastric cancer is far rarer than AFP-producing gastric cancer, and almost all PIVKA-Ⅱ-producing gastric cancers also produce AFP. The reason for this is unclear, but the following possibilities are conceivable: (1) AFP can be produced not only via hepatocellular metaplasia but also via retrodifferentiation to fetal gastrointestinal tract or yolk sac in gastric cancer; (2) hepatoid carcinoma of the stomach may possess different cytological characteristics from those of HCC; and (3) gastric cancer producing only PIVKA-Ⅱ might be overlooked because PIVKA-Ⅱ is not measured so frequently as AFP.

PIVKA-Ⅱ-producing cancers other than HCC and gastric cancer

Malignant tumors other than HCC and gastric cancer that produce PIVKA-Ⅱ have been reported although the frequency is extremely low. To date, PIVKA-Ⅱ-producing cancers in the lung[36,37], colon[38], adrenal cortex[39], ovary[40], and pancreas[41] have been reported. Serum AFP levels were increased in all of those cases. Immunohistochemically, all of those cases were positive for PIVKA-Ⅱ and all but one case were positive for AFP. Histologically, hepatoid structure was observed in all but one case. Liver metastasis was observed in 2 (33%) of the 6 cases. Four patients died and the median survival period was 14.5 mo. Two patients were alive for 4 and 48 mo respectively and liver metastasis was not present in either of those cases.

CONCLUSION

PIVKA-Ⅱ-producing gastric cancer is a very rare subtype of gastric cancer, and AFP is also produced in almost all cases. The hepatoid pattern is often detected histologically, and the production of PIVKA-Ⅱ by tumor cells is usually confirmed immunohistochemically. Liver metastasis and portal vein tumor thrombus are frequently observed, and almost all patients with liver metastasis die within 6 mo. Hepatocellular metaplasia of tumor cells is suggested to be the mechanism of PIVKA-Ⅱ production. Analysis of a larger number of cases is needed to clarify the clinicopathological features of this very rare subtype of gastric cancer.

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REVIEW

Ischemic post-conditioning to counteract intestinal ischemia/reperfusion injury

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Abstract

Intestinal ischemia is a severe disorder with a variety of causes. Reperfusion is a common occurrence during treatment of acute intestinal ischemia but the injury resulting from ischemia/reperfusion (IR) may lead to even more serious complications from intestinal atrophy to multiple organ failure and death. The susceptibility of the intestine to IR-induced injury (IRI) appears from various experimental studies and clinical settings such as cardiac and major vascular surgery and organ transplantation. Whereas oxygen free radicals, activation of leukocytes, failure of microvascular perfusion, cellular acidosis and disturbance of intracellular homeostasis have been implicated as important factors in the pathogenesis of intestinal IRI, the mechanisms underlying this disorder are not well known. To date, increasing attention is being paid in animal studies to potential pre- and post-ischemia treatments that protect against intestinal IRI such as drug interference with IR-induced apoptosis and inflammation processes and ischemic pre-conditioning. However, better insight is needed into the molecular and cellular events associated with reperfusion-induced damage to develop effective clinical protection protocols to combat this disorder. In this respect, the use of ischemic post-conditioning in combination with experimentally prolonged acidosis blocking deleterious reperfusion actions may turn out to have particular clinical relevance.

Key words: Acidosis; Intestinal ischemia/reperfusion injury; *In vivo* models; Ischemic post-conditioning

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INTRODUCTION

Reperfusion following ischemia (IR) causes severe injury (IRI) to the intestine that is life threatening. Here we overview methods to prevent or at least diminish these deleterious effects of IR. Special attention is being paid to ischemic post-conditioning (POC) with manipulation of the intracellular pH (pH_i). Ischemia occurs when an organ lacks sufficient blood supply as a result of, for example, shock, vascular disease or organ transplantation. Complete cessation of oxygenation for more than 20 min typically

results in irreversible organ damage causing cell death within hours[1]. The intestine is particularly susceptible to ischemia because its high rate of oxygen use renders it relatively incapable of increasing oxygen transport in the face of hypoxic stress. Intestinal ischemia can result from intestinal intussusception, acute mesenteric arterial occlusion, hemodynamic shock and bowel disease[1,2]. The compromising effects of ischemia concern various aspects of intestinal physiology including impaired capillary blood flow[3,4], acidosis[5], changed villus structure[6,7], increased mucosal permeability[8,9] and reduced mitochondrial activity leading to decreased NADPH production[10,11]. During the ischemia event, aside from inadequate oxygen supply that compromises mitochondrial oxidative phosphorylation, there is an accumulation of metabolites that, directly or through mediators, may lead to cellular injury[12]. Prolonged ischemia (10-12 h) causes the affected intestinal area to die which evokes wide-spread systemic adverse effects due to the intestinal release of toxic substances into the circulation that subsequently affects other organs like the heart, lungs, liver and kidney and eventually results in sepsis and multiple organ failure[13]. Therefore, acute intestinal ischemia can be a devastating disease with a high mortality rate, depending to some extent on the underlying cause: venous thrombosis 32%; arterial embolism 54%; non-occlusive ischemia 73%; and arterial thrombosis 77%[14].

Paradoxically, restoration of blood flow by reperfusion may intensify rather than decrease organ damage ("oxygen paradox")[15] depending on the duration and intensity of the ischemia and on the timing of oxygen reintroduction to the tissues[1,16,17]. The intestine is, with the heart, lungs, brain and kidney, among the organs most sensitive to IR. In the intestinal mucosa, IR induces damage that is characterized by altered microvascular and epithelial permeability as a result of complex interactions between the endothelium and various cell types and cellular necrosis and/or apoptosis of villous cells[18]. In this injury process, activation of neutrophils, mast cells and platelets and increased release of endothelial factors are involved[19,20]. Cytokines such as TNF-α, IL-1 and IL-6 and oxygen free radicals are assumed to be important pathogenic mediators in IRI, as is capillary no-reflow[1,21]. A neurotransmitter believed to be released from the injured intestine and playing a main role in the aggravation of intestinal IRI is serotonin which controls intestinal movement, platelet activity and vasoconstriction[22].

As a result of reperfusion, the injured intestine may increase the release of toxic substances into the circulation that subsequently cause sepsis and multiple organ failure[23-25]. Eventually, IR may cause loss of mucosal barrier function, bacterial translocation and strong activation of inflammatory responses leading to endothelial destruction[23,26]. This inflammatory aspect of IR includes both cellular and humoral components and increasing evidence highlights the role of leukocytes and leukocyte adhesion molecules in intestinal IRI[27,28].

For some time, necrosis has been considered to be the main effect of ischemia on intestinal epithelial cells but, to date, apoptosis seems to be the principal contributor to IR-induced cell death[29]. The main executors of this "programmed cell death" are the endoprotease cysteines called caspases[30].

TREATMENTS TO COUNTERACT IRI

For the reasons given above, there is a strong and increasing interest in understanding and counteracting the damaging effects of IR on the intestinal mucosa. Various approaches to diminish the deleterious consequences of IR have been tested in animal models and *in vitro*. Many of these involve pretreatment (before the start of experimentally induced ischemia) with exogenous substances to interfere with the various processes that underlie the IRI syndrome such as intracellular signaling pathways, free radical dynamics and inflammation. The vast number of drugs tested in such pretreatment studies is steadily increasing and include anti-cytokine-induced neutrophil chemoattractant antibody[31], propofol[32], curcumin[33], NMDA receptor antagonists[34], carnitine[35], peroxisome proliferator-activated receptor-gamma ago nist[36] and erythropoietin[37]. Although these pre-treatment studies do not have immediate clinical applications, they have increased our understanding of the IRI disease process. This is exemplified by pretreatment studies with nitroglycerin as follows. Due to its strategic location at the luminal surface of vessels, the vascular endothelium is particularly sensitive to IR. Endothelial functioning is impaired by the sudden increase in oxygen free radical species upon reperfusion. Paradoxically, free radicals (including oxygen free radicals and nitric oxide) are also involved in the protective process of ischemic preconditioning whereby a given stimulus increases tissue tolerance to IR damage[38]. Interestingly, it has been shown in both human and animal studies[39,40] that nitroglycerin can induce a protective phenotype that limits tissue damage by IR. It appears that nitroglycerin protects the endothelium against post-ischemic endothelial dysfunction via a mechanism that is mediated by oxygen free radical release and opening of mitochondrial permeability transition pores[41].

However, no pretreatments have found clinical application yet because treatment should be initiated shortly before the onset of ischemia, a moment that, obviously, cannot be precisely anticipated in a clinical setting. For this reason, attention is being increasingly focused at other protective treatments of the intestine, namely directly after ischemia, of which we will summarize the most characteristic ones below.

Melatonin, applied intraperitoneally in rat at the start of reperfusion, appears to exert a strong antioxidant effect that prevents intestinal IRI in a dose-dependent manner[42] and the administration before mesenteric reperfusion of allopurinol, a xanthine-oxidase inhibitor[43], offered protection against IRI as well. Although use of allopurinol to inhibit xanthine oxidase prior to intestinal reperfusion is utilized by some clinicians, substantial clinical evidence to support this practice has not been generated. Intestinal

regional hypothermia applied during mesenteric ischemia reduced the pro-inflammatory responses induced by IR[44]. Also, whereas no significant effect was found on microvascular barrier function during reperfusion in dogs, it has been claimed that hypothermia does protect the rat intestine against IRI. Apparently, this protection is associated with diminished NF-kB activity, induction of iNOS and expression of heme oxygenase-1[45]. After mesenteric IR, moderate hypothermia may have beneficial effects on the intestine, heart and liver[2].

Reperfusion of ischemic tissue is generally associated with intense inflammation-mediated tissue injury. In animal models, the *in vivo* anti-inflammatory actions of physalins, natural steroidal compounds, appear to be mostly due to the activation of glucocorticoid receptors[46]. Following IR, dexamethasone and physalin B and F markedly prevented neutrophil influx and increased vascular per meability in the intestine and the lungs. Moreover, hemorrhage was prevented in the intestine of reperfused ani mals. Dexamethasone and physalins effectively suppressed the increase in organ (intestine and lungs) and serum concentrations of TNF-alpha. Interestingly, treatment with these compounds was associated with enhancement of IL-10. The anti-inflammatory effects of dexamethasone and physalins were reversed by pretreatment with the corticoid receptor antagonist RU486. Therefore, the *in vivo* anti-inflammatory actions of physalins, natural steroidal compounds, appear to be mostly due to the activation of glucocorticoid receptors. Compounds derived from these so-called secosteroids may represent novel therapeutic options for the treatment of inflammatory diseases including IRI[46].

Another way to reduce neutrophil infiltration during IRI has been pursued by luminal treatment with special amino acid-based solutions of rodent small bowel throughout reperfusion after 60 min ischemia. This reduced neutrophil infiltration while electrophysiology and histology revealed good preservation of mucosal structure and barrier function[47]. Finally, post-treatment with simvastatin, which has anti-inflammatory and antioxidant actions, resulted in a significant increase in bowel and mucosal weight in ileum and in villus height and crypt depth in jejunum and ileum in rat. Moreover, simvastatin reduced intestinal injury score as well as the apoptosis index, indicating that this drug inhibits programmed cell death following intestinal IR[48].

Unfortunately, up to now these animal studies have not resulted in protocols that can be reliably applied in the clinic. Below we will consider the implications of the particularly interesting recent finding that brief intermittent episodes of ischemia and reperfusion after a prolonged period of ischemia could reduce the deleterious effects of IR on the intestine, a phenomenon called ischemic "post conditioning" (POC).

ISCHEMIC POSTCONDITIONING AND pH_i

Post-conditioning has been shown to protect against various pathological events including necrosis, apoptosis and microvascular injury[49]. More specifically, it attenuates IRI in the heart, spinal cord, brain, kidney, liver, muscle and lung in the experimental setting[50,51]. Recently, POC was tried as a protection paradigm against IRI in the intestine[52,53]. However, semiquantitative histopathological evaluation and measurement of wet-to-dry weight ratios did not reveal a significant difference between the ischemic and post-conditioned rabbit intestine in the degree of necrosis, tissue wet-to-dry weight ratios or blood flow[54], a negative result casting doubt on the potential efficacy and reliability of POC applications in the clinical setting. These ambiguous effects of POC on the animal intestine may be due to the high complexity by which POC changes ischemic tissue including delaying realkalinization of tissue pH, triggering release of auta coids, modulating the activity of ion channels and activa ting kinases[51]. Since these processes together may act on multiple cellular and molecular targets and may severe ly affect intestinal functioning. However, at the same time, these multiple actions of POC differ from the monotherapy approach by drugs that have failed to consistently reduce IRI and therefore might be promising in human trials provided that current POC protocols are adequately improved[51]. Such an improvement might be obtained by strengthening the delaying action of POC on realkalinization by combining POC with prolonged acidosis. This approach appears to substantially limit heart infarct size in animal models[41,55], raising the possibility that this modification of the POC protocol might also be effective in blocking the deleterious effects of IR in the (human) intestine. Lowering the pH of the reperfusion medium might prevent activation of Na^+/H^+ exchange processes, as was shown for reperfusion of ischemia-exposed astrocytes[56] and heart[57]. This notion opens avenues to protocols preventing or curing IRI. Therefore, here we will pay attention to the way the intracellular pH (pH_i) is controlled by cellular factors and can be manipulated *in vivo*.

The pH_i is essential for maintaining cellular homeostasis in the intestinal mucosa. Even small changes in pH_i (less than 0.1 units) may alter ion channel properties and depress the activity of key enzymes involved in glycolysis and ATP synthesis. Consequently, mucosal cells possess homeostatic mechanisms to stabilize their pH_i. If such mechanisms become impaired by prolonged ischemia, cell death will be the result. As we have shown for the mouse jejunum[58], cellular inactivation and subsequent cell death are concomitant with intracellular acidosis. Consequently, restoration of the pH_i by reperfusion will help intestinal epithelial cells to recover. However, this recovery will only take place when ischemia-induced acidosis is not too severe; otherwise acidosis will remain and reperfusion will cause cells to die[58]. This condition of recovery might be improved by experimentally intervening with the pH_i.

A particular role in the maintenance of the pH_i is played by short-chain fatty acids (SCFAs) produced by the bacterial flora in the intestinal lumen and by the various types of Na^+/H^+ exchanger protein (NHEs) res-

ponsible for controlling pH_i homeostasis during the continuously changing luminal environment. SCFAs include N-butyrate, acetate, propionate and isobutyrate and induce acidification of the rat colon, probably by non-ionic diffusion and other means of cellular absorption of the acid moiety[59]. After exposure to acetate, propionate or n-butyrate, all colonocytes acidify rapidly and over 90% reveal pH_i alkalinization[60]. NHE proteins exchange extracellular sodium ions for intracellular protons with a 1:1 stoichiometry. Identified functions of NHE include regulation of the pH_i, in particular during recovery from an acid load, maintenance of cell volume in response to an osmotic load and transepithelial Na^+ absorption[61,62]. Out of the 10 known NHE isoforms, NHE2 and NHE3 are present in the brush border of the duodenum[63-65], in the apical membrane of villus cells of the small intestine and in surface cells of the colon. NHE1 is found in the basolateral membrane of epithelial cells in all intestinal segments[66]. A primary role of mammalian NHE is to regulate the cytosolic pH. NHEs are activated by decreased pH_i and upon activation raise the pH_i to normal value. The ubiquitous NHE1 is a major regulator of pH_i. Through its coordinate functions in H^+-efflux, actin anchoring and scaffolding, NHE1 is assumed to promote protein activities and interactions, assembling signaling complexes in specialized plasma membrane domains and coordinating divergent signaling pathways[67]. NHE3 is an important contributor to the regulation of pH_i, Na^+ and water homeostasis of the organism as it catalysis Na^+ and fluid (re)absorption across epithelia. NHE3-null mice show a disturbed acid-base balance[65].

MECHANISM OF POC WITH PROLONGED ACIDOSIS

The mechanism by which POC with prolonged acidosis would protect organs against IRI is largely unknown. In the rat heart, the beneficial effect of delaying the recovery of pH_i during reperfusion is possibly due to inhibition by low pH of the activity of calpain[55], the calcium-binding protein that leads to cardiac contractile dysfunction following ischemic insult. Whereas IR causes endothelial dysfunction, reoxygenation at low pH (6.4) enhances the recovery of acetylcholine-induced vasorelaxation, an improvement of endothelial functioning that likely acts through preservation of cGMP signaling[68]. Recently, a comparative proteomics approach revealed that the IR-protective action of POC on the rat intestinal mucosa involves the expression of proteins functionally concerned with cellular energy metabolism, anti-oxidation and anti-apoptosis[69,70].

Therefore, lowering the pH_i during POC might protect against the deleterious effects of intestinal IR. In view of this latter data, it might be investigated if SCFAs, as natural regulators of the pH_i and inhibitors of NHEs[71], could be used to fine-tune the pH_i of intestinal epithelial cells as a component of POC.

In the heart, recovery from ischemia is enhanced by reintroducing blood flow through repeated intermittent occlusions and reperfusions. The mechanism by which this "IR cycling" protects against IRI is not known but it has been supposed that the intermittent ischemia prevents formation of mitochondrial permeability transition pores by maintaining an acidic myocardial pH_i for several minutes until survival kinases can be activated[41]. This is another argument for assuming that regulating the pH_i may be of importance in preventing IRI.

CONCLUSIONS AND PERSPECTIVES

IRI is a major clinical problem because of the sequelae of a number of clinical conditions. To protect against IRI, some compounds successfully tested in animals and *in vitro* systems might give rise to the development of novel IRI therapeutics but most of them may not have substantial clinical relevance as they have to be administered well before ischemia has started. Therefore, post-ischemic treatments seem to be more promising. Among these, of the various approaches under study, the most promising one may be POC, especially when combined with manipulation of the pH_i. Since POC with prolonged acidosis was recently shown to protect the heart against IRI, it would be interesting to use an intestinal *in vivo* model and real-time imaging techniques to study the action of POC on intestinal structure and functioning[58,72].

In general, the development of successful POC protocols would strongly benefit from better knowledge about the molecular mechanisms of action and optimal ways of the application of POC. For some organs, such information is now becoming available. Ischemic POC probably reduces myocardial apoptosis by increasing BCL-2 protein expression via activation of opioid receptors and the JAK-STAT signaling pathway[73] and neuroprotective POC action on the spinal cord seems to involve phosphatidylinositol 3-kinase and ERK pathways[74]. Furthermore, cardioprotective effects of POC depend critically on the duration of reperfusion and reocclusion episodes[75]. More information of this kind may support the development of sophisticated POC protocols in which fine-tuned periods of intermittent ischemia and reperfusion are combined with drugs that have proven their potential use in pretreatment studies and act specifically on molecular POC targets at the proper cell physiological conditions (pH, temperature *etc*).

Another interesting perspective offered is the intriguing fact that transient non-lethal IRI of one organ confers resistance to a subsequent episode of lethal IRI in a remote organ ("remote preconditioning")[76]; a POC protocol successfully applied to the intestine might exert remote, protective effects on other organs such as the heart, lungs, kidney and brain, finding widespread clinical application in protecting the whole body against the devastating effects of ischemia and IR.

Finally, while emphasis in this review has been placed on the potential protective effects against IRI of POC, especially in combination with pH_i manipulation, a quite

different, but nevertheless promising, approach to solve at least partly the IRI problem is worth mentioning. As inflammation is a main aspect of IRI, controlling the attraction of leukocytes would be a clinically highly relevant tool in counteracting the damaging effects of IRI. Such control could be effectuated by pharmacological manipulation of Toll-like receptors (TLRs) which regulate the organism's defense against infections and sense host tissue injury by recognizing products of dying cells. Better understanding of TLR involvement in IRI may enable the invention of novel TLR-based therapies for IRI in the intestine[77,78].

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CASE REPORT

Granulocytic sarcoma of the rectum: Report of one case that presented with rectal bleeding

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Abstract

Granulocytic sarcoma is an uncommon and localized extramedullary tumor composed of immature granulocytic cells. It may present in association with acute myeloid leukaemia, myelodysplastic syndrome and chronic myelogenous leukaemia. Granulocytic sarcoma may occur in any anatomical site but involvement of the gastrointestinal tract is rare, especially in the rectum. We report on the case of a 17 year old female who presented with rectal bleeding, abdominal pain and weight loss one mo prior to admission. Rectosigmoidoscopy revealed a rectal polypoid and ulcerated mass. The histological examination of the mass showed granulocytic sarcoma. Bone marrow examination was compatible with acute promyelocytic leukaemia (FAB type M3). This case report is a reminder of this peculiar sign of tumoral syndrome in acute myeloid leukaemia. We also discuss diagnostic methods and analyze the disease course.

INTRODUCTION

Granulocytic sarcoma (GS), also known as one variant of myeloid sarcoma in the World Health Organization classification, is an extramedullary solid tumor composed of myeloid precursor cells. The World Health Organization classification of hematopoietic tumors divides myeloid sarcoma into 2 major categories. The more common form is GS, composed mainly of myloblasts, neutrophils and myeloid precursors. The less common form is monoblastic sarcoma[1]. This tumor occurs commonly in patients with acute myeloid leukaemia (AML) and less commonly in those with myelodysplastic syndrome or chronic myeloproliferative disorders. Incidence of GS varies from 2% to 7% in AML patients and most frequently occurs in acute myeloblastic leukaemia with maturation [French-American-British (FAB) M2][2,3]. However, other subtypes including FABM4 or M5 and

M7 have also been described[3]. GS can occur in any organ but the most common sites are the skin, bone/spine and lymph nodes[1]. However, gastrointestinal tract involvement is relatively rare, especially in the rectum. We present a case of rectal GS in a patient with AML (FAB type M_3) who presented with rectal bleeding.

CASE REPORT

A 17 year old female was admitted in July 2005 to our department of Internal Medicine complaining of rectal bleeding, abdominal pain especially located in the left lower quadrant and loss of appetite one month prior to admission. She had no significant medical history. On admission, she was in a stable clinical condition with normal vital signs but looked pale. No hepatosplenomegaly, lymphadenopathy, petechiae or ecchymotic areas were noted. Rectal examination confirmed the presence of blood in the stools and revealed a polypoid and ulcerated mass measuring 2 cm in great diameter budding through the anal verge.

A full blood count showed a hemoglobin of 60 g/L, hematocrit of 20%, white cell count of 4×10^9/L with 20% of blasts and platelet count of 49×10^9/L. Blood chemistry profile showed levels of total protein of 53 g/L, serum albumin of 25 g/L and lactate dehydrogenase of 743 IU/L. Bone marrow examination revealed hypercellularity with an excess of myeloblasts (80%) and markedly depleted hematopoietic cells. The blasts were large with large and irregular nuclei, small basophilic cytoplasm and several azurophilic granules inside. Auer rod bodies and faggot formation were noted in some myeloblasts. Karyotype of the bone marrow cells was 46XX, t(15;17)(q22;q21). The diagnosis was acute promyelocytic leukaemia (FAB type M_3). Rectosigmoidoscopy revealed a rectal polypoid and ulcerated mass measuring 2 cm in great diameter budding through the anal verge (Figure 1). Biopsy specimens of the mass showed a massive infiltration of the rectal mucosa by large atypical myeloid cells. Their cytoplasm was eosinophilic and granular. Nuclei were round or convoluted with fine chromatin and one or more fine nucleolus (Figure 2). In addition, lymphocytes, plasma cells and neutrophils were also present. Immunohistochemical stains showed that the infiltrating cells were reactive for myeloperoxidase (Figure 3), lysozyme and CD43. T- and B- cells markers (CD3, CD45) as well as CD30 were negative. These features confirmed the diagnosis of a GS infiltrating the rectum.

The patient received chemotherapy according to the AIDA protocol (All- trans Retinoic acid (ATRA) + Idarubicin). Induction treatment consisted of oral ATRA of the dose of 45 mg/m^2 per day associated to intravenous idarubicin at the dose at 12 mg/m^2 per day on d 2, 4, 6 and 8 until complete remission. Bone marrow examination at 1 mo follow-up revealed no blast cells and rectosigmoidoscopy showed no mass in the rectum. The patient tolerated the treatment well. After 2 wk of complete remission, treatment was consolidated with 3 courses chemotherapy. At the 4 year follow-up, the patient remained in complete clinical, cytogenetic (karyotype of the bone marrow cells was 46xx) and molecular (transcript PML/RARA was negative) remission.

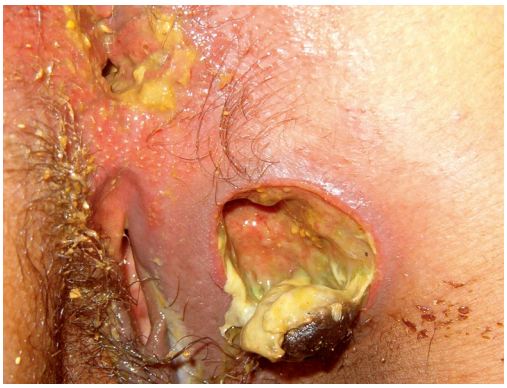

Figure 1 A rectal polypoid and ulcerated mass budding through the anal verge.

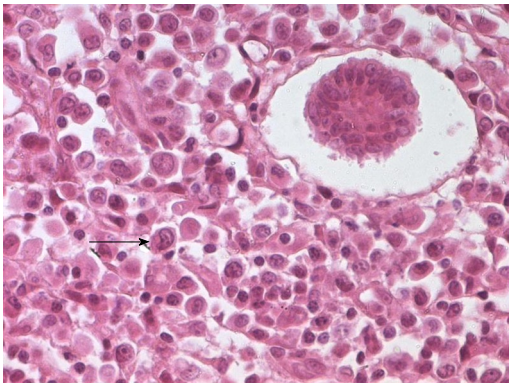

Figure 2 Tumoral cells with eosinophilic cytoplasm, round or convoluted nuclei with fine chromatin and one or more fine nucleolus (HE stain × 400).

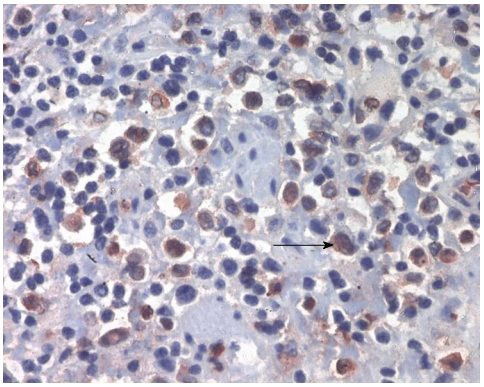

Figure 3 Cytoplasmic expression of myeloperoxydase (brown staining × 400).

DISCUSSION

GS represents an extramedullary tumor of myeloblasts and /or immature myeloid cells. It was first described by Burns in 1811 and given the name chloroma in 1853 by King because of a characteristic greenish color caused by

myeloperoxidase in the tumor cells[4]. This unusual tumor can present in four clinicopathological situations: firstly, an additional manifestation in a patient known to have leukaemia; secondly, as a sign of AML in a non leukemic patient at presentation as in the present case; thirdly, as a sign of impending blast crisis in chronic myeloid leukaemia or leukemic transformation in myelodysplastic disorders; and fourthly, as an isolated event[5]. GS involves any site but most commonly affects the skin (13%-22%), bone/spine (9%-25%) and lymph nodes (15%-25%)[3]. Involvement in the gastrointestinal tract is relatively rare and occurs mostly in the small bowel. However, involvement of the colon and rectum is exceedingly rare. To our knowledge, only five patients with isolated colonic and three with rectal granulocytic sarcoma have been described in the literature[6-8].

The most common presentation of GS is abdominal pain, change in bowel habit, small bowel obstruction and less commonly, hemorrhage[9]. In a patient with AML, gastrointestinal bleeding may result from the complication of thrombocytopenia or other coagulopathy disorders caused by leukaemia, especially in FAB M_3 type. But this can also be the consequence of infiltration of the gastrointestinal tract by GS.

GS may range from well differentiated tumors on histological examination to those with virtually no evidence of myeloid differentiation. Immunohistochemical study can increase the accuracy of the diagnosis[8]. In fact, the chloroacetate esterase, lysozyme, CD34 and myeloperoxidase stain confirm the granulocytic nature of the tumor cells[7]. In our patient, tumor cells were immunohistochemically positive for lysozyme, myeloperoxidase and CD43. B-cell and T cell lymphomas were excluded by negative stains for CD3 and CD20. The chromosome rearrangement in our patient was t (15;17) which has not been reported with GS previously in the literature. GS has been treated with systemic chemotherapy, surgical resection and radiation therapy. Although only a few large series comparing treatment modalities of GS are available in the literature, systemic chemotherapy seems to offer the most benefit[2]. In our patient, clinical symptoms and GS improved 3 wk after chemotherapy.

In conclusion, GS of the large intestine has seldom been described; this involvement is usually asymptomatic but patients may present with acute abdominal pain, change in bowel habit and rectal bleeding. In addition to thrombocytopenia, rectal involvement of GS must be considered in a patient with AML who presents with rectal bleeding.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
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The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic Surgery

April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
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June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

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Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

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10th OESO World Congress on Diseases of the Oesophagus 2010

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International Liver Association's Fourth Annual Conference

September 11-12
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New Advances in Inflammatory Bowel Disease

September 12-15
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ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
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October 07-09
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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]

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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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