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REVIEW

Differential role of Hedgehog signaling in human pancreatic (patho-) physiology: An up to date review

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Abstract

Since the discovery of the Hedgehog (Hh) pathway in drosophila melanogaster, our knowledge of the role of Hh in embryonic development, inflammation, and cancerogenesis in humans has dramatically increased over the last decades. This is the case especially concerning the pancreas, however, real therapeutic breakthroughs are missing until now. In general, Hh signaling is essential for pancreatic organogenesis, development, and tissue maturation. In the case of acute pancreatitis, Hh has a protective role, whereas in chronic pancreatitis, Hh interacts with pancreatic stellate cells, leading to destructive parenchym fibrosis and atrophy, as well as to irregular tissue remodeling with potency of initiating cancerogenesis. *In vitro* and *in situ* analysis of Hh in pancreatic cancer revealed that the Hh pathway participates in the development of pancreatic precursor lesions and ductal adenocarcinoma including critical interactions with the tumor microenvironment. The application of specific inhibitors of components of the Hh pathway is currently subject of ongoing clinical trials (phases 1 and 2). Furthermore, a combination of Hh pathway inhibitors and established chemotherapeutic drugs could also represent a promising therapeutic approach. In this review, we give a structured survey of the role of the Hh pathway in pancreatic development, pancreatitis, pancreatic carcinogenesis and pancreatic cancer as well as an overview of current clinical trials concerning Hh pathway inhibitors and pancreas cancer.

Key words: Pancreatic cancer; Hedgehog; Pancreatitis; Pancreas; Development

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Core tip: The Hedgehog (Hh) pathway is a ligand-dependent and evolutionary conserved cellular signaling mechanism with various physiologic (development) and pathogenetic functions (especially carcinogenesis). Concerted Hh signaling is essential for human pancreatic development and homeostasis of the gastrointestinal tract. Aberrant expression within the Hh signaling pathway results in malformations like annular pancreas. The Janus aspect of Hh in pancreatitis is reflected by the protective role of Hh in acute pancreatitis *vs* the disease-progressive function of Hh in chronic pancreatitis (CP), whereby CP is linked to pancreatic cancerogenesis *via* pancreatic intraepithelial neoplasia (PanIn). Starting with PanIn and ending up at metastatic disease, Hh pathway is expressed in ductal pancreatic cancer thereby influencing and being paracrine influenced by the tumor microenvironment.

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INTRODUCTION

Hedgehog (*Hh*) genes were originally identified in drosophila melanogaster as regulators of body patterning during embryonic development[1]. Today it is known that the Hh pathway plays a central role in diverse biological processes in mammals, such as embryonic development, cell proliferation, differentiation, tissue repair and maintenance of stem cell status in the adult[2].

In general, activation of the Hh pathway relies on the binding of a secreted ligand to its receptor. Three ligand homologues are known in mammals: Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*) and Sonic hedgehog (*Shh*). The ligands are produced as precursors and are secreted after extensive modifications to bind to their membrane bound receptor, called Patched. In mammals, two homologues exist, Patched1 (Ptch1) and Patched2 (Ptch2). After signal transduction *via* the co-receptor Smoothened (Smo), the executing transcription factors of the Hh pathway are the Gli proteins, of which three homologues are known in mammals: Gli1, Gli2 and Gli3[3]. Using a simplified model, the canonical Hh signaling can be described as follows[2,4]: In the absence of a Hh ligand, Ptch inactivates Smo - probably by preventing its localization into the primary cilium, a cell organelle that is thought to be essential for proper Hh signaling[5,6]. As a consequence, the Gli proteins are processed in such a way that they act as transcriptional repressors of the Hh target genes. However, upon binding of the Hh ligand to the receptor Ptch, inactivation of Smo is ended, allowing Smo to translocate to the primary cilium and initiate a cascade of events that ultimately lead to the conversion of Gli factors into their active form. The latter then shuttle into the nucleus and enable transcription of Hh target genes, including components of the pathway itself, such as *Ptch* and *Gli1,* indicating a built-in feedback loop within the Hh signaling cascade[2]. In addition to the "classical and canonical" Hh signaling described above, also non-canonical (Gli-independent), non-classical (ligand-independent) and aberrant Hh signaling (driven by activating mutations) have been identified at different stages of carcinogenesis (Figure 1)[7].

The pancreas is a fundamental organ of the digestive system with specialized endocrine and exocrine functions. The acinar cells within the exocrine pancreatic compartment produce and secrete numerous digestive enzymes into the duodenum. In the endocrine compartment, specialized cells produce hormones and directly release them into the blood stream - most importantly to control and regulate the blood glucose concentration. It is known from previous studies that physiologic Hh pathway signaling is crucial for correct development of the pancreas[8,9]. With this review, we give an overview of the current understanding of the role of Hh signaling in pancreatic development, cell differentiation and functional specialization. In a second part, the pathomechanistic implications of deregulated Hh signaling are discussed for the clinically most important pancreatic pathologies.

Hh SIGNALING IN HUMAN PANCREATIC DEVELOPMENT

Development of the pancreas

Pancreatic development is based on: (1) The fusion of two evaginations of the foregut to one single organ; and (2) endodermal growth by dichotomy branching. According the classical Carnegie stages[10,11], in stage 13, the dorsal pancreatic bud arises at first as a thickening of the endodermal tube, which proliferates, into the dorsal mesogastrium. In close, in stage 14, the ventral pancreatic bud evaginates to the liver primordium. As a result of differential growth of the duodenum, which rotates 90 degrees clockwise and becomes "C"-shaped, the ventral pancreatic bud comes to lie below and behind the dorsal pancreatic bud in stage 15. Until stage 17, both pancreatic buds have fused: The ventral pancreatic bud forms the posterior part of the head and the posterior part of the uncinated process, whereas the rest of the pancreas is formed by the dorsal pancreatic bud (the anterior part of the head, the body and the tail). Failure of the ventral pancreatic bud to migrate will result in an annular pancreas with consequent duodenal stenosis[12]. The main pancreatic duct (of Wirsung) is formed by the fusion of the distal part of the dorsal pancreatic duct and the entire ventral pancreatic duct and enters the duodenum combined with the bile duct at

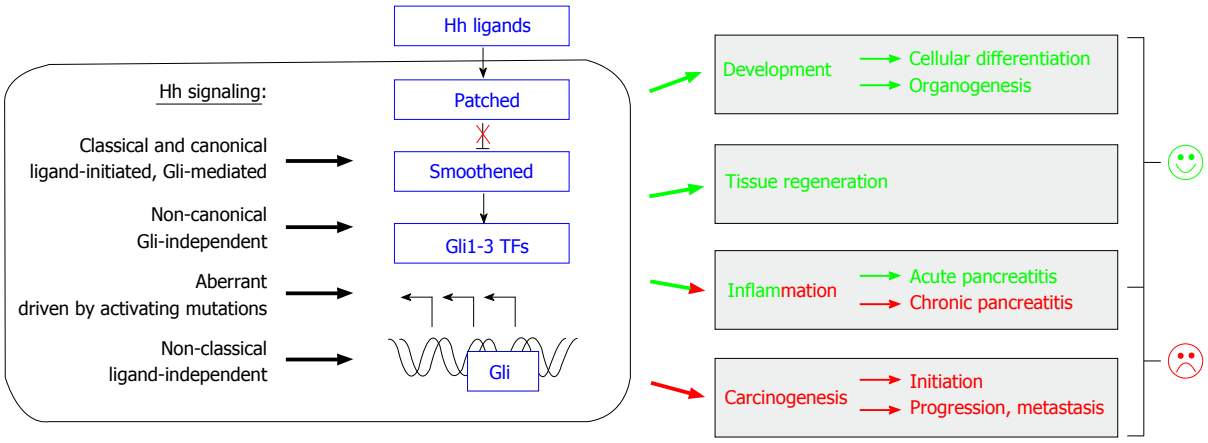

Figure 1 Overview of the Hedgehog signaling cascade and different activation modes (for details see ref. [7]). As described in detail in chapters Ⅱ to Ⅳ, the Hh pathway exerts positive and negative (labeled with green and red color, respectively) functions during development, regeneration, inflammation and cancerogenesis in the pancreas. Hh: Hedgehog; TF: Transcription factors.

the major papilla. Until the postnatal period, the proximal portion of the dorsal pancreatic duct either obliterates or persists as an accessory duct (of Santorini), entering the duodenum at the minor papilla (10% adults), so-called pancreatic divisum.

Cellular development of the pancreas

Differentiation and early specification of pancreatic endoderm is induced by fibroblast growth factor 2 and activin [a transforming growth factor beta (TGF-β) family member], both produced by the notochord and endothelium of the dorsal aorta. Both repress the expression of the transcription factor *Shh* locally in the gut endoderm, destined to form the dorsal pancreatic bud. Endoderm lying caudally to the pancreatic region does not respond to those signals[13]. The ventral bud is induced by upregulation of the pancreatic and duodenal homeobox 1 *(PDX1)* gene from the splanchnic mesoderm.

From 10th to 15th week, the primitive endodermal ductal epithelium provides the stem cell population for all the secretory cells, which are initially located in the duct walls or in the buds, from which they arise. Islet differentiation proceeds in two phases[13]: Phase Ⅰ (9th-15th week) is characterized by the proliferation of polyhormonal cells, whereas the differentiation of monohormonal cells is seen from week 16 onwards, referred to as phase Ⅱ. Later, these endocrine cells accumulate in pancreatic islets (of Langerhans) and scatter throughout the pancreas, starting with insulin and amylin secretion by β-cells approximately at the 5th month until neonatal period. The dorsal bud gives rise mostly to α-cells, which produce glucagon; however, most of the pancreatic polypeptide producing γ-cells develop from the ventral bud. After week 30, somatostatin-producing δ-cells are seen. The remaining primitive duct cells will either differentiate into definitive duct cells with microvilli and cilia or into acinar cells in which zymogen granules or acinar cell markers can be detected at weeks 12-16[13].

Correct ductal branching pattern and formation of acinar structures is determined by pancreatic mesenchyme which gives rise to connective tissue between the ducts resulting in pancreatic proliferation and maintaining the relative proportions of acinar, α-and β-cells. Additionally, it provides cell lines for smooth muscle within the pancreatic tissue, and angiogenic mesenchyme produces blood and lymphatic vessels.

Molecular regulation of pancreatic development by Hh signaling

Pancreas development is regulated by the activation/inactivation of Hh signaling members, which are ex-/repressed either within pancreatic tissue (*e.g., Ihh*) or in adjacent tissue (*e.g., Shh*)[14]. Initial absence of *Shh* signaling is required for regular pancreatic development, because ectopic expression of *Shh* leads to transformation of pancreatic mesoderm into intestinal mesenchyme in mice[15]. In single mutant mice (*i.e.*, *Shh*$^{-/-}$ or *Ihh*$^{-/-}$), gastrointestinal defects of the developing endoderm like annular pancreas or other malformations have been reported, suggesting similarities to human gut malformations[8,16].

It was shown that the graded response to Hh-signaling controls regular pancreatic development in mice, in which Hh signaling occurs at low levels during early organogenesis to ensure the correct establishment of organ boundaries and tissue architecture, and is up-regulated at later developmental stages to promote proliferation and maturation of the tissue[9,17-19]. Nielsen *et al*[20] confirmed the suggested concerted Hh signaling also in human pancreatic organogenesis: In early pancreatic development (7.5 wk), Gli3 was highly expressed in developing pancreatic ducts - while Smo and Gli2 were absent. In contrast, Smo and Gli2 were highly expressed between weeks 14 to 18, whereas the expression of Gli3 was reduced.

PDX1 (a pancreatic-promoting transcription factor; syn.: Insulin promotor factor 1) is also expressed in the preduodenal endoderm - including the sites of dorsal and ventral pancreatic bud formation. Total absence of the

pancreas is observed in homozygous *PDX1* mutant mice that suggest that *PDX1* is necessary for the formation of the pancreas and may be essential in the differentiation of pancreatic precursor cells[21,22]. Although all of the involved downstream effectors of human pancreas development have not been determined in detail yet, it appears that expression of the paired homeobox genes *PAX4* and *PAX6* specifies the endocrine cell lineage: Cells expressing both become β-, δ-, and γ-cells; whereas those expressing only *PAX6* become α-cells.

Hh IN PANCREATITIS

The cellular and molecular processes in acute pancreatitis (AP) and chronic pancreatitis (CP) were intensively elucidated in the last years providing valuable detailed insights which could be important in the next years for a further therapeutical approach in this partially lethal disease entity (reviewed in detail in[23-25]). In short, in the phase of AP, the major cellular key players are neutrophils, monocytes and macrophages which interact by building and secretion of cytokines and inflammatory mediators, mainly tumor necrosis factor α, interleukin (IL) 1β and 6, and monocyte chemotactic protein. In the phase of CP, pancreatic stellate cells (PSC), tissue infiltrating myeloid cells, and particularly macrophages are coming to the fore by induced and increased progressive fibrosing of the pancreas tissue, being mediated mainly by nuclear factor (NF)-κB[26,27]. Finally, the crosstalk of the mentioned cells is linked to T-subsets (CD-8$^+$/central memory cells as well as T-regulator cells) which are involved in the pathogenesis of CP[28,29]. Additionally, CP is commonly regarded as a relevant risk factor for ductal pancreatic cancer (DPC) by irregular ductal changes leading to acino-ductal metaplasia and pancreatic intraepithelial neoplasia (PanIn)[30,31].

Focusing on the linkage between the Hh pathway and AP as well CP, experimental investigations demonstrated that the members of the Hh pathway could be detected in different amounts in AP and CP, whereby the definitive functional role of Hh in AP and CP seems to be very different. Additionally, in the process of CP forward to DPC an irregular expression pattern of the Hh members are observed compared to the normal and structured embryonic development of the pancreas[9,32,33].

AP

Compared to CP, the role of Hh in AP has been dealt with only in few studies. Summarizing these data, activation of the Hh signaling is linked to injury and repair using the cerulean-mediated model, whereby the unequivocal conclusion of the available experimental data is that the Hh has protective function in AP. In 2008, Fendrich *et al*[33] presented a functional analysis of the Hh pathway in AP using pharmacologic and genetic techniques (like Ptch1-LacZ reporter mice and two different *Cre-driven* pancreas-specific depletion mice models of Smo) demonstrating that Hh is essentially involved in effective regeneration of the exocrine pancreas. By this approach, *Shh*, *Ihh*, and Gli1 are increasingly expressed in caerulein treated mice, whereby the pharmacologic and genetic inhibition lead to persistence of PDX1 expressing metaplastic intermediates and impaired tissue repair. Additionally, the group of Zhou *et al*[34] used a Cerulein-induced AP model in mice to show elegantly that: (1) *Shh*, not *Ihh* or *Dhh*, is involved in this model; (2) *Shh*-inhibition aggravates the AP; and (3) the anti-inflammatory autocrine effect of *Shh* is mediated by IL-10. A recent experimental study from 2014 showed that Gli1, the downstream member of the Hh cascade, could essentially influence the inflammatory reaction in the circumstances of remodeling processes of the pancreas. Based on genetic analysis of deletion of a single allele of Gli1, the authors postulated that the canonical Hh pathway, respectively the transcription factor Gli1, is essential for pancreatic recovery in inflammatory processes *via* Gli1 targeted cytokines, including IL-6, murine homolog of IL8, monocyte chemoattractant protein-1, and Macrophage colony-stimulating factor M-csf, leading to pancreatic tumorgenesis *via* improper stromal remodeling and persistence of the inflammatory infiltrate[35].

Chronic pancreatitis

Empiric studies in humans with CP demonstrated a heterogeneous upregulated expression of *Ihh*, its receptors Ptch and Hedgehog-Interacting Protein, and Smo in different histological distribution and cellular localization of human tissue with CP using Northern blotting, immunohistochemistry and Western-blotting[32,36,37]. Interestingly, the members of the Hh pathway were localized mainly in the islet cells, whereas the Hh signaling members were present in degenerated acinar and tubular complexes of CP[36,37]. In addition, Kayed *et al*[37] could show that the inhibition of the Hh pathway *via* Cyclopamin led to growth inhibition of TAKA-1 pancreatic ductal cells through cell cycle arrest *in vitro*.

Based on cDNA microarrays, Bhanot *et al*[31] could support the findings, that the Hh pathway is altered in microdissected ectatic ducts of CP whereby dysregulation of Hh could enhance the probability of DPC *via* duct ectasia, acino-ductal metaplasia or intraepithelial neoplasia as reviewed by Bahnot *et al*[31] in 2008.

As mentioned above, PSCs are essentially involved in the pathogenesis of the CP, whereby the main question is, how the Hh pathway regulates the activation of these PSCs.

The experimental analysis of the group of Shinozaki *et al*[38] revealed that *Ihh* has no evident effect on expression of collagen-1 or alpha-smooth muscle actin or on proliferation of PSCs, but *Ihh* modulates the migration potency by changing the amount of membrane-type 1 matrix metalloproteinase and its localization on the plasma membrane leading to a pro-migration status of PSCs. Although the *Ihh* effects are mediated by Gli1, experimental overexpression of Gli1 using an adenovirus-mediated or RNA interference techniques revealed a negatively regulation by Gli1 to *Ihh* effects *in vitro*.

But the question remains: Why is Hh pathway up-

Table 1 Summary of the role of Hedgehog signaling in pancreatitis, indicating the protective role in acute pancreatitis *vs* disease-progressive function in chronic pancreatitis as well the possible association to pancreatic cancerogenesis[32-38,42,43]

	Acute pancreatitis	Chronic pancreatitis
Pathogenetic effect of Hh	Protective	Progressive
Detected members of Hh	↑ *Shh* (*Ihh*, *Dhh*), Gli1	↑ *Ihh* (*Shh*), Ptc, Hip, SMO, Gli1, Gli2
Interactive cells (auto-and paracrine effects)	Acinar/ductal cells with; acute inflammatory cells	Acinar/ductal cells with; PSC
(Immune) mediators of inflammation	IL-10, IL-6, mIL-8, Mcp-1, and M-csf (Csf1)	MT1-MMP, MMP9, TGF-β1, smooth muscle actin, fibronectin 1, type Ⅰ collagen
Association to cancerogenesis	No	Yes, possibly *via* ADM and PanIn

Hh: Hedgehog; *Shh*: Sonic Hh; *Ihh*: Indian Hh; *Dhh*: Desert hedgehog; IL: Interleukin; mIL-8: Murine homolog of IL8; Mcp-1: Monocyte chemoattractant protein-1; M-csf: Macrophage colony-stimulating factor; Ptc: Patched; Hip: Hh-interacting protein; SMO: Smoothened; MT1-MMP: Membrane-type 1 matrix metalloproteinase; MMP-9: Matrix metallopeptidase 9; TGF-β: Transforming growth factor beta; ADM: Acino-ductal metaplasia; PanIn: Pancreatic intraductal neoplasia; PSC: Pancreatic stellate cells.

regulated within the fibrogenic process of CP? Based on *in vitro* and *in situ* studies with xenografts as well as in humans with pancreatitis, it is postulated that para- and partially autocrine activation of stromal cells by Hh ligands from epithelial components and vice versa are responsible[39-41]. The experimental data of Jung *et al*[42] are based on transgenic phenotypes in zebrafish with over-expression of either *Ihh* or *Shh* along with green fluorescence protein. Consecutive analysis of these transgenic phenotypes using quantitative and qualitative investigations of mRNA and protein levels including PCR, *in situ* hybridization, and immunohistochemistry revealed that myofibroblasts and ductal cells are activated and proliferate which is triggered by paracrine Hh signaling in a restricted expression of Ptc1, Smo and Gli1/2. Additionally, Hh ligands could induce matrix metallopeptidase 9 and TGF-β1 in this animal model[42].

Recent investigations by Tsang *et al*[43] could support the published findings of pro-fibrinogenic effects of Hh in CP by using an *in vivo* model. The application of Rhein, a natural anthraquinone derivative, reduces the activation of PSCs in mice with experimental induced CP. The morphological effect of Rhein in reduced pancreatic fibrosis was paralleled by reduced molecular expression of fibrogenic markers including alpha-smooth muscle actin, fibronectin 1, type Ⅰ collagen as well as the members of the Hh pathway *Shh* and Gli1.

Interestingly, the promoting fibrotic effect of Hh signaling is not only existent in pancreas, but also could be observed in other organs like lung, bile duct and liver implicating a tissue independent overriding principle of the Hh pathway in this pathogenesis[44-46].

CP and pancreatic carcinogenesis

Since chronic recurrent inflammation has been linked to carcinogenesis, especially in pancreas, some findings of Hh in AP/CP and pancreatic carcinogenesis are presented in the following for supporting this already emphasized linkage[47,48]. First of all, Hh modulates the axis between inflammation and cancerogenesis *via* activation and production of cytokines by human peripheral $CD4^+$ T cells[49]. Furthermore, experimental studies of Hh in AP and CP revealed morphological changes like ductal metaplasia promoted by *Shh*, which are *per se* no pre-tumorous conditions[33,50]. Nevertheless, during progression of CP, morphological changes of the ductal pancreatic tissue like papillary lesions with nuclear atypia resulting in PanIn lesions could be observed which have a high association to aberrant Hh expression and pancreatic cancer[31,50].

In conclusion (summarized in Table 1), members of the Hh pathway have protective properties in case of AP, whereby the face of Hh changes to a progressive and disease-promoting function in CP. Especially in CP, the negative effects of Hh on tissue remodeling and repair favored the possibility of cancerogenesis *via* de- and trans-differentiation[51-54].

Hh IN PANCREATIC CANCER: FROM *IN VITRO* TO *IN SITU*

In vitro: Findings in cell culture experiments and xenografts

Hh signaling in the normal pancreas and in pancreatic ductal adenocarcinoma is exclusively paracrine with expression of *Shh* (tumor cell and stroma signal circle as shown in Figure 2)[55]. The silencing of Smo in pancreatic cancer epithelium in mice showed no altered tumor spread or development, so the Hh signaling does not occur in an autocrine way[55].

In paracrine signaling, the Hh ligand sends signals directly to the stroma and provides a selective tumor growth advantage. This was established through a pancreatic cancer model where Hh signal was needed for overall tumor growth while the particular tumor cells themselves did not respond to Hh ligand[56].

The existence of cancer stem cells (CSC) in different tumors, including pancreatic cancer, offers an explanation why some therapy assessments are ineffective[57,58]. Therefore, a good knowledge base for new therapies, which target pancreatic CSCs, is very important. The Hh signaling pathway plays a vital role in pancreatic and embryonic development; autocrine or paracrine secreted *Shh* activates a signal transduction cascade that includes other Hh members like Ptch and Smo, which then activates the canonical Hh pathway through Gli.

This leads to transcription of multiple targets like

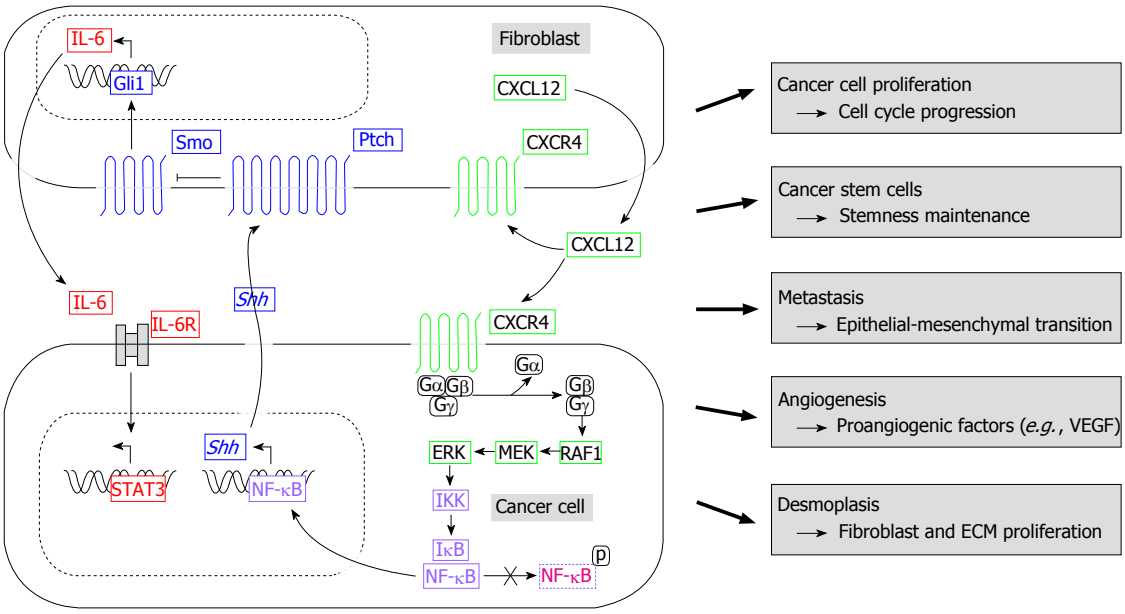

Figure 2 Illustrated summary of Hedgehog pathway and its effetcs at different stages in the formation and progression of ductal pancreatic carcinoma. Stromal cells secrete CXCL12 that binds to its receptor CXCR4 of ductal pancreatic cancer (DPC) cells (paracrine) and of stromal cells themselves (autocrine) resulting in *Shh* expression. *Shh*, secreted by DPC cells, binds in a paracrine manner Smo on stromal cells of the tumor microenvironment ending up in IL-6 secretion, which is known to regulate the progression of precursor lesions and tumor formation. For details see chapter Ⅳ. Based on[63,77,85]. CXCL12/CXCR4: CXC-motif-chemokine 12/CXC chemokine receptor type 4; ECM: Extracellular matrix; ERK: Extracellular signal-regulated kinases; Hh: Hedgehog; IκB: Inhibitor of kappa B; IKK: Inhibitor of nuclear factor kappa-B kinase; IL-6: Interleukin-6; MEK: Mitogen-activated protein kinase; NF-κB: nuclear factor-κB; Ptch: Patched; RAF1: V-Raf-1 murine leukemia viral oncogene homolog 1; *Shh*: Sonic Hh; Smo: Smoothened; STAT3: Signal transducer and activator of transcription 3; VEGF: Vascular endothelial growth factor.

Nanog, Cyclin D1, *Ptch*, Gli1 and Gli2. Activation of *Shh* signaling seems to precede the transformation of pancreatic tissue stem cells to cancerous stem cells. This was shown in mice, which were treated with sulforaphane to inhibit the growth of these stem cells. Sulforaphane is a natural compound found in cruciferous vegetables like broccoli that as an inhibitor acts on various receptors and pathways with anti-cancerous properties like apoptosis induction and cell proliferation[59]. This experimental study showed that human pancreatic stem cells need the activity of the Hh-Gli pathway for proliferation, survival, self-renewal and tumorigenicity[60].

In 2002, Chen *et al*[61] modulated mammalian embryonic pancreas development *in vitro* using cyclopamine treated pancreatic explants. A recombinant form of *Shh* was added to pancreatic buds to activate the Hh signaling pathway. The fluorescently labeled epithelium of the pancreatic explants underwent extensive growth and branching when treated by cyclopamine, which indicates that Hh inhibition did not block branching in the epithelium[61].

Walter *et al*[62] isolated pancreatic fibroblasts from benign and malignant primary pancreatic resection specimens by immunohistochemistry marker selection through vimentin. Together with two fibroblast cell lines, SC2 and SC3 (from non-neoplastic pancreas), the cancer-associated fibroblasts (CAF) were characterized for Hh activity. The fibroblast cell lines and the isolated CAFs where treated with *Shh* ligands to observe any expression changes on Gli mRNA. As a result they detected overexpression of Smo in pancreatic CAFs, which could transduce the *Shh* signal followed by Gli1 activation. The Hh pathway has been identified as activated in cancer associated stromal fibroblasts in mouse models of pancreatic cancer. CAFs can actively transduce the Hh signal to induce Gli expression. CAFs expressing Smo respond to exogenous Hh ligand, whereas control fibroblasts lacking Smo expression are unresponsive to Hh ligand, and downregulation of Smo in CAFs inhibits transduction of the Hh signal[62].

In human tumor xenografts, expression of *Shh* by tumor cells correlated with increased expression of GLi1 and Ptch1 in the stromal compartment. Pathway inhibition affected only stromal *Gli1* and *Ptch1* expression and resulted in decreased tumor growth exclusively in Hh ligand-expressing tumors[63].

Tian *et al*[64] demonstrated that the expression of an oncogenic allele of Smo (SmoM2) in mouse pancreas neither activated Hh signaling in epithelial cells nor promoted their neoplastic transformation. In murine pancreatic cancer models as well as in human pancreatic cancer specimens, activation of the Hh pathway was observed only in stromal cells surrounding Hh ligand-expressing tumor cells[64].

In-situ: Findings in human specimen of pancreas

Tumors of the pancreas can develop either from ductal, neuroendocrine or acinar cell populations. Due to a lack of information about the role of the Hh signaling pathway in acinar and neuroendocrine tumors of the pancreas, the following paragraphs will concentrate on DPC.

Among all cancers, DPC has one of the worst pro-

gnoses among all cancers with an overall 5-year survival rate of less than 5%[65]. Chemo- and radiotherapy are largely ineffective; furthermore, metastatic spread frequently occurs even after complete surgical resection[66]. The Hh pathway is one highly promising signaling transduction pathway for a better understanding of the origin of DPC.

Expression of Hh pathway members is usually not present in healthy adult pancreatic tissue[67]. In 2008, a global sequencing analysis revealed that the Hh pathway is one of the core signaling pathways that undergoes somatic alterations in nearly all pancreatic cancers[68]. Kayed *et al*[37] showed an aberrant activity of the Hh pathway in chronic pancreatitis and pancreatic cancer. Later on, it was recognized that *Shh* expression enhances the proliferation of pancreatic duct epithelial cells[69] and is not only up-regulated in the setting of pancreatic injury, but also in noninvasive precursor lesions of DPC: (1) PanIn; and (2) intraductal papillary mucinous neoplasia (IPMN) starting with rising expression values up to Hh pathway persistence in metastatic state[67,70,71]. Additionally, it was stated that up-regulation of the *Shh* ligand is sufficient to misdirect the pancreatic ductal epithelium towards a gastrointestinal metaplastic phenotype, which explains the involvement in IPMN formation[50,63].

However, dysfunction, or rather re-activation of the Hh pathway is not the only reason for the development of PanIn and DPC. Lauth *et al*[72] described a synergistic molecular crosstalk between Hh pathway and activated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS) signaling pathway[72]. Over 90% of patients suffering from DPC showed a *K-RAS* mutation, thus identifying the K-RAS pathway as another key mediator of pancreatic carcinogenesis[68,73]. Patients with a K-RAS mutation developed PanIn; and an additional P53 loss of function leads to subsequent development of the lesion towards DPC[74]. According to various studies, the crosstalk between Hh and K-RAS takes place *via* the RAF/MEK/MAPK pathway[75,76].

In 2013, Mills *et al*[77] were able to identify Gli1 as an effector of K-RAS at early stages of pancreatic carcinogenesis. They showed in a mouse model that loss of Gli1 impairs K-RAS-induced carcinogenesis. Although the mice still developed PanIn, the incidence of PanIn decreased and as a result, no mice suffered from DPC[77].

In recent studies, another central role in pre-neoplastic lesions of the pancreas is awarded to the signal transducer and activator of transcription 3 (STAT3) and its upstream cytokine IL-6. It is supposed that STAT3 activation is involved in driving early changes in the microenvironment promoting PanIn formation in the presence of oncogenic K-RAS[78,79]. Mills *et al*[77] stated that Gli1 also acts on CAF by paracrine regulation of the IL-6/STAT3 pathway in stromal cells of the tumor microenvironment (TME) and, thus, regulating the progression of precursor lesions and tumor formation (Figure 2).

DPC pathogenesis is characterized by a desmoplastic reaction to invading tumor cells, including a dense extracellular matrix that was recently shown to be the result of epithelial to mesenchymal transition (EMT)[80,81]. The epithelial-mesenchymal interaction, especially in the paracrine model of the Hh pathway, plays a distinctive role in different carcinoma entities as well as in DPC. Deregulated Hh pathway in PanIn and DPC leads to the secretion of Hh ligands *Shh* and *Ihh*, followed by a paracrine activation of CAFs in the surrounding stroma leading to expansion and desmoplasia[40,82,83]. In detail, neoplastic epithelium secretes *Shh*, which binds to the cognate Ptch-receptor on stromal cells, followed by desmoplastic stromal expansion and microenvironment remodeling. Moreover, supporting the paracrine action model of Hh pathway in DPC, Yauch *et al*[83] showed that treatment with Hh pathway antagonist results in downregulation of *Hh* target genes only in the tumor stroma but not in the epithelial cancer cell. In the same way, Smo expression decreases in mesenchymal cells in the pancreas resulting in Hh pathway activation. However, Lee *et al*[80] described that Hh pathway activity controls the balance between epithelial and stromal elements: Pathway activation causes stromal hyperplasia and reduced epithelial growth, whereas Hh inhibition causes accelerated growth of epithelial elements and suppression of desmoplasia.

It is suggested that the TME and extensive desmoplasia are partly responsible for chemoresistance in DPC by creating a "fence" around the tumor cells, which protects them against therapeutic compounds[84]. Therefore, tearing down this barrier could be a promising strategy to improve therapeutic approaches. Singh *et al*[85] already showed that inhibition of Hh pathway depleted tumor-associated stromal tissue.

There are many other different tumor specific characteristics that are influenced by the interrelation of Hh pathway and the TME. Bailey *et al*[86] identified paracrine *Shh*-mediated fibroblasts within the TME as source of Hypoxia-inducible factor 1 alpha (HIF-1α), which is known to be a regulator of angiogenesis and metastasis in cancer. Another example is the CXC-motif-chemokine 12/CXC chemokine receptor type 4 (CXCL12/CXCR4) pathway, which is on the one hand critical for normal cellular processes, but on the other hand contributes to metastasis, growth, survival and stem cell characteristics of cancer cells[87-89]. CXCL12, the sole ligand for CXCR4, is produced by tumor-associated stromal cells, is increased in DPC; and after binding to its receptor CXCR4, leads to activation of extracellular signal-regulated kinases resulting in release and nuclear translocation of NF-κB, which then directly binds the *Shh* promotor[85,90,91]. In summary, Hh pathway acts in a predominantly paracrine manner, thereby influencing and being influenced by the TME (for an overview of Hh-dependent interactions between tumor and stroma cells in DPC, Figure 2).

CSC, also called tumor initiating cells are suggested to be responsible for cancer initiation, progression and chemo-resistance in several malignancies including DPC[92]. The transcription factors Nanog, octamer-binding transcription factor 4 and BMI1 Proto-Oncogene, Polycomb Ring Finger (BMI-1) are essential for the

Table 2 Clinical trials of Hedgehog inhibitors for pancreatic cancer (https://clinicaltrials.gov/)

Drug	Combination	Phase	Status	Trial ID
GDC-0449	Gemcitabine	0	N	NCT01713218
		1/2	A	NCT01195415
		2	A	NCT01064622
	Erlotinib, gemcitabine	1	A	NCT00878163
	Gemcitabine, nab-paclitaxel	2	A	NCT01088815
LDE-225	Gemcitabine, nab-paclitaxel	1/2	R	NCT01431794
		1/2	R	NCT02358161
	BKM120	1	C	NCT01576666
IPI-926	Gemcitabine	1/2	C	NCT01130142

A: Active, not recruiting; C: Completed; N: Not yet recruiting; R: Recruiting.

"stemness", including characteristics like self-renewal of CSC[93-95]. The Hh pathway is implicated in the maintenance of pancreatic CSCs: For example, Li *et al*[96] stated that *Shh* expression was 46-fold greater in pancreatic CSCs ($CD24^+/CD44^+/ESA^+$) as in other DPC cells ($CD24^-/CD44^-/ESA^-$). Additionally, *Gli1* is known to up-regulate genes that are crucial for many properties for stemness of CSC - like *Nanog* and *BMI-1*[97-99].

Recapitulating this chapter, Hh pathway plays an important role in DPC, beginning from PanIn precursors to progressed metastatic disease. Hh signaling cross talks with a variety of other signaling pathways, like K-RAS, requires the interaction with the EMT in particular *via* paracrine pathway stimulation in order to contribute to the development of DPC (Figure 1).

Hh-BASED CLINICAL TRIALS FOR PANCREATIC CANCER

At present, clinical trials using Hh inhibitors enroll patients with pancreatic malignancies including advanced, metastatic, recurrent or resectable pancreatic cancer. Currently, no trials are listed within the United States National Institutes of Health database (www.clinicaltrials.gov) which target pancreatitis or other pancreatic non-neoplastic conditions. As summarized in Table 2, most trials in the phase 1 or 2 setting use GDC-0449 (vismodegib) which is a small molecular weight inhibitor of Smo[100] thereby interfering with Hh signaling at the plasma membrane level similarly to cyclopamine, a naturally occurring Smo antagonist[101]. Other Hh-targeting drugs in current clinical trials on pancreatic cancer are the Smo-inhibitors LDE-225 (Sonidegib)[102] and IPI-926 (Saridegib)[103].

For the latter, a preclinical study on pancreatic cancer in mice demonstrated that IPI-926 depletes tumor-associated stromal tissue and facilitated the delivery and increased the intratumoral concentration of gemcitabine[84]. In line with these results, all currently ongoing clinical trials combine selective Hh antagonists with established chemotherapies (gemcitabine, paclitaxel) or other targeted drugs (erlotinib epidermal growth factor receptor inhibitor) or BKM120 (Phosphatidylinositol-4,5-bisphosphate 3-kinase inhibitor) to investigate possible therapeutic benefits of these drug combinations. Taken together, current clinical studies employ inhibitors of the Smo co-receptor in combination with established chemotherapeutic drugs. Novel experimental inhibitors targeting the Hh pathway at the level of the transcriptional regulation (*e.g.*, Gant-61, Gant-58) have not yet entered the stage of clinical evaluation[104].

CONCLUSION

Besides its physiologic functions in human pancreatic development, the Hh pathway is activated in numerous pathological conditions, including carcinogenesis. However, the data on its functional aspects currently available draw a more nuanced picture. Progression from pancreatic cancer precursors lesions (PanIn) to DPC and metastatic disease is strongly influenced by a paracrine Hh signal modulating the interaction between DPC cells and CAFs. This Hh driven signaling predominantly includes the IL-6/STAT3 and the CXCL12/CXCR4 pathways resulting in disease progression by invasion, angiogenesis, metastasis formation and chemoresistance as well as gaining of stem cell like characteristics. Therefore, therapeutic targeting of the Hh pathway may provide new therapeutic approaches to improved disease control and prognosis for both, chronic pancreatitis and pancreatic carcinogenesis.

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REVIEW

Insulin resistance in development and progression of nonalcoholic fatty liver disease

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Abstract

Although insulin resistance (IR) is strongly associated with nonalcoholic fatty liver disease (NAFLD), the association of IR and NAFLD is not universal and correlation between IR and severity of NAFLD is still controversial. In this review, we summarize recent evidence that partially dissociates insulin resistance from NAFLD. It has also been reported that single-nucleotide polymorphisms in the diacylglycerol acyltransferase gene, rather than IR, account for the variability in liver fat content. Polymorphisms of the patatin-like phospholipase 3 gene have also been reported to be associated with NAFLD without metabolic syndrome, which suggests that genetic conditions that promote the development of fatty changes in the liver may occur independently of IR. Moreover, environmental factors such as nutrition and physical activity as well as small intestinal bacterial overgrowth have been linked to the pathogenesis of NAFLD, although some of the data are conflicting. Therefore, findings from both genetically engineered animal models and humans with genetic conditions, as well as recent studies that have explored the role of environmental factors, have confirmed the view that NAFLD is a polygenic disease process caused by both genetic and environmental factors. Therefore, IR is not the sole predictor of the pathogenesis of NAFLD.

Key words: Nonalcoholic fatty liver disease; Insulin resistance; Metabolic syndrome; Diabetes; Nonalcoholic steatohepatitis

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Core tip: Insulin resistance is considered as the major contributor for the development and progression of nonalcoholic fatty liver disease (NAFLD). However, recent evidence that has shown that non-obese individuals from developing countries are also affected by NAFLD, thus the conventional paradigm of NAFLD as the "hepatic manifestation of metabolic syndrome" has become outdated. Recent studies have highlighted novel pathophysiological mechanisms for the development and progression of NAFLD. Insulin resistance contributes to the disease process, but it is evident that environmental and genetic factors also contribute for development of necroinflammation and subsequent

progression to fibrosis. This review provides a summary of current knowledge of the pathogenesis of NAFLD and discusses factors that dissociate insulin resistance from NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an emerging public health problem[1] due to increasing prevalence in developed and developing countries. NAFLD is the second leading cause of chronic liver diseases after hepatitis C in Western countries and affects individuals of all age groups[2]. NAFLD includes a wide spectrum of conditions that range from a simple steatosis to nonalcoholic steatohepatitis (NASH) which may further progress to cirrhosis and its complications, in absence of alcohol consumption; or a low daily consumption of alcohol (< 30 g/d for men, < 20 g/d for women)[3-5]. NAFLD has been linked to insulin resistance (IR) and other components of metabolic syndrome such as diabetes mellitus, central abdominal obesity and dyslipidemia[6]. Patients with NAFLD are at an increased risk for all-cause mortality, including liver-related deaths and non-liver-related deaths such as death due to cardiovascular disease and diabetes[7].

Recent evidence that has shown that non-obese individuals from developing countries are also affected by NAFLD; thus, the conventional paradigm of NAFLD as the "hepatic manifestation of metabolic syndrome" has become outdated[8]. Recent studies have highlighted novel pathophysiological mechanisms in the development and progression of NAFLD. IR contributes to the disease process, but it is evident that environmental and genetic factors also have the contribution in the development of necroinflammation and subsequent fibrosis. The dogma of a sequential progression of simple steatosis to NASH to cirrhosis in NAFLD is currently under scrutiny.

The pathogenesis of NAFLD is now conceptualized as a complex and multifaceted process that requires further understanding. This review provides a summary of our current understanding of these processes, particularly the evidence that IR is not the lone predictor for NAFLD, but rather, the disease is multifactorial and may be caused by the involvement of genetic and environmental factors.

RESEARCH

We searched MEDLINE, EMBASE, and PubMed using the MeSH terms "insulin resistance", "nonalcoholic fatty liver disease", and "nonalcoholic steatohepatitis". The reference lists of the articles selected for inclusion were also reviewed for additional relevant papers. The search was limited to studies that were reported in the English language and that were published between 1995 and March 2015. Articles that are specifically related to the epidemiology, diagnosis and current treatment strategies for NAFLD and NASH are summarized.

Burden of NAFLD

The reported prevalence of NAFLD from Western countries is 20%-30%, from Asian countries is approximately 15%[9-11]. In normal-weight individuals without any known metabolic risk factors, the prevalence of NAFLD is reported to be approximately 16%. However, the prevalence is much higher among high-risk groups such as diabetics (60%), patients with hyperlipidemia (90%) and obese patients undergoing bariatric surgery (91%)[9-13]. Only 20% of patients under the age of 20 have NAFLD, but among patients aged 60 and above, the prevalence is more than 40%[14]. This findings further strengthened in another study where older age is identified as an independent risk factor for disease progression from simple steatosis to NASH and for the development of fibrosis and cirrhosis[15]. Hamabe *et al*[16] showed that smoking is an independent risk factor for NAFLD. A few studies have also reported ethnic variation in the prevalence of NAFLD, but these reports present contrasting data[17,18]. The risk of mortality is higher in NASH and advanced fibrosis compared with simple steatosis[19]. The progression to advanced fibrosis has been shown to be associated with the patient's age and the degree of inflammation[20]. In a long-term longitudanal study of 129 patients with NAFLD, Ekstedt *et al*[19] explored that mortality was not increased in patients with simple steatosis but was increased in NASH patient. Although the mortality was primarily due to cardiovascular disease, liver-related deaths were more common in patients with NASH-related cirrhosis[21].

Pathogenesis

Traditional concept: The two-hit hypothesis: Day *et al*[22] first proposed the current concept of the "two-hit hypothesis in NAFLD" in 1998 (Figure 1). The first hit is primarily as a result of IR, increased dietary intake and enhanced hepatic lipogenesis there is accumulation of free fatty acids (FFAs) and triglycerides (TGs) in hepatocytes[22]. The second hits is a combination of oxidative stress, lipid peroxidation, mitochondrial dysfunction and the release of inflammatory mediators, which leads to progressive liver injury which constitute steatohepatitis and fibrosis[22]. The activation of proinflammatory pathways and toll-like receptors merge at the junction of two main intracellular signaling pathways known as nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK)[23,24]. NF-κB activation has been reported in NASH and can lead to increased transcription of many proinflammatory genes, whereas JNK activation causes IR *via* the direct phosphorylation and degradation of insulin receptor substrate 1 (IRS1); this in turn reduces the intracellular signaling pathway activity downstream of the insulin receptor[23]. Lipid peroxidation can promote

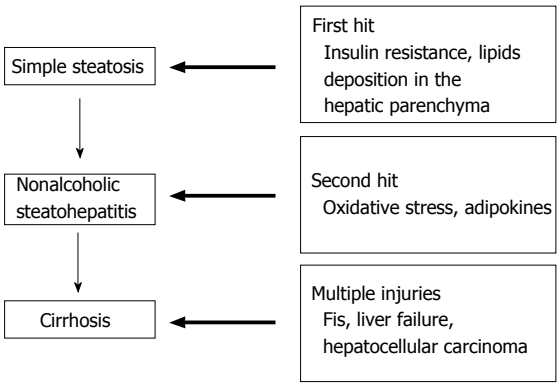

Figure 1 Two-hit hypothesis of nonalcoholic fatty liver disease (traditional view).

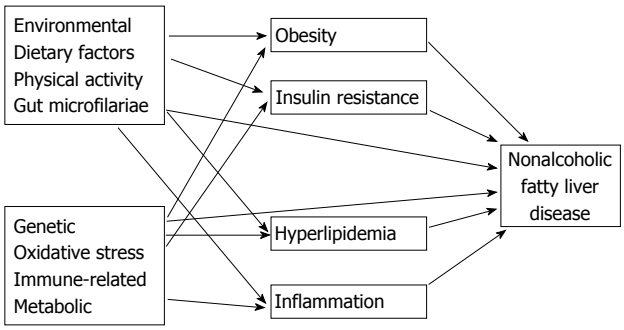

Figure 2 Interplay among environmental and genetic factors in the development of nonalcoholic fatty liver disease.

the proliferation of stellate cells, which contributes to fibrogenesis[25]. Reactive oxygen species induce the release of cytokines from hepatocytes, which leads to the initiation of various immune-mediated mechanisms that contribute to further liver cell injury. The combination of hyperinsulinemia, hepatic iron and lipid peroxidation induces oxidative stress[17], which can cause mitochondrial dysfunction in NASH and can contribute to TG accumulation and eventually to cell necrosis[11].

Multiple-hit pathogenesis

Accumulations of knowledge in recent years have challenged the traditional "two-hit" pathogenesis. Knowledge of interaction between insulin resistance, adipokines, adipose tissue inflammation and other less recognized pathogenic factors has been argued that multiple hits from adipose tissue and the gut occur at the same time and promote liver inflammation (Figure 2). This process suggests that cellular inflammation and insulin resistance occur concurrently[26,27]. Progression of NAFLD to NASH is explained by subsequent "two-hit" theory. In the "multiple-hit" model[28,29] hepatic steatosis may represent an epiphenomenon of several distinct injurious mechanisms including IR rather than a true "first hit"[30]. Hyperinsulinemia, results in increased hepatic *de novo* lipogenesis and increased adipose tissue lipolysis; leads to an increased efflux of free fatty acids to the liver[31,32]. After the initial development of steatosis, the liver becomes extremely vulnerable. Multipe series of pathogenic and injurious factors including oxidative damage, activation of transforming growth factor-beta pathway, dysregulation of multiple adipokines and apoptpsis and activation of hepatic stellate cell may lead to hepatocyte injury and finally to the progression from simple steatosis to NASH and fibrosis[33]. So multiple factors ineract in the complicated ways for development and progression of steatosis, NASH and fibrosis[14,34,35].

Distinct-hit hypothesis

A more recent model has proposed that the development of simple steatosis and NASH follows distinct pathways. The activation of these pathways is a complex process and is not only the result of a simple hepatic insult. Many other factors promote the activation of the pathways that lead to the development of steatosis and NASH[34]. The most important factors include genetic factors, the activation the hedgehog pathway and hepatic progenitor cells[36].

Role of IR in NAFLD

Studies have demonstrated that NAFLD is associated with higher IR compared with controls, even after the exclusion of overweight and obese subjects, and that IR increases with increasing degrees of steatosis[37-40]. IR in NAFLD is predominantly peripheral and occurs in the skeletal muscle and adipose tissue. Peripheral IR in the skeletal muscle causes reduced glucose uptake, which leads to hyperglycemia. In adipose tissue, IR impairs the anti-lipolytic action of insulin, which leads to an increased release of FFA. Elevated plasma concentrations of insulin, glucose, and fatty acids then impair the β-oxidation of fatty acids by negative feedback and promote the uptake of hepatic fatty acids and triglycerides, *de novo* lipid synthesis (*via* SREBP) (sterol-regulatory element-binding protein) and the expression of C/enhancer-binding protein (CCAAT/EBP). Insulin resistance also increases the amount of intra-hepatocytic fatty acids *via* an increase in glycolysis and a decrease in apolipoprotein B-100, which blocks the export of VLDL. The development of IR in NAFLD is most likely related to the imbalance between pro-insulin (adiponectin) and anti-insulin (TNFα) cytokines, specially, those secreted by adipose tissue. Alterations in several molecules, including FFAs, TNFα, membrane glycoprotein PC-1, and leptin, interfere with the insulin signaling pathway. FFAs are both the result and cause of IR. Excess FFAs cause hepatic IR *via* the down regulation of IRS1 signaling and by the activation of the inhibitor kappa B kinase (IKK-B)/NF-κB pathway. Patients with NAFLD have increased insulin resistance not only in muscle but also in liver and adipose tissue[41], and this reduced insulin sensitivity plays a major role in the pathogenesis of NAFLD. This IR, increases peripheral lipolysis in adipose tissue that leads to increase in the delivery of FFAs to the liver and *de novo* lipogenesis[17,35]. In addition, lipid overload in pancreatic-B cells leads to dysregulated insulin secretion and changes in the expression of peroxisome proliferator-activated receptor(PPAR)-α, glucokinase, the glucose

transporter-2, pre-pro-insulin and pancreatic duodenal homeobox-1, which can lead to IR as a result of FFA-induced B-cell apoptosis[12]. It has been suggested that IR in the liver is sufficient to produce dyslipidemia and increase the risk of atherosclerosis[42]. However, current evidences are not sufficient to demonstrate a consistent association between any particular type of adipokine and the histological severity of NAFLD[43].

IR IS THE CAUSE OR A CONSEQUENCE?

Although the development and progression of NAFLD is strongly associted with metabolic syndrome and IR, several studies have evidenced that all obese and diabetic dividuals dont have NAFLD. There are also evideces that NAFLD can occur in nonobese, as well as persons without metabolic syndrome[44]. Therefore, it could be hypothesized that factors other than IR could be the determinant of the development and severity of NAFLD. Familial lustering[45,46] and in the ethnic variation in the prevalence of NAFLD strengthen the initial concept[17]. Single-nucleotide polymorphisms in the adiponectin, interleukin-6, *TNFα* and *apoE* genes has been studied[47-49]. Multiethnic genome-wide association study with NAFLD revealed that the patatin-like phospholipase domain containing protein 3 (also known as adiponutrin) gene is strongly associated with hepatic TG content[50]. Allelic variants of the patatin-like phospholipase domain containing protein 3 (*PNPLA3*) genes have been found to be correlated with amounts of hepatic fat in Hispanics and African-Americans, and to be associted with prevalence of NAFLD. *PNPLA3* has also been independently identified in a separate population-based genome-wide study that influences the alanine aminotransferase (ALT) livel[51]. Environmental factors like; sedentary life styles, excess food intake, constituents of food and intestinal bacterial overgrowth have evidences to contribute in the pathogenesis of NAFLD. Obesity resulting from excess food intake and lack of exercise has been proven to contribute to the progression of fibrosis in patients with NAFLD[19]. An increased consumption of meat, soft drinks, saturated fat and cholesterol and a low consumption of fish and polyunsaturated fat (PUFA) were found to be associated with NAFLD[52-55]. Dietary supplementation with PUFA has been demonstrated in randomized control trial to be benifical in regression of fatty liver and reduction of ALT compared to dietery advice alone[56,57]. On the other hand highcarbohydrate and lowfat diets are associated with more progressive disease[58,59]. Conversely, studies in mice[60] and non-human primates[61], exposure to a maternal high-fat diet associated with development and progression of NAFLD in the offspring. Small intestinal bacterial overgrowth increases gut permeability, which leads to portal endotoxemia and increased numbers of circulating inflammatory cytokines, both of which have crucial role in the progression of NAFLD to NASH[62]. Several studies have reported an association between small intestinal bacterial overgrowth and the progression of NAFLD[63-65]. Dietary supplimentatation of probiotics and treatment with antibiotics resulted in benificial effects in NAFLD, which has furter sterngthen the concept[65].

FROM SIMPLE STEATOSIS TO NASH

Linear progression vs different entity

Although simple steatosis and NASH are currently classified as two histological subtypes of NAFLD, the two conditions are likely distinct from both a histological and a pathophysiological standpoint[34]. The American Association for the Study of Liver Diseases has recently suggested the classification of patients within the NAFLD spectrum into two main categories: NASH and "not steatohepatitis, with steatosis" ("simple steatosis")[66]. Differentiation is on histological variation where NASH is defined by the findings of lobular inflammation, portal inflammation, cellular ballooning, and fibrosis. In contrast, "not steatohepatitis, with steatosis" is characterized by simple fat infiltration with minimal/no inflammation[66]. NASH is a progressive disease, may progress to cirrhosis upto 9%-20% over a period of 5-10 years[67-69]. Vernon *et al*[9] explored that, only NASH is progressive and associated with the development of cirrhosis and hepatocellular carcinoma. In contrast, "simple steatosis" tends to be stable over time[69]. Though there is recent study of progression of steatosis to NASH and also there is progression to fibrosis[70], In agreement with these findings, Musso *et al*[71] in a meta-analysis concluded that a minority of patients with pure fatty liver will progress to NASH and only NASH seems to be associated with an increased risk of progressive liver disease[71]. Along these lines, a community based study of NAFLD outcomes has shown that no patients with simple steatosis died during a 7.6-year follow-up, whereas 35% of patients with NASH died during[69]. All these results established that NASH and "not steatohepatitis, with steatosis" are two distinct entities rather than a real progression of histological changes that can progress over time. For this reason, simple steatosis and NASH should be considered as a separate disease entity that develops along a distinct pathogenic pathway with multiple hits. The conceptualisation of these pathophysiological mechanisms would not only improve our biological understanding of NAFLD but may also allow clinicians to interven the patogenesis more accurately in future.

TREATMENT OF NAFLD

Considering that IR is a primary factor in the pathogenesis of NAFLD, several insulin sensitizers have been used in different settings. Table 1 summarizes a few of these trials. According to these trials, none of these drugs was effective, and thus further studies are warranted to identify their role. Notably, metformin was shown to improve liver injury, but this medication, which is typically used in the treatment of type 2 diabetes, could not prevent fibrosis in patients with steatosis[72]. Additionally, glitazones, which are PPARγ agonists, were found to be efficient in the management

Table 1 Insulin-sensitizing agents and anti-diabetic drug trials for halting nonalcoholic fatty liver disease progression

Insulin-sensitizing agent	Results of the study	Relevance to NAFLD	Ref.
Metformin	Improvements in liver histology and ALT levels in 30% of patients with NASH	Appears to be beneficial for NAFLD patients but not for non-obese patients with early-stage NAFLD	Loomba *et al*[72]
Pioglitazone	Improvement in the biochemical and histological features of NASH	Could be used as a treatment for NAFLD	Promrat *et al*[73]
Pioglitazone	Improvement in insulin resistance but not in hepatic fibrosis and ALT levels	Not adapted to treat NAFLD	Sanyal *et al*[74]

NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; ATL: Alanine aminotransferase.

of NAFLD *via* a notable decrease in liver fibrosis[73]. In contrast, another study revealed that pioglitazone does not promote beneficial effects with respect to liver fibrosis, but it diminished inflammation and steatosis[74]. Therefore, further studies are required to elucidate these contradictory results. Additionally, salsalate, a potential anti-diabetic drug that is currently under development, has been shown to improve glycemia in diabetic patients through a downregulation of the proinflammatory IKKβ/NFκB pathway[75]. Additionally, this agent likely improves NAFLD through an induction of adiponectin[76].

CONCLUSION

Hepatic steatosis is recognized to be the consequence of a complex interplay among diet, environment and liver and adipose tissues, although a comprehensive understanding of pathogenesis of NAFLD has not yet been complete. Therefore, NAFLD is currently perceived as multifactorial pathogenic disease with both genetic and environmental factors. Genome-wide association studies have identified specific genetic associations that are involved in NAFLD. From a therapeutic point of view, pathogenic-based interventions aimed at the reversal of NAFLD are likely to be a rational approach to the prevention and treatment of hepatic IR, metabolic syndrome and related complications. Further studies are required to explore the relationship among adiponutrin mutations, steatosis and IR. A better understanding of the different factors involved in the pathophysiology of NAFLD will open the opportunity to intervene its progression in future.

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Clinical impacts of mesothelin expression in gastrointestinal carcinomas

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Abstract

Mesothelin, C-ERC/mesothelin is a 40-kDa cell surface glycoprotein that is normally present on normal mesothelial cells lining the pleura, peritoneum, and pericardium. Moreover, mesothelin has been shown to be overexpressed in several human cancers, including virtually all mesothelioma and pancreatic cancer, approximately 70% of ovarian cancer and extra bile duct cancer, and 50% of lung adenocarcinomas and gastric cancer. The full-length human mesothelin gene encodes the primary product, a 71-kDa precursor protein. The 71-kDa mesothelin precursor is cleaved into two products, 40-kDa C-terminal fragment that remains membrane-bound *via* glycosylphosphatidylinositol anchor, and a 31-kDa N-terminal fragment, megakaryocyte potentiating factor, which is secreted into the blood. The biological functions of mesothelin remain largely unknown. However, results of recent studies have suggested that the mesothelin may play a role of cell proliferation and migration. In pancreatic cancer, mesothelin expression was immunohistochemically observed in all cases, but absent in normal pancreas and in chronic pancreatitis. Furthermore, the expression of mesothelin was correlated with an poorer patient outcome in several human cancers. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy. The present review discusses the expression and function of mesothelin in cancer cells and the utility of mesothelin as

a target of cancer therapy.

Key words: Mesothelin; Luminal membrane expression; Cytoplasmic expression; Tumor marker; Cancer therapy

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Core tip: Mesothelin is a 40-kDa cell surface glycoprotein expressed on normal mesothelial cells lining the pleura, pericardium, and peritoneum. Moreover, mesothelin has been shown to be overexpressed in several cancer types. Recent studies have suggested that the overexpression of mesothelin increases cell proliferation and migration. Furthermore, the expression of mesothelin was related to an unfavourable patient outcome in several human cancers. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy.

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INTRODUCTION

Mesothelin is a 40-kDa cell surface glycoprotein that is normally present on normal mesothelial cells lining the pleura, peritoneum, and pericardium[1,2]. Moreover, mesothelin has been shown to be overexpressed in several human cancers, including virtually all mesothelioma and pancreatic cancer, approximately 70% of ovarian cancer and extra bile duct cancer, and 50% of lung adenocarcinomas and gastric cancer[3-6] (Table 1). The full-length human mesothelin gene (Full-ERC/mesothelin) encodes a 71-kDa precursor protein. The 71-kDa mesothelin precursor is cleaved into two products, 40-kDa C-terminal fragment (C-ERC/mesothelin) that remains membrane-bound *via* glycosylphosphatidylinositol anchor[7], and a 31-kDa N-terminal fragment (N-ERC/mesothelin, megakaryocyte potentiating factor), which is secreted into the blood (Figure 1)[1]. The function of mesothelin in cancer is still unclear. However, results of recent studies have suggested that the mesothelin may play a role of tumor progression *in vitro*[8-11] and *in vivo*[11,12].

MESOTHELIN EXPRESSION IN GASTROINTESTINAL CANCERS

Co-expression of mesothelin and CA125 correlates with unfavorable patient outcome in pancreatic ductal adenocarcinoma

Mesothelin could play a role of the binding to CA125[13-15]. Mesothelin and CA125 binding may be important in the peritoneal spread[13,15]. In ovarian cancer, advanced clinical stage and/or high histological grade patients showed mesothelin expression and CA125 expressions[15]. Our group showed that the co-expression of mesothelin and CA125 group was a higher histological grade and a higher level of blood vessel permeation and correlated with recurrence rate and poor patient outcome in pancreatic ductal adenocarcinoma[16]. These findings suggest that the co-expression of mesothelin and CA125 may lead to tumor development, metastasis, and a poorer patient prognosis.

Luminal membrane expression of mesothelin is a prominent poor prognostic factor

The expression of mesothelin was related to an unfavorable patient outcome in pancreatic ductal adenocarcinoma[16,17]. Our group investigated mesothelin expression in gastric cancers by using immunohistochemistry, especially focusing on the localization of mesothelin, *i.e.,* "luminal membrane-positive" and/or "cytoplasm-positive" (Figure 2)[18].

The overall survival revealed that the "luminal membrane-positive" group showed a significantly poorer outcome compared to the "luminal membrane-negative" group. On the other hand, the "mesothelin-positive" group and the "cytoplasmic-positive" group were not correlated with overall survival in the gastric cancer patients.

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas has a histological spectrum ranging from benign adenoma to invasive cancer. We performed an immunohistochemical analysis of mesotheelin expression in IPMN. Mesothelin was absent in all of the normal pancreatic tissues. But, mesothelin was expressed in both adenoma and carcinoma cells. Most of mesothelin expressed adenoma cells exhibited slight "cytoplasmic-positive", and the "luminal membrane-positive" group has a tendency of poor prognosis and high recurrence rate[19].

Based on these results, the "luminal membrane-positive" of mesothelin is a useful prognostic factor, implying that membrane-localized mesothelin might have the significant function of the aggressive behavior in the cancer cells.

THE ROLE OF MESOTHELIN EXPRESSION IN TUMOR BIOLOGY

Our study generated the novel finding, the potential role of the "luminal membrane-positive" mesothelin in the malignant behavior of tumor cells[18-21]. The human mesothelin gene encodes a 71-kDa precursor protein (Full-ERC/mesothelin). This precursor protein is cleaved by furin-like proteases into a 31-kDa N-terminal secreted form (N-ERC/mesothelin) and a C-terminal fragment, 40-kDa mesothelin (C-ERC/mesothelin)[1,7,22]. The 5B2 anti-mesothelin antibody, which we used in our studies,

Table 1 Mesothelin expression in human cancer detected by immunohistochemistry

Tumour	Mesothelin expressions (%)	Comments	Ref.
Pancreatic cancer	86-100	Co-expression of mesothelin and CA125 group associated with a poorer patient prognosis	[6,16,17]
Gastric cancer	29-59	Luminal membrane expression is one of the poor prognostic factors	[6,18,27,35]
Extrahepatic bile duct cancer	72-100	Luminal membrane expression or cytoplasmic expression of mesothelin could be a reliable prognostic factor	[6,21]
Colorectal cancer	28-58	Luminal membrane expression was associated with lymphatic invasion	[6,20]
Intraductal papillary mucinous neoplasm	57	Luminal membrane expression was correlated with the histological classification of the tumor and the recurrence rate	[19]

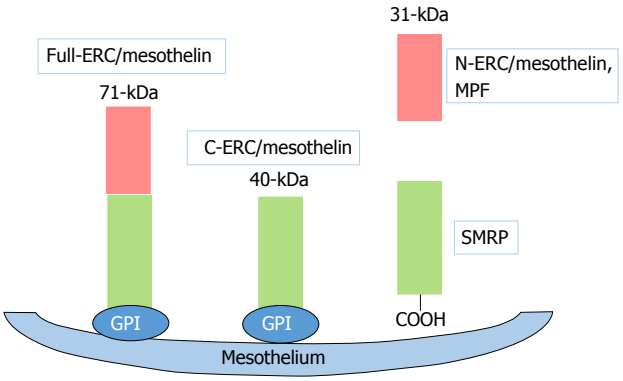

Figure 1 Schematics showing the maturation of mesothelin protein. The primary product of the full-ERC/mesothelin gene is a 71-kDa precursor protein. This protein is physiologically cleaved, releasing a 31-kDa fragment, N-ERC/mesothelin, into the blood. MPF: Megakaryocyte potentiating factor; SMRP: Soluble mesothelin-related peptide; GPI: Glycosylphosphatidylinositol.

can detect the 71-kDa precursor protein (Full-ERC/mesothelin) and the 40-kDa C-terminal fragment (C-ERC/mesothelin), but not the 30-kDa N-terminal fragment (N-ERC/mesothelin). Based on the specificity of this antibody, the "luminal membrane-positive" mesothelin observed in our study might have indicated the existence of 40-kDa mesothelin (C-ERC/mesothelin) membrane-bound form, while the "cytoplasmic-positive" mesothelin might have indicated the presence of the the 71-kDa precursor protein (Full-ERC/mesothelin). To demonstrate the mechanism of the membranous localization of mesothelin, we enforced the expression of Full-, C-, and N-ERC/mesothelin in human colorectal cancer (CRC) cell lines[20]. The 7E7 antibody, which recognizes the 30-kDa N-terminal fragment (N-ERC/mesothelin), revealed the diffuse cytoplasmic expression of Full- and N-ERC/mesothelin in Full-WiDr and N-WiDr. In contrast, the 22A31 antibody, which recognizes 40-kDa mesothelin (C-ERC/mesothelin), demonstrated a dot-like expression of Full- and C-ERC/mesothelin in Full-WiDr and C-WiDr. Moreover, some of the dot-like spots along with the cellular membrane were merged with actin, showing yellow signals. According to these results, we confirmed the membranous expressions of C-ERC/mesothelin in CRC cell lines.

To demonstrate the biological role of Full-, C-, and N-ERC/mesothelin in the lymphatic invasion of CRC, we performed an *in vitro* lymphatic invasion assay. C-ERC/mesothelin, the 40-kDa membrane-localized fragment, promoted the lymphatic invasion by increasing cell adhesion to lymphatic endothelial cells.

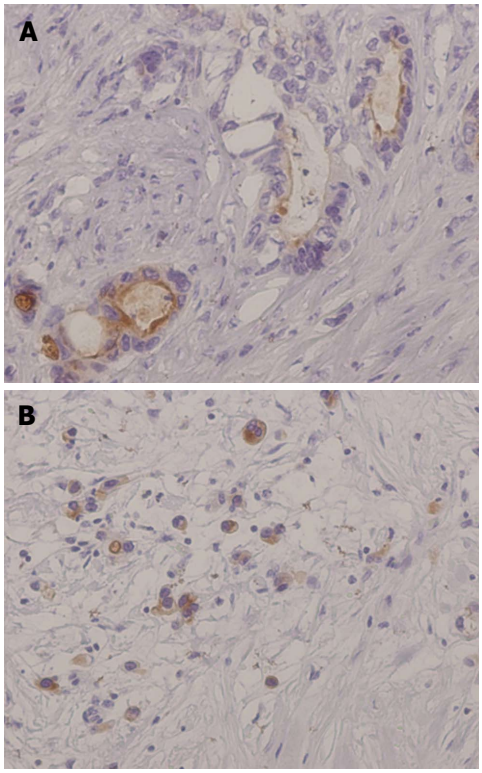

Figure 2 The expression of mesothelin in gastric cancer. A: Luminal membrane expression. The entire circumference of the cell membrane was stained; B: "Cytoplasmic expression" with granular cytoplasmic staining in cancer cells.

THE PATHWAYS OF MESOTHELIN INVOLVED IN CANCER

Recent studies reported that mesothelin is not only associated with increased cell proliferation and the migration of pancreatic cancer cells *in vitro*[11,23], but also contributes to tumor progression *in vivo*[11]. Mesothelin protects cancer cells from paclitaxel-induced apoptosis through both the concomitant activation of PI3K/Akt and MAPK/ERK pathways[24]. Overexpression of mesothelin in pancreatic cancer cells leads to constitutive activation of signal transducer and activator of transcription 3, which results in enhanced expression of cyclin E and cyclin

E/cyclin-dependent kinase 2 complex formation as well as increased G1-S transition[23]. Mesothelin expression correlated closely with interleukin (IL)-6 in human pancreatic cancer specimens and cell lines. Cancer cell with forced mesothelin expression grow faster than control cells by producing higher quantities of IL-6[9,10].

BLOOD TEST FOR MESOTHELIN

Several ELISAs have been developed to measure the levels of soluble mesothelin-related peptide (SMRP) and megakaryocyte potentiating factor (MPF, N-ERC/mesothelin). The soluble form of mesothelin is likely due to an abnormal splicing event resulting in a frameshift mutation and premature termination at amino acid 600 deleting the amino acids at the COOH terminus that are responsible for its association with the cell membrane. The full-length human mesothelin gene encodes the primary product, a 71-kDa precursor protein. It can be physiologically cleaved by some furin-like proteases into a 40-kDa C-terminal fragment that remains membrane-bound, and a 31-kDa N-terminal fragment, which is secreted into the blood. The C-terminal 40-kDa fragment is referred to as mesothelin. In contrast, the N-terminal 31-kDa fragment is a secreted protein identified as MPF. SMRP has proven to be a promising cancer biomarker in the sera of patients with tumors of mesothelial origin[25,26]. MPF has been reported to be expressed in gastrointestinal cancers[27,28].

Wu *et al*[29] revealed that SMRP performs better than CA125 as a tumor marker for epithelial ovarian cancer, it increases only in malignant patients and not in benign patients or healthy volunteers. Furthermore, the sensitivity is enhanced when combined with CA125. Hassan *et al*[30] identified a positive correlation with the tumor burden and SMRP levels, as a marker for monitoring the response to treatment o malignant mesothelioma.

MESOTHELIN TARGET IMMUNOTHERAPY

Because of the high expression of mesothelin in many malignancies and its limited expression in normal tissues, mesothelin has been suggested as an attractive target for immunotherapy. Several therapeutic agents that target mesothelin have been developed and some are being evaluated in preclinical and clinical studies. SS1P is an immunotoxin being clinically tested as a systemic agent in solid tumor patients. Two phase Ⅰ trials of single-agent SS1P have been performed[31,32]. The majority of patients developed antidrug antibodies by the end of their first cycle, resulting in non-therapeutic drug levels if any additional cycles were given. MORAb-009 (amatuximab) is a chimeric antibody. A phase Ⅰ clinical trial of MORAb-009 for mesothelioma, pancreatic cancer, and ovarian cancer patients has been completed[33]. Eleven of 24 subjects had stable disease. Phase Ⅱ studies of MORAb-009 in different mesothelin-expressing cancers are ongoing. The mesothelin tumor vaccine in clinical development is CRS-207. The safety of this vaccine was established in a phase Ⅰ clinical trial of patients with mesothelin-expressing refractory cancers[34].

CONCLUSION

Mesothelin is an attractive antigen that is expressed in several gastrointestinal cancers. Recent studies have revealed oncogenic functions of mesothelin in cancer proliferation and invasion and drug resistance. Also, soluble mesothelin could be useful as a tumor marker. The limited mesothelin expression in normal tissues and high expression in many cancers makes it an attractive candidate for cancer therapy.

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ORIGINAL ARTICLE

Basic Study

Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine

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Abstract

AIM: To study whether the inflammatory bowel disease (IBD) colon which exhibits varying severity and cytokine levels across its mucosa create varying types of transepithelial leak.

METHODS: We examined the effects of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1-β (IL1β) and hydrogen peroxide (H_2O_2) - singly and in combinations - on barrier function of CACO-2 cell layers. Our focus was on the type (not simply the magnitude) of transepithelial leak generated by these agents as measured by transepithelial electrical resistance (TER) and transepithelial flux of ^{14}C-D-mannitol, ^{3}H-Lactulose and ^{14}C-Polyethylene glycol as radiolabeled probe molecules. The isoquinoline alkaloid, berberine, was then examined for its ability to reduce specific types of transepithelial leak.

RESULTS: Exposure to TNF-α alone (200 ng/mL; 48 h) induced a 50% decrease in TER, *i.e.*, increased leak of Na^+ and Cl^- - with only a marginal but statistically significant increase in transepithelial leak of ^{14}C-mannitol (J_m). Exposure to TNF-α + IFN-γ (200 ng/mL; 48 h) + IL1β (50 ng/mL; 48 h) did not increase the TER change (from TNF-α alone), but there was now a 100% increase in

J_m. There however was no increase in transepithelial leak of two larger probe molecules, ^{3}H-lactulose and ^{14}C-polyethylene glycol (PEG). However, exposure to TNF-α + IFN-γ + IL1β followed by a 5 h exposure to 2 mmol/L H_2O_2 resulted in a 500% increase in ^{14}C-PEG leak as well as leak to the luminal mitogen, epidermal growth factor.

CONCLUSION: This model of graded transepithelial leak is useful in evaluating therapeutic agents reducing IBD morbidity by reducing barrier leak to various luminal substances.

Key words: Intestine; Crohn's disease; Tight junction; Ulcerative colitis; CACO-2; Berberine; Micronutrient; Cytokine

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Core tip: A cell culture model of graded transepithelial leak can be very valuable in evaluating the various types and magnitudes of leak that can exhibit across the inflammatory bowel disease mucosa. This graded leak can be achieved through various combinations of proinflammatory cytokines and peroxide. Berberine provides an example of a micronutrient that can be more effective against one type of induced leak than another.

DiGuilio KM, Mercogliano CM, Born J, Ferraro B, To J, Mixson B, Smith A, Valenzano MC, Mullin JM. Sieving characteristics of cytokine- and peroxide-induced epithelial barrier leak: Inhibition by berberine. *World J Gastrointest Pathophysiol* 2016; 7(2): 223-234 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/223.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.223

INTRODUCTION

The idiopathic inflammatory bowel diseases (IBD), the major types being Crohn's disease (CD) and ulcerative colitis (UC), are autoimmune diseases affecting the gastrointestinal tract and causing chronic intestinal inflammation. CD and UC have similar key characteristics, perhaps the most important being an observed compromise of epithelial barrier function. The crucial role of the mucosal layer of the gastrointestinal tract is to actively separate gut luminal contents from the underlying interstitium. The epithelial cell layer that lines the gastrointestinal (GI) tract functions as a selectively permeable barrier. A major component of this barrier is the tight junctional (TJ) protein complex, which prevents free diffusion along the paracellular pathway. In the case of IBD, the integrity of the TJ barrier is compromised in part as a result of the inflammatory response increasing local and systemic pro-inflammatory cytokine [tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1-β (IL1β)] production. Luminal antigens now able to traverse the "leaky" barrier exacerbate the inflammatory response in the mucosa and submucosa, which in turn further worsens the integrity of the TJ complex and the cell layer overall[1-6]. It is debated whether intestinal leak is causal or simply a result of the disease. A study by Hollander *et al*[7] found a two-fold increase in permeability to ^{14}C-polyethylene glycol (PEG)-400 of CD patients and their healthy relatives as compared to normal controls, suggesting intestinal barrier leak may precede clinical intestinal inflammation and be an etiologic factor in IBD. However the overall involvement of compromised barrier function in IBD is not debated.

TNF-α is a key proinflammatory cytokine involved in intestinal inflammation in IBD[6]. Experimentally it has been shown to increase epithelial TJ permeability in several cell types including the human intestinal epithelial monolayers: CACO-2, T84, and HT29/B6[1,8-11]. In CACO-2, TNF-α decreased transepithelial electrical resistance in both a dose- and time- dependent manner[1,9,10]. A decrease in transepithelial electrical resistance (TER) was shown after 24 h incubation with 100 ng/mL TNF-α which reached a maximum at 48 h, and was sustained for up to 8 d after TNF-α removal[10]. TNF-α reduction of TER was associated with an increase in mannitol (182 MW) as well as inulin (5000 MW) permeability[9]. The exact characteristics of the TNF-α effect on barrier function vary across different cell lines. In the renal epithelium model, LLC-PK1, TNF-α produces a rapidly reversible reduction of TER within 2 h and is accompanied by increased permeability to molecules as large as PEG (4000 MW)[11]. TNF-α was also capable of producing a pronounced effect on HT29/B6 cell layers, reducing TER by 81% of the control[12]. The mechanism by which TNF-α generates leak in CACO-2 cell layers has been shown not to be simply a direct result of apoptosis, but rather attributed to TNF-α's ability to activate NF-κB, induce myosin light chain kinase (MLCK) protein expression and activity, and engender TJ leak[9].

The proinflammatory cytokine IFN-γ has also been reported to increase TJ permeability across T84, HT29/B6, and CACO-2 cell layers[1,13]. T84 cells treated basal-laterally with IFN-γ for 24 h showed a decrease in TER that continued for five days after exposure[13]. Watson *et al*[14] (2005) demonstrated that in T84 cell layers, IFN-γ caused a greater increase in permeability to larger-sized than to smaller-sized molecules, proposing that IFN-γ selectively activates specific permeation pathways within the TJ. In several studies, using both HT29/B6 and CACO-2 cell layers, TNF-α has been used in combination with IFN-γ to produce a synergistic effect on TER[11,15]. In the CACO-2 model, IFN-γ (10 ng/mL) and TNF-α (2.5 ng/mL) individually did not have an effect on TER or paracellular flux of 3 kD dextran. However, when the cell layers were first primed with IFN-γ for 24 h followed by treatment with TNF-α for 8 h, the cells exhibited both a significant decrease in TER and an increase in 3 kD dextran flux[15].

Increased levels of IL1β in IBD patients have also

been associated with increased intestinal inflammation[16]. In CACO-2 cell layers, IL1β caused a drop in TER that was maximal after 48 h treatment. This decrease in TER was accompanied by approximately a 20-fold increase in paracellular permeability to inulin. IL1β was also shown to affect TJ proteins, inducing a decrease in occludin protein expression and an increase in claudin-1 expression. The CACO-2 TJ permeability increase as a result of IL1β exposure involved NF-κB activation and *MLCK* gene regulation, not induction of apoptosis. Studies by Al-Sadi *et al*[17,18] suggest a role for p38-kinase dependent activation of the nuclear transcription factor, activating transcription factor-2.

Increased production of mucosal-damaging oxygen radicals by white blood cells has been shown in IBD[19]. Decreased nutritional intake of a variety of antioxidants in IBD patients can lead to an imbalance that causes an additional increase in reactive oxygen species levels resulting in exacerbated oxidative stress of the inflamed intestinal tissue[20,21]. Furthermore, Strus *et al*[22] (2009) demonstrated that hydrogen peroxide-producing bacteria, present in samples from IBD patients, may be another contributing factor behind increased hydrogen peroxide in the IBD mucosa. In the literature, oxidative stress of CACO-2 cells induced by treatment with H_2O_2 causes increased paracellular permeability as evidenced by a decrease in TER, as well as increases in both mannitol and inulin flux. Hydrogen peroxide is capable of disrupting the TJ through a specific mechanism that involves protein tyrosine phosphorylation[23-25].

The objectives of the following study were to: (1) Observe the effects of the proinflammatory cytokines, TNF-α, IFN-γ, IL1β, and H_2O_2, alone and in combination, on CACO-2 barrier function in order to create an *in vitro* model of graded leak that can reflect the clinical situation in IBD at different sites along the intestinal mucosa; (2) determine if this leak allows for barrier breakdown to biologically active proteins such as epidermal growth factor (EGF); and (3) determine if a previously described nutraceutical capable of barrier protection can in fact reduce barrier compromise under these extreme conditions.

MATERIALS AND METHODS

Cell culture

The CACO-2 cell culture, an epithelial cell line derived from human colon adenocarcinoma[26], was used between passages 52 and 64. Upon confluence, cells were passaged on a weekly basis by trypsinization [0.25% trypsin, 2.2 mmol/L EDTA (Corning Cellgro, Manassas, VA)] and were seeded at 7.5 × 10^5 cells/Falcon 75-cm^2 culture flask with 25 mL of Dulbecco's Modified Minimum Essential Medium (Corning Cellgro) supplemented with 2 mmol/L L-Glutamine, 1% non essential amino acids, 1 mmol/L Sodium Pyruvate (all culture medium additives, Corning Cellgro) and 10% defined fetal bovine serum (HyClone, Logan, UT). Cultures were incubated at 37 ℃ in 95% air-5% CO_2 atmosphere.

Treatment with cytokines and/or hydrogen peroxide

Human recombinant proteins TNF-α, IL1β and IFN-γ were obtained from Life Technologies (Frederick, MD). For individual exposures and combinations of cytokine treatment, 200 ng/mL TNF-α, 50 ng/mL IL1β, and between 100 and 200 ng/mL IFN-γ were applied (in complete medium) to both the apical and basal-lateral compartments for 48 h. This combination of three cytokines is referred to in this manuscript as "cytomix" for purposes of brevity. Media was first filter sterilized with a 0.2 μm disc filter unit (Corning). Seven- and twenty-one day post-confluent CACO-2 cell layers were used in our studies, as barrier function at these days is highly similar in CACO-2 monolayers, and we did not observe a difference between their responses to cytokines. For hydrogen peroxide (Sigma Life Science, St. Louis, MO) exposure, with or without a prior 48 h incubation with cytokines, the CACO-2 cells were treated both apically and basal-laterally with 2 mmol/L hydrogen peroxide in Dulbecco's Phosphate Buffered Saline containing calcium and magnesium (Corning Cellgro) supplemented with 5 mmol/L glucose for 5 h. The dosages of cytokines and hydrogen peroxide were fixed in a given experiment; however, over the course of experiments, concentration and exposure time were reduced, yet conditions remained capable of achieving the maximum effect, as we had been using saturating levels of cytokines in a receptor-mediated response.

Transepithelial electrophysiology and permeability

Cells were seeded into sterile Millipore Millicell polycarbonate (PCF) permeable supports (30 mm diameter with 0.4 μm pore size) on day 0 at a seeding density of 5 × 10^5 cells/insert. Four sterile Millicell PCF inserts were placed into a 100 mm petri dish. On day 1, all cell layers were refed (2 mL apical/15 mL basal-lateral) with control medium containing penicillin (50 U/mL) and streptomycin (50 mcg/mL), followed by refeedings every 2-3 d until exposure. Depending on the specific exposure combination, cells were fed medium supplemented with the appropriate cytokines for 48 h treatment, followed in certain experiments with 5 h of peroxide exposure, then followed by transepithelial electrophysiological measurements and radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol (PerkinElmer, Boston, MA), 0.1 mmol/L, 0.25 μCi/mL ^{3}H-Lactulose (American Radiolabeled Chemicals, Inc., St Louis, MO) and/or 0.1 mmol/L, 0.3 μCi/mL ^{14}C-Polyethylene glycol (PerkinElmer, Waltham, MA).

On the day of transepithelial experiments (for cells treated with cytokines only), the cell layers were refed with fresh control medium and allowed to incubate at 37 ℃ for 1 to 1.5 h prior to electrophysiological readings. Potential difference, TER, and short-circuit current (I_{sc}) were measured using 1 s, 40 μA direct current pulses, with TER calculated using Ohm's law. As soon as electrical measurements were completed, the basal-lateral medium was aspirated and replaced with

15 mL of medium containing the appropriate radioisotope and incubated at 37 ℃. Triplicate basal-lateral medium samples were taken for liquid scintillation counting (LSC) for specific activity (cpm/micromole) determination. Duplicate samples were taken from the apical medium at 60 and 120 min for LSC to determine radioisotope flux rates. The media lost due to sampling from the apical compartment were replaced with fresh medium of the same sample volume. The flux rate (in cpm/min per square centimeter and pmol/min per square centimetre) was calculated for the radioisotope diffusing across the cell layer. In experiments that included exposure to hydrogen peroxide, the cell layers were rinsed in saline before being refed with saline, with or without hydrogen peroxide. After five hours of incubation, the basal-lateral saline was aspirated and replaced with 15 mL of saline containing 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol. Triplicate basal-lateral samples and duplicate apical samples were taken at 75 min, and the flux rate was calculated as before.

Paracellular flux of ^{125}I-EGF

CACO-2 cell layers, treated as described above prior to exposure, were refed in control medium or medium containing 50 ng/mL TNF-α, 100 ng/mL IFN-γ, and 50 ng/mL IL1β in the apical and basal-lateral compartments for 48 h. On the day of experimental measurements, the cell layers were exposed to control saline or saline containing 1 mmol/L hydrogen peroxide for 3 h. The apical saline was then replaced with medium containing 0.5 μCi/mL, 10 mmol/L ^{125}I-EGF and the basal-lateral saline was replaced with control medium. After a 2 h incubation period, apical and basal-lateral samples were taken for LSC to determine EGF flux rates. Basal-lateral medium was also sampled for column (G-25) chromatography analysis. Total ^{125}I-EGF flux rates (as cpm/min per square centimetre) were adjusted based upon the percent of intact ^{125}I-EGF in the basal-lateral compartment.

Pretreatment with berberine chloride prior to cytokine exposure

Seven-day post-confluent CACO-2 cell layers were refed in control medium or medium containing 100 μmol/L berberine chloride in the apical and basal-lateral compartments. A berberine chloride (Sigma-Aldrich) stock solution (2.7 mmol/L) was prepared in deionized distilled water, but was made each day at the time of use. Following a 24 h berberine pretreatment, the appropriate cell layers were continued in control medium or berberine medium and additionally exposed to either no cytokines, TNF-α, or cytomix for 48 h prior to transepithelial electrophysiology and permeability measurements. For studies that included hydrogen peroxide exposure, on the day of the experiment the cell layers were treated for 5 h with control saline or saline containing 1 mmol/L hydrogen peroxide ± berberine.

Statistical analysis

For electrophysiology and radiotracer flux studies, cytokine- and/or hydrogen peroxide-exposed cell samples were compared against appropriate matched controls within the same experiment. All data are expressed as the mean ± standard error of the mean with the number of replicates provided for each set of studies. Differences between means are evaluated by two-sided Student's *t* tests for two groups or by one-way ANOVA followed by Tukey's *post hoc* testing where multiple conditions existed.

RESULTS

Exposure to TNF-α

Treatment for 48 h of 7-d post-confluent CACO-2 monolayers with apical and basal-lateral 200 ng/mL TNF-α resulted in a 50% decrease in TER (Figure 1A). The reduction in TER was associated with only a marginal statistically significant increase in transepithelial leak of ^{14}C-D-mannitol (Figure 1B). Unlike the consistent decrease in TER, this increase in mannitol flux did not always achieve statistical significance within each individual experiment, as exemplified by the lack of statistical significance in Figure 2A.

Exposure to TNF-α and IFN-γ

Forty-eight hours combined exposure to 200 ng/mL TNF-α and 100-200 ng/mL IFN-γ (apical and basal-lateral) also caused a significant decrease in TER. Interestingly, this 35% reduction of TER was consistently less than that produced by TNF-α alone (50%) (Figure 2B). A simultaneous slight increase in mannitol flux (J_m) was again observed (Figure 2A), although not quite achieving statistical significance. Exposure to IFN-γ alone reduced TER by only 20% and did not have a significant effect on J_m.

Exposure to TNF-α, IFN-γ, and IL1β

Exposure of 21-d post-confluent CACO-2 cell layers to TNF-α, IFN-γ and 50 ng/mL IL1β (cytomix) on both cell surfaces led to a similar decrease of TER as seen with the combination of TNF-α and IFN-γ (approximately 30%-35% decrease) (Figure 3A). IL1β alone did not generate leak any greater than TNF-α or IFN-γ achieved individually (data not shown). Upon adding IL1β to the mixture of TNF-α and IFN-γ, there was now however a dramatic and consistent 100% increase in J_m (Figure 3B). Further investigation into the impact of cytomix on paracellular permeability showed that the leak pathway produced did not however allow for an increase in flux of the larger probe molecules, ^{3}H-lactulose (MW 342) or ^{14}C-PEG (MW 4000) (Figure 4).

Exposure to cytomix and hydrogen peroxide

Treatment of both 7- and 21-d post-confluent CACO-2 cell layers with cytomix for 48 h followed by 5 h exposure to 2 mmol/L H_2O_2 (apical and basal-lateral) induced on average a 500% increase in PEG transepithelial leak. H_2O_2 alone caused only a 35% increase in PEG leak (Figure 5). In the PEG flux studies, column (G-25)

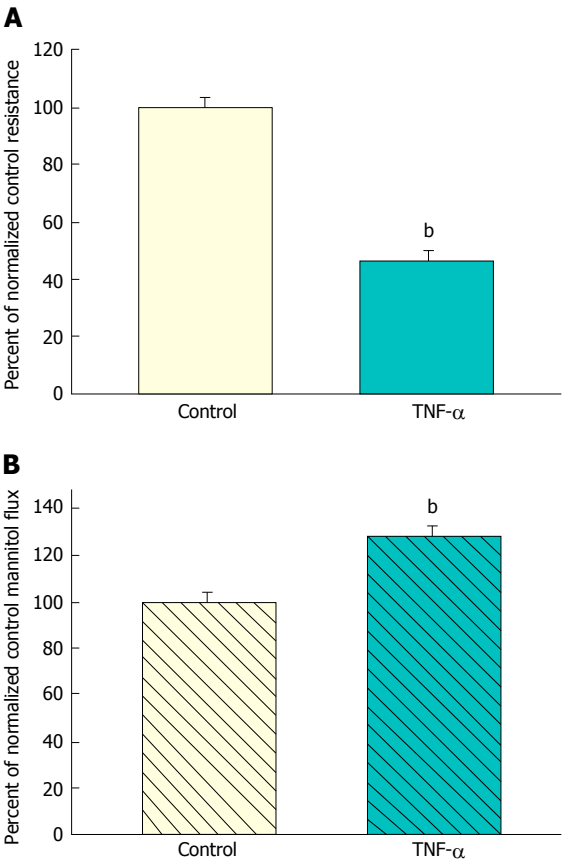

Figure 1 The effect of tumor necrosis factor-α on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 200 ng/mL tumor necrosis factor-α (TNF-α) (apical and basal-lateral compartments) 48 h prior to electrical measurements. Data shown represent the mean ± SE of 16 cell layers per condition. Data represent the percent of control resistance normalized across 3 experiments; B: After electrical measurements, radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL 14C-D-mannitol were performed on CACO-2 cell layers, as described in Materials and Methods. Data represent the percent of control flux rate normalized across 4 experiments, and is expressed as the mean ± SE of 20 cell layers per condition. $^{b}P < 0.001$ *vs* control (Student's *t* test, one-tailed).

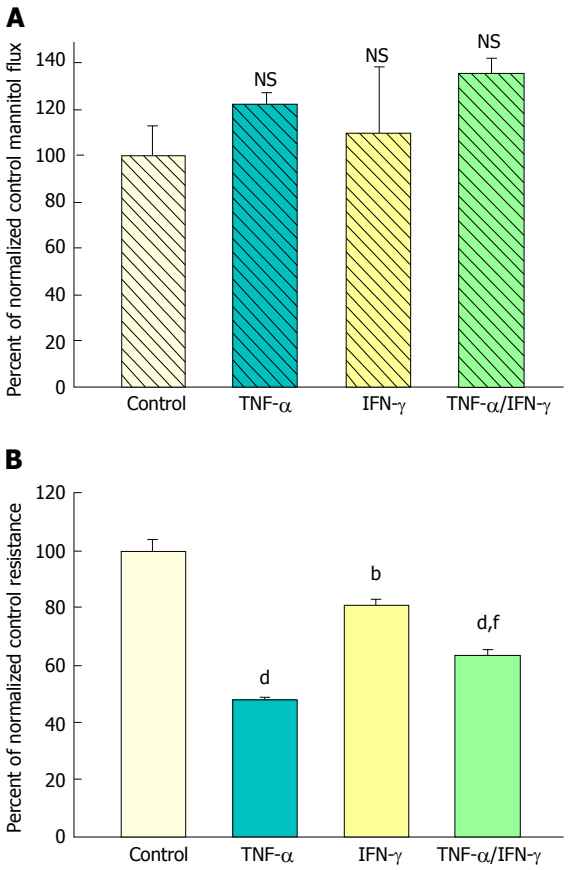

Figure 2 The effect of tumor necrosis factor-α and interferon-γ on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Radiotracer flux studies were conducted as described in Figure 1 with the treatment conditions listed above. Data represent the percent of control flux rate, and is expressed as the mean ± SE of 4 cell layers per condition. NS indicates non significance *vs* control; B: CACO-2 cell layers were cultured and treated as described in Figure 1, using the following conditions: Control medium; medium containing 200 ng/mL tumor necrosis factor-α (TNF-α); medium containing 200 ng/mL Interferon-γ (IFN-γ); or medium containing a combination of 200 ng/mL TNF-α and 200 ng/mL IFN-γ. Data shown represent the mean ± SE of 4 cell layers per condition, with data expressed as the percent of control resistance. $^{b}P < 0.01$ *vs* control; $^{d}P < 0.001$ *vs* control; $^{f}P < 0.01$ *vs* TNF-α alone (one-way ANOVA followed by Tukey's *post hoc* testing).

chromatography was used to verify leak of a 4000 MW species of PEG (data not shown). After the 5 h H_2O_2 exposure, the combination of cytomix and H_2O_2 resulted in an 80%-90% decrease of TER (data not shown).

Hematoxylin and eosin-stained cross sections of CACO-2 cell layers were used to evaluate the histological effects of the various cytokine/H_2O_2 exposure regimens. As shown in Figure 6, cytomix alone produced no observable morphological changes in cross sections of the epithelial cell layer. Exposure to 2 mmol/L H_2O_2 resulted in increased blebbing of membranes from the apical surface of occasional cells. Exposure to both cytomix and peroxide induced not only blebbing of apical membranes in occasional cells but also frequent apoptotic nuclei and rare, though occasional, sites of cell detachment.

Transepithelial leak of EGF

CACO-2 cell layers exposed for 48 h to 50 ng/mL TNF-α, 100 ng/mL IFN-γ, and 50 ng/mL IL1β followed by a 3 h 1 mmol/L H_2O_2 treatment manifested a transepithelial leak pathway that allowed for not only a leak to 4000 MW PEG, but also an over 30-fold increase in ^{125}I-EGF permeation. This EGF flux was performed in an apical to basal-lateral direction to mimic the diffusion gradient for EGF that would exist *in vivo* (Table 1). In these EGF studies, the leak of ^{125}I isotope across the cell layer was analyzed by gel filtration chromatography to determine the amount of transepithelial isotope diffusion that corresponded solely with the compound of interest, 6100 MW EGF. Transepithelial leak of actual 6100 MW EGF - and not simply ^{125}I-EGF degradation products - was thus verified.

Evaluation of berberine as a potential therapeutic agent using the graded transepithelial leak model

A major benefit of this *in vitro* model of graded epithelial barrier leak is the capability of evaluating a great number

Table 1 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β and hydrogen peroxide on transepithelial flux of ^{14}C-polyethylene glycol and ^{125}I-epidermal growth factor

	^{14}C-PEG flux		^{125}I-EGF flux	
	cpm/min per square centimeter	pmol/min per square centimeter	cpm/min per square centimeter	fmol/min per square centimeter
Control	3.74 ± 0.07	7.54 ± 0.20	43.7 ± 2.0	0.034 ± 0.005
Cytomix	3.40 ± 0.12	6.87 ± 0.27	30.3 ± 1.4	0.015 ± 0.001
Cytomix/H_2O_2	9.80 ± 0.28[a]	20.97 ± 0.64[a]	192.0 ± 4.0[c]	1.21 ± 0.029[c]

[a]$P < 0.05$ *vs* control; [c]$P < 0.05$ *vs* Cytomix-only condition (one-way ANOVA followed by Tukey's *post hoc* testing). CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing the combination of 50 ng/mL tumor necrosis factor-α, 100 ng/mL interferon-γ, and 50 ng/mL interleukin-1β (apical and basal-lateral compartments) for 48 h. On the day of radiotracer flux studies, the cell layers were exposed to control saline or saline containing 1 mmol/L hydrogen peroxide for 3 h. These studies were performed using 0.1 mmol/L, 0.025 μCi/mL ^{14}C-polyethylene glycol (MW 4000) and 10 nmol/L, 0.5 μCi/mL ^{125}I-EGF (MW 6100), as described in Materials and Methods. Total ^{125}I flux rates (as cpm/min per square centimeter) were adjusted, based upon the percent of intact ^{125}I-EGF in the basal-lateral compartment (using column chromatography), and expressed finally as fmol/min per square centimeter. Similar gel chromatography analyses were performed for ^{14}C-PEG experiments, but here all isotopes that diffused across the epithelial cell layer was found to be 4000 MW PEG. Data shown represent the mean standard error for an $n = 8$ in all cases. PEG: Polyethylene glycol; EGF: Epidermal growth factor.

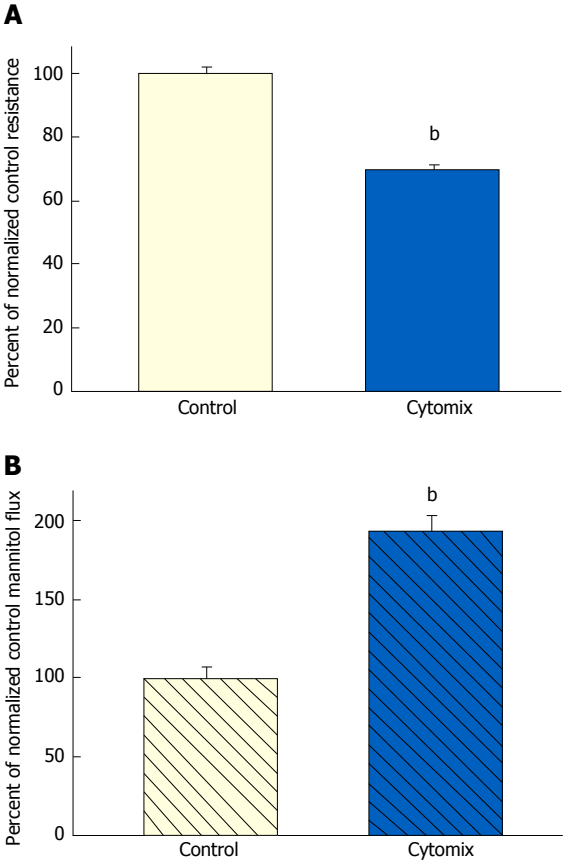

Figure 3 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β on CACO-2 transepithelial electrical resistance and transepithelial flux of ^{14}C-D-mannitol. A: Twenty-one day post-confluent CACO-2 cell layers cultured and treated as described in Figure 1, were refed in control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 150 ng/mL interferon-γ, and 50 ng/mL interleukin-1β. Data shown represent the mean ± SE of 16 cell layers per condition. Data represent the percent of control resistance normalized across 4 experiments; B: Radiotracer flux studies were conducted as described in Figure 1, with the same conditions listed above for panel A. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE of 8 cell layers per condition. [b]$P < 0.001$ *vs* control (Student's *t* test, one-tailed).

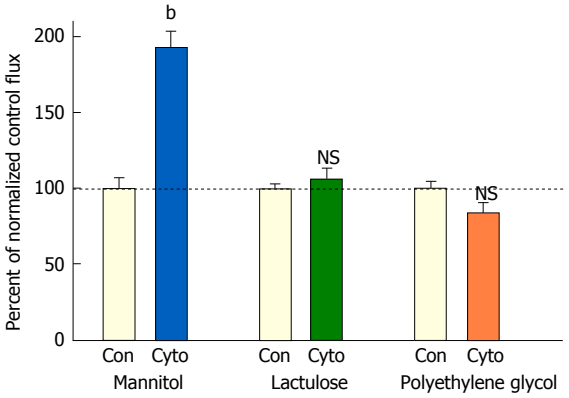

Figure 4 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β on transepithelial flux of ^{14}C-D-mannitol, ^{3}H-lactulose, and ^{14}C-polyethylene glycol across CACO-2 cell layers. Twenty-one day post-confluent CACO-2 cell layers on Millipore PCF filters were refed in control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 150 ng/mL interferon-γ, and 50 ng/mL interleukin-1β (apical and basal-lateral compartments) 48 h prior to radiotracer flux studies. These studies were performed using 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol; 0.1 mmol/L, 0.25 μCi/mL ^{3}H-lactulose; and 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol as described in Materials and Methods. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE of 8 cell layers per condition for the mannitol flux and 4 cell layers per condition for both the lactulose and polyethylene glycol fluxes. NS indicates non significance. [b]$P < 0.001$ *vs* control (Student's *t* test, one-tailed).

of potentially efficacious micronutrients - or combinations thereof - for reducing each type of leak that may occur across the surface of the IBD mucosa. In this study, 100 μmol/L berberine chloride pretreatment and simultaneous exposure was evaluated for its effects on the ability of TNF-α and cytomix to impair CACO-2 barrier function. Berberine treatment not only increased basal TER, but also reduced both the TNF-α- and cytomix-induced decrease in TER (Figure 7A). Additionally, berberine reduced the TNF-α- and cytomix-induced increase in J_m (Figure 7B). Berberine also effectively - and significantly - reduced the macromolecule leak resulting from cell layer exposure to cytomix and H_2O_2 (Figure 8A). Berberine likewise reduced both the H_2O_2- and cytomix + H_2O_2-induced decrease in resistance (Figure 8B).

DISCUSSION

Colon mucosa in active IBD is rarely homogeneously inflamed; rather, there is typically much heterogeneity

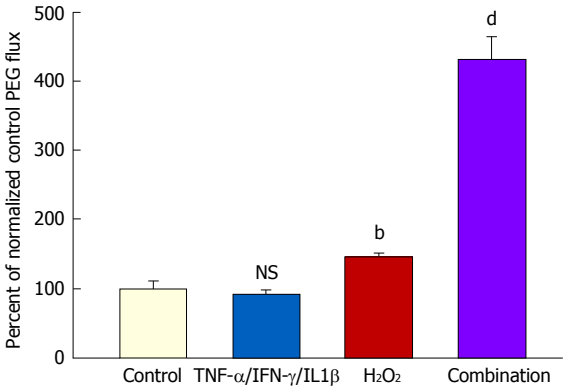

Figure 5 The effect of tumor necrosis factor-α + interferon-γ + interleukin-1β plus hydrogen peroxide on transepithelial flux of ^{14}C-polyethylene glycol. Seven-day and 21-d post-confluent CACO-2 cell layers on Millipore PCF filters were refed in control medium or medium containing the combination of 200 ng/mL TNF-α, 200 ng/mL IFN-γ, and 50 ng/mL IL1β (apical and basal-lateral compartments) 48 h prior to radiotracer flux studies. On the day of the experiment, the cell layers were treated for 5 h with control saline or saline containing 2 mmol/L H_2O_2. Paracellular permeability was assessed using 0.1 mmol/L, 0.3 μCi/mL ^{14}C-polyethylene glycol as described in materials and methods. Data represent the percent of control flux rate normalized across 2 experiments, and is expressed as the mean ± SE for 8 cell layers per condition. NS indicates non significance *vs* control. $^{b}P < 0.01$ *vs* control; $^{d}P < 0.001$ *vs* control (one-way ANOVA followed by Tukey's *post hoc* testing). IFN-γ: Interferon-γ; IL1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α.

across the apical surface. This can range from grossly normal, non-inflamed tissue, to normal-appearing tissue but with histological evidence of inflammation (seen, *e.g.*, in white blood cell infiltration in stained tissue sections), to tissue that appears obviously inflamed grossly. In addition, there can be granulomas, pseudopolyps and even micro-ulceration areas that are denuded of epithelium[27-29]. It is well known that inflammation can lead to increased epithelial barrier leak through elevated cytokine levels and other mechanisms[30]. Such grossly observed heterogeneity reflects itself in different degrees of barrier compromise. This variability in barrier compromise can manifest itself not only in the quantitative magnitude of leak, but also in the types of solutes able to leak across the epithelial barrier. As described in Mullin *et al*[31] (1997) and Watson *et al*[14] (2005), induction of transepithelial paracellular leak by different agents can result in leak to only small molecules or it can extend to paracellular permeation of macromolecules.

A decrease in TER typically signifies (in "low resistance" epithelial tissues like ileum or colon or CACO-2 cell layers) increased paracellular conductance or diffusion of Na^+ and Cl^- ions. It implies nothing about potential leak to larger (or uncharged) molecules. And as shown in Figure 1, such was the situation that we observed when CACO-2 cell layers were treated with TNF-α - a significant decrease in TER with only a marginal (and variable) increase in leak to D-mannitol [and no significant leak to the disaccharide lactulose (MW 342) (data not shown)]. This implies that paracellular pathways to Na^+ and Cl^- ions were increased by TNF-α treatment of the cell layers, but the paracellular pathways that would allow D-mannitol (MW 182) (or larger molecules) to pass, were hardly affected.

However, when cell layers were also treated with IL1β and IFN-γ as well as TNF-α, a sizable increase (100%) in D-mannitol leak was combined with the TER decrease. Obviously, paracellular leak pathways that would admit D-mannitol, were now being induced. This signifies a different type of paracellular pathway that would likely allow for transepithelial paracellular leak of monosaccharides and perhaps neutral amino acids as well as inorganic salts. This could have an effect on the efficiency of gastrointestinal nutrient absorption, as well as ATP consumption by gastrointestinal mucosa. But it is difficult to see how this type of leak would induce inflammation in GI mucosa, since luminal molecules (antigens) capable of eliciting an inflammatory response are typically much larger. It is noteworthy that in our study, even with the increased leak of D-mannitol, leak to the larger probe molecules, lactulose and PEG (4000 MW), was unaffected (Table 2). This not only implies distinct paracellular leak pathways for these different molecules, but also shows that induction of paracellular leak can be a staged, graded phenomenon. Only when exposure of cell layers to cytokines was combined with subsequent treatment with hydrogen peroxide (a situation that reflects certain inflamed tissue in IBD) was a paracellular leak to large molecules observed.

Transepithelial leak can be both a manifestation of morbidity as well as a driver of morbidity, depending upon the nature of the molecules that are leaking. If leak is induced only to Na^+ and Cl^- ions (as we observed with TNF-α treatment of CACO-2 cell layers), a situation exists that may not generate serious morbidity. Induced leak to D-mannitol along with decreased TER, however, has implications for the physiological efficiency of nutrient absorption that may then have metabolic/bioenergetic implications for the organism. However, induced leak to molecules larger than D-mannitol, as we observed only for combined treatment with cytokines and peroxide, is the situation most problematic in IBD, because now there can be leak of peptides/proteins present in the GI lumen, into the interstitial fluid compartment, where activation of inflammatory cascades is possible. Substances normally sequestered in the GI lumen such as bacterial toxins and antigens, could - at least on a basis of size - now leak across the epithelial barrier into the interstitial compartment under the epithelium. Bacterial toxins such as *Clostridium perfringens* enterotoxin (CPE) - which is active from only the abluminal compartment[32] - as well as simple lipopolysaccharide endotoxin, might begin to permeate and either simply raise an immune response (and more cytokine production) in the interstitium or further damage the epithelial barrier directly (in the case of CPE) and compound the barrier compromise even further.

A protein not often considered in the paracellular leak scenarios out of the GI lumen and across a compromised GI barrier is the potent mitogenic growth factor, EGF. EGF

Table 2 Summary of the magnitude of effect of combinations of cytokines and hydrogen peroxide on transepithelial electrical resistance, mannitol leak, lactulose leak and polyethylene glycol leak

	Transepithelial electrical resistance decrease	Mannitol leak increase	Lactulose leak increase	Polyethylene glycol leak increase
TNF	++	[1]	[0]	ND
TNF/IFN	+	[1]	[0]	ND
TNF/IFN/IL1β	+	++	[0]	[0]
TNF/IFN/IL1β + hydrogen peroxide	++++	++++	++++	++++

CACO-2 cell layers having been exposed to the above combinations of cytokines and hydrogen peroxide were evaluated for type (salts, mannitol, lactulose, PEG) and magnitude of leak produced. All treatments increased leakage of salts as seen by a decrease in TER, but large molecule leak was seen only in the presence of peroxide alone or both cytokines and hydrogen peroxide, never in the presence of cytokines alone. [1]Indicates that the observed effect did not routinely achieve statistical significance; [0]Indicates that an effect was measured but no significant change was observed. ND: Not determined; IFN-γ: Interferon-γ; IL1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; PEG: Polyethylene glycol.

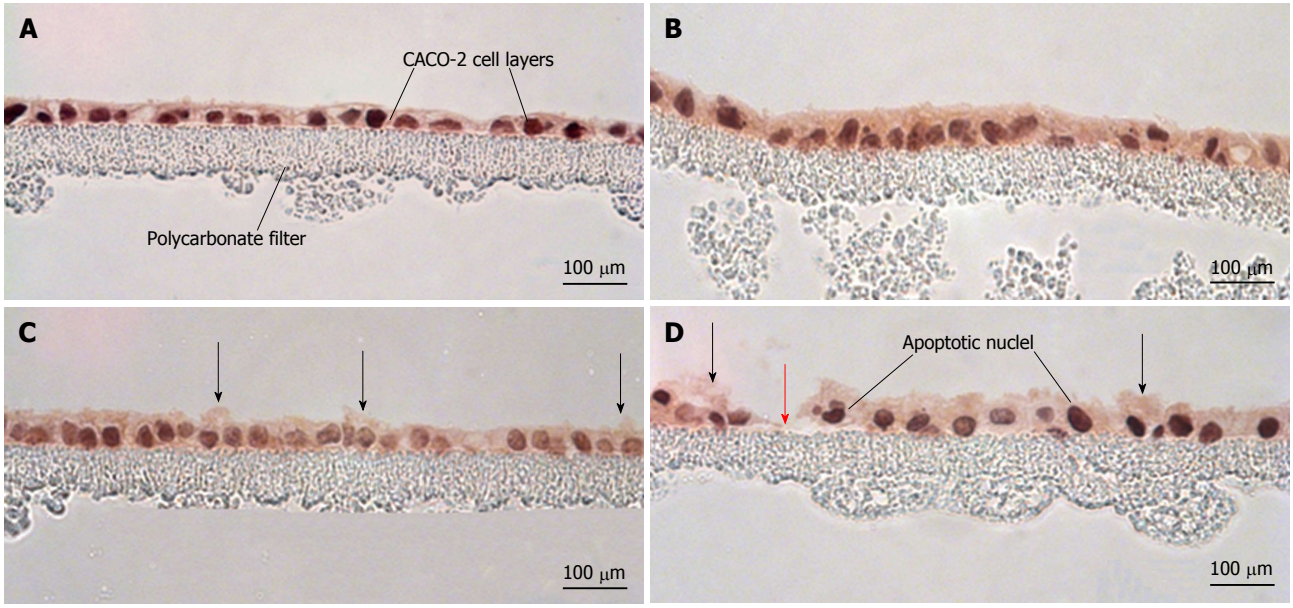

Figure 6 Morphological effects of cytokines and hydrogen peroxide on CACO-2 cell layers. CACO-2 cell layers were cultured at confluent density onto Millipore polycarbonate filters. Seven day post-seeding, the cell layers were refed with control medium or medium containing the combination of 200 ng/mL tumor necrosis factor-α, 200 ng/mL interferon-γ, and 50 ng/mL interleukin-1β ("cytomix") (apical and basal-lateral compartments) for 48 h, followed by exposure to saline or saline containing 2 mmol/L H_2O_2 for 5 h. Cell layers were then fixed in formalin and stained with hematoxylin and eosin. A: CACO-2 cell layers exposed to control medium and control saline; B: CACO-2 cell layers exposed to cytomix medium and control saline; C: CACO-2 cell layers exposed to control medium and saline containing hydrogen peroxide; D: CACO-2 cell layers exposed to cytomix medium and saline containing hydrogen peroxide. In C and D, black arrows indicate instances of cytoplasmic blebbing. The red arrow points at a gap in the epithelial barrier arising from cell death and detachment.

(MW 6100) exists in the GI lumen at concentrations over a thousand fold greater than that in the bloodstream, as a result of EGF synthesis and vectorial secretion by salivary glands and Brunner's glands[33,34]. This EGF is typically biologically inactive however because its receptors are found on the abluminal side of GI epithelia and on interstitial fibroblasts - not on the apical surface of epithelia[35]. However, we show (Table 1) that combined treatment with cytokines and peroxide allows for EGF leak out of the luminal compartment - a situation predicted from the increased leak of PEG (Figure 5). This leakage of luminally situated EGF (down a very steep concentration gradient) into the interstitium may be a major contributing cause for the increased risk of neoplasia in UC, because one could now be putting colonic epithelial cells (whose DNA may be compromised by increased free radical generation) under a near constant replication stimulus.

The greatest utility of the *in vitro* model of graded transepithelial leak being presented here may derive however from the recent research surrounding improvement/recovery of epithelial barrier function by a diverse - and growing - array of natural (*e.g.*, micronutrients) and synthetic enhancers of TJ barrier function. There have been numerous recent reviews and publications focused on the ability of certain micronutrients like zinc, berberine, quercetin, butyrate, indole, *etc*., to modify TJ protein composition and in the process yield a TJ - and epithelial barrier - that is less leaky[36-39]. The list of naturally occurring compounds with this capability being reported in the biomedical literature is expanding year by year. One must consider not only the action of these agents in isolation, but also contend with the possibility that certain combinations of these agents can

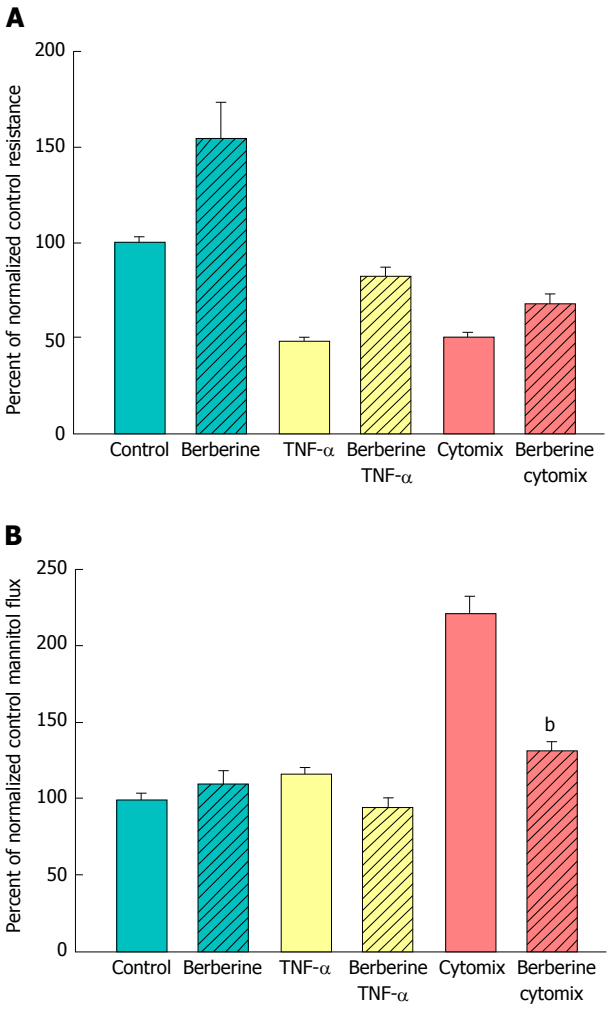

Figure 7 The effect of berberine on cytokine-induced leak of CACO-2 cell layers. A: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 100 μmol/L berberine. After 24 h treatment with berberine alone, the cell layers were given either control or berberine medium in addition to being exposed to no cytokines, TNF-α alone, or cytomix (apical and basal-lateral compartments) for 48 h prior to electrical measurements. Data shown represent the mean ± SE of 8 cell layers per condition. Data represent the percent of control resistance normalized across experiments; B: After electrical measurements, the same CACO-2 cell layers represented in A were used to perform radiotracer flux studies with 0.1 mmol/L, 0.1 μCi/mL ^{14}C-D-mannitol, as described in Materials and Methods. Data represent the percent of control flux rate normalized across experiments and is expressed as the mean ± SE of 8 cell layers per condition. $^{b}P < 0.001$ *vs* cytomix alone (one-way ANOVA followed by Tukey's *post hoc* testing). TNF-α: Tumor necrosis factor-α.

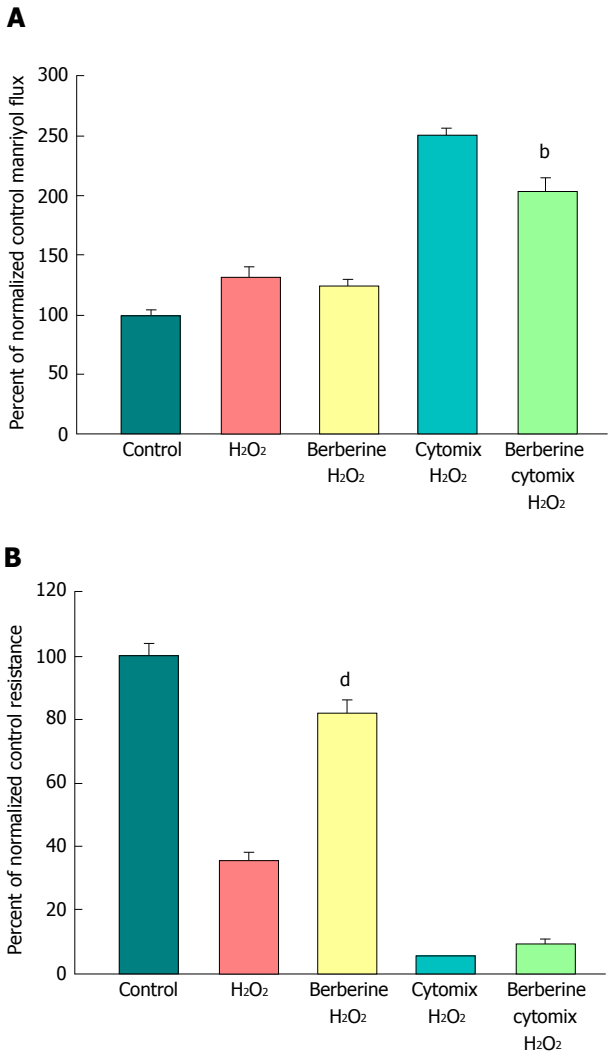

Figure 8 The effect of berberine on cytokine- and peroxide-induced leak of CACO-2 cell layers. A: After electrical measurements, the same CACO-2 cell layers represented in B were used to perform radiotracer flux studies with 0.1 mmol/L, 0.025 μCi/mL ^{14}C-PEG. Data represent the percent of control flux rate, and is expressed as the mean ± SE of 4 cell layers per condition (3 cell layers for the condition of cytomix + peroxide, due to removal of one outlier data point, as determined by a 90% confidence level in the Dixon's Q test); B: Seven-day post-confluent CACO-2 cell layers on Millipore polycarbonate filters were refed in control medium or medium containing 100 μmol/L berberine. After 24 h treatment with berberine alone, the cell layers were given either control or berberine medium, in addition to being exposed to either cytomix (50 ng/mL tumor necrosis factor-α, 100 ng/mL interferon-γ, 50 ng/mL interleukin-1β) or no cytokines (apical and basal-lateral compartments) for 48 h. On the day of the experiment, the cell layers were treated for 5 h with control saline or saline containing 1 mmol/L hydrogen peroxide ± berberine. Data shown represent the mean ± SE of 4 cell layers per condition, with data expressed as the percent of control resistance. $^{b}P < 0.01$ *vs* cytomix + H_2O_2; $^{d}P < 0.001$ *vs* H_2O_2 alone (one-way ANOVA followed by Tukey's *post hoc* testing). Experiment was repeated with similar results.

display even greater efficacy than single agents alone in remodeling and enhancing TJs[40]. In short, it is a situation that demands the testing of a large number of agents and permutations of agents. Moreover, one needs to test not only for improvement of basal barrier function, but also for ability to offset the action of proinflammatory proteins and molecules that compromise barrier function. And as we described above, those agents can induce different states of leak, further complicating the testing situation. An *in vitro* system like what we describe here is ideal for the testing of a large number of different enhancers of barrier function in a range of distinct leak states. This is particularly needed because agents that successfully enhance barrier function in one permeability state of a barrier may or may not be effective in yet another permeability state. As an example, the effectiveness of berberine in reducing leak was tested in this *in vitro* system and found to not only enhance basal CACO-2 barrier integrity but also to reduce the proinflammtory cytokine - induced compromise in epithelial barrier function (Figure 7). It is the first demonstrated effectiveness of berberine in the context of selective

leak induction by treating epithelial cell layers with combinations of cytokines + peroxide. Berberine's effectiveness in attenuating not only cytokine-induced leak to small molecules but also the transepithelial leak to larger molecules that is seen with cytokines + peroxide, portends potential clinical therapeutic value for IBD.

In our studies, berberine was presented to both cell surfaces simultaneously, as has also been done in earlier studies with berberine and CACO-2 cell layers, studies also showing berberine effectiveness in reducing cytokine-induced barrier disruption[41]. Future studies by our group will evaluate potential sidedness aspects to berberine's effectiveness in this model. Basal-lateral effectiveness of berberine has been shown pointedly by Taylor *et al*[42,43] in colon tissue studies. *In vivo* studies where berberine has been proven effective when given orally[44] may suggest an action from the apical surface, but it is equally possible that berberine is diffusing across damaged epithelial mucosa and engaging the cell from the basal-lateral surface. This is in fact suggested in studies where mucosal barrier damage is modeled through the use of cytochalasin-D treatment[43], and studies showing poor intestinal absorption of berberine into the bloodstream[45].

In conclusion, the expanding array of compounds that are effective in reducing leak across epithelial barriers, both basal as well as induced leak, may be the source of an entirely new class of therapeutics in IBD, therapeutics that could work in complementarity to agents that reduce inflammatory response directly, such as the anti-TNF-α drugs or the salicylates. A need exists for a cell culture model system that could allow large numbers of such compounds (and their combinations) to be tested *in vitro* before animal model and human studies are undertaken.

COMMENTS

Background

In inflammation and inflammatory diseases impacting epithelial cell layers, there are many molecular agents assaulting the epithelial barrier. Pro-inflammatory cytokines such as tumor necrosis factor-α, interferon-γ and interleukin-1β, and the chemical hydrogen peroxide, are four very common entities in the inflammatory microenvironment that impact an epithelium. In this study, the authors considered that each agent and combinations of agents may have unique effects in terms of the barrier leak that they produce. The authors asked the question of whether these varying effects would confer leakiness to different sizes of molecules. The authors then asked if an agent capable of improving barrier integrity and resisting leak would confer its benefits on specific types of leak.

Research frontiers

It has been well established by numerous research groups that the above three cytokines and hydrogen peroxide all possess ability to induce transepithelial leak, but the nature of the leak produced by the different agents-and especially their combinations-has not been clearly delineated.

Innovations and breakthroughs

In a barrier-related disease such as inflammatory bowel disease (IBD), transepithelial leakage of small molecules (salts, water, sugars, amino acids) will have different medical implications than leak of macromolecules such as protein growth factors, food antigens, and bacterial toxins and antigens. In the testing of various agents capable of barrier protection it is important to note what types of leak are reduced by the agent under study. Berberine, for instance, is shown here to be able to redress the most severe form of leak, namely the leak to macromolecules produced by the combination of cytokines and peroxide.

Applications

Certain aspects of IBD could be alleviated specifically by shutting down or reducing the unregulated leak of molecules across the inflammation-damaged mucosal lining. This manuscript highlights the issue that specific therapeutic agents will be uniquely able to target certain types of leak.

Terminology

Transepithelial leak is not a monolithic entity. It can result from altered tight junctions giving rise to greater leak to small molecules. Or it can result from full disappearance of tight junctions, giving rise to unrestricted paracellular leak, as can happen in epithelial-to-mesenchymanl transition. Or it can result from the disappearance of whole cells (by death and/or detachment), which gives rise to similarly unrestricted leak, as in tight junction disappearance, but here requiring very different mechanisms (including cell replication and motility) to close the leak. All forms are likely in play in IBD, and each-by being regulated differently-can be affected by unique agents capable of restoring aspects of barrier function.

Peer-review

This paper indicated hyper permeability of colon cancer cell layer by cytokine and inhibition by berberine. The results are interesting and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

Visualization of sphingolipids and phospholipids in the fundic gland mucosa of human stomach using imaging mass spectrometry

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Abstract

AIM: To analyze the lipid distribution in gastric mucosae.

METHODS: Imaging mass spectrometry (MS) is a useful tool to survey the distribution of biomolecules in surgical specimens. Here we used the imaging MS apparatus named iMScope to identify the dominant molecules present in the human gastric mucosa near the fundic glands. Five gastric specimens were subjected to iMScope analysis. These specimens were also analyzed by immunohistochemistry using MUC5AC, H(+)-K(+)-ATPaseβ Claudin18 antibodies.

RESULTS: Three major molecules with *m/z* 725.5, 780.5, and 782.5 detected in the gastric mucosa were identified as sphingomyelin (SM) (d18:1/16:0), phosphatidylcholine

(PC) (16:0/18:2), and PC (16:0/18:1), respectively, through MS/MS analyses. Using immunohistological staining, SM (d18:1/16:0) signals were mainly co-localized with the foveolar epithelium marker MUC5AC. In contrast, PC (16:0/18:2) signals were observed in the region testing positive for the fundic gland marker H(+)-K(+)-ATPaseβ. PC (16:0/18:1) signals were uniformly distributed throughout the mucosa.

CONCLUSION: Our basic data will contribute to the studies of lipid species in physical and pathological conditions of the human stomach.

Key words: Imaging mass spectrometry; iMScope; Sphingomyelin; Phosphatidylcholine; Gastric mucosa

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Core tip: Imaging mass spectrometry (MS) is a useful tool to survey the distribution of biomolecules in surgical specimens. Here we used the imaging MS apparatus named iMScope to identify the dominant molecules present in the human gastric mucosa near the fundic glands. Three major molecules with *m/z* 725.5, 780.5, and 782.5 detected in the gastric mucosa were identified as sphingomyelin (d18:1/16:0), phosphatidylcholine (PC) (16:0/18:2), and PC (16:0/18:1), respectively.

Kurabe N, Igarashi H, Ohnishi I, Tajima S, Inoue Y, Takahashi Y, Setou M, Sugimura H. Visualization of sphingolipids and phospholipids in the fundic gland mucosa of human stomach using imaging mass spectrometry. *World J Gastrointest Pathophysiol* 2016; 7(2): 235-241 Available from: URL: http://www.wjgnet.com/2150-5330/full/v7/i2/235.htm DOI: http://dx.doi.org/10.4291/wjgp.v7.i2.235

INTRODUCTION

The wall of the stomach is composed of mucosa, submucosa, muscularis propria, and subserosa[1]. Except for the mucosa and proper glands, the structures of these layers are the same throughout the gastrointestinal tract. The mucosa of the stomach contains two structurally different layers: A superficial layer with foveolae and a deep layer with coiled glands. The lamina propria exists beneath the foveolar epithelium and harbors the proper gastric glands. The gastric mucosa possesses the ability to protect itself from numerous internal and external stimuli. Various intrinsic factors and systems, such as acid, mucus, bicarbonate, prostaglandins, biotin, blood flow, and the self-renewal of the epithelium as well as extrinsic infections, contribute to this defense mechanism. Loss of gastric mucosa causes gastric ulceration, erosion, or gastritis.

Imaging mass spectrometry (MS) is a recently developed modality that combines microscopy and MS[2-6]. Using this technique, the spatial distribution and molecular profiling of the analytes can be assessed simultaneously in a non-targeted manner. In fact, some lipids and proteins can be identified solely through imaging MS[7-9]. Because antibodies against lipids are difficult to generate, imaging MS is the most suitable option for the study of the lipid "metabolome". Shimadzu Co. (Shimadzu, Kyoto, Japan) has developed a novel application for imaging MS named iMScope[10]. Because of its higher resolution compared with other imaging MS apparatuses, it enables us to visualize the localization of many lipids at one time. Using iMScope, we have already demonstrated the exact spatial distribution of lung surfactant and also discovered a specific phosphatidylcholine that is a potential biomarker in colorectal cancer tissue[11,12].

In this study, to investigate the molecular profile of human gastric mucosa in detail, iMScope was used to analyze the lipid distribution in the human gastric mucosa near the fundic glands. We identified, for the first time, the exact localization of lipids, including phospholipids and sphingolipid, in the human gastric mucosa near the fundic glands.

MATERIALS AND METHODS

Sample preparation

Five gastric samples were retrieved from the archives of Hamamatsu University Hospital. Non-disease portions (fundic gland area) of gastric tissues obtained from gastric surgical specimens were snap-frozen in liquid nitrogen and stored at -80 ℃. The tissue blocks were put in the cryostat (CM1950; Leica, Microsystems, Wetzlar, Germany) at -20 ℃ for 30 min. The tissue blocks were then sectioned to a thickness of 8 μm at -20 ℃. Then, the tissue sections were subjected to hematoxylin and eosin (HE) staining. The adjacent sections were mounted on indium-tin-oxide (ITO)-coated glass slides (Bruker Daltonics, Billerica, MA, United States) for imaging MS and on MAS coated glass slides for immunohistochemistry. The tissue sections on the ITO-coated glass slides were then kept at room temperature. Next, 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonics) was deposited on the sections using a deposition apparatus[11].

Imaging MS and MS/MS analysis

An iMScope (Shimadzu) instrument, which consists of an atmospheric pressure matrix-assisted laser desorption/ionization system equipped with a quadrupole ion trap-time of flight analyzer, was used to obtain the imaging MS data[10]. The sample was scanned with a focused laser (a diode-pumped 355-nm Nd:YAG laser) to acquire the mass spectrum of each spot with a laser shot number of 200 per pixel and a 1000 Hz frequency. The reflection mode was applied to each measurement. The mass range was set to *m/z* 700-900 with a scan pitch of 7.5 μm (for 20 × magnification) or a 20 μm (for 2.5 × magnification) pixel size. The BioMap software (freeware: www.maldi-msi.org) graphical interface was used to visualize the ion images[13].

Table 1 Summary of averaged peak intensities in gastric mucosae

m/z	Averaged intensity
723.4	5.02 ± 1.75
725.5	29.09 ± 13.48
741.4	8.24 ± 5.30
756.5	7.11 ± 2.70
772.4	5.99 ± 3.03
780.5	22.16 ± 23.27
781.5	10.69 ± 9.95
782.5	22.55 ± 16.04
796.5	12.69 ± 8.95
798.5	14.90 ± 9.58
804.5	8.09 ± 4.87
806.5	6.55 ± 3.02
808.5	9.61 ± 6.41
820.5	5.92 ± 2.40
824.5	8.26 ± 4.54

For each spectrum, baseline subtraction, smoothing, normalization to the total ion current, and recalibration were conducted using ClinProTools 2.2 software (Bruker Daltonics)[12]. The total ion currents were the sum of all spectrum intensities. The spectra processing parameters were as follows: Baseline correction [Top Hat algorithm (minimal baseline width set to 10%), resolution (500 ppm), and smoothing (Savitzky Golay, 5 cycles with a 2 *m/z* width)]. Recalibration was performed to reduce mass shifts. Peak picking was also performed based on the overall average spectrum for the whole mass range (signal to noise threshold of 5). The treated data was the average spectrum of input data sets. MS/MS analyses were performed to assign the molecular species using QSTAR Elite (Applied Biosystems, Foster City, CA, United States)[12]. The MS/MS spectral data were then verified using the LIPID MAPS database (http://lipidmaps.org).

Immunohistochemistry

Tissue preparation and immunohistochemical procedures were performed as previously described[14,15]. The 5 μm-thick sections were treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity. To identify the structure of the gastric mucosa, antibodies against MUC5AC (1:50, clone CLH2; Novocastra Laboratories, United Kingdom), claudin-18 (1:200, clone 5G7F2; proteintech, IL, United States), and H(+)-K(+)-ATPaseβ (1:1600, clone 2G11; Abcam, United Kingdom) were used to indicate the foveolar epithelium, fundic glands and foveolar epithelium, and fundic glands, respectively. For antigen retrieval, the slides were heated at 96 ℃ for 30 min in Tris-HCl-EDTA (TE) buffer (pH 9.0), followed by incubation at room temperature for 30 min. The sections were then incubated with a peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO; Nichirei, Japan) at room temperature for 30 min. Next, the sections were treated with diaminobenzidine (DAB) substrate-chromogen solution (DAKO Cytomation; Carpinteria, CA, United States), followed by counterstaining with 0.1% hematoxylin. Images of these sections were obtained using Keyence BZ-9000 (Keyence, Tokyo, Japan). The stained sections were histologically evaluated by experienced pathologists[11,12].

RESULTS

We used the imaging MS modality called iMScope to analyze the spatial distribution of lipids in the gastric mucosae from five individuals. The gastric mucosal region (Figure 1A; inset) was subjected to imaging MS analysis. Table 1 presents the list of ions obtained in the five gastric mucosae using imaging MS analysis. A representative mass spectrum obtained from the gastric mucosa near the fundic gland is shown in Figure 1B. Three major peaks were observed (*m/z* 725.5, 780.5, and 782.5) among these ions. The most intense peak was the ion at *m/z* 725.5. We subsequently used BioMap software to image the spatial distribution of these ions. Figure 1C presents the region of interest (ROI) of the gastric mucosa used to perform imaging MS. The strong signals from these ions were observed in the mucosal region of the gastric wall (Figure 1D for *m/z* 725.5, E for *m/z* 780.5, and F for *m/z* 782.5).

Identification of gastric mucosa specific lipids

MS/MS analyses were performed to assign these ion species. Figure 2A presents the MS/MS spectrum obtained for the ion at *m/z* 725.5. This spectral pattern was identical to the one previously reported by Sudano *et al*[16]. Thus, this ion was shown to be sphingomyelin (SM) [SM (d18:1/16:0) + Na]$^+$. The ions at *m/z* 780.5 and 782.5 were identified as phosphatidylcholoine (PC) [PC (16:0/18:2) + Na]$^+$ and [PC (16:0/18:1) + Na]$^+$, respectively, because of the neutral losses of 59 Da and 183 Da (Figure 2D and G).

Detailed spatial distribution of the identified lipids

To specify the spatial distribution patterns of these lipids more precisely, we compared the ion images with the staining of three gastric mucosal markers. Figure 2B, E, and H are the low-power field ion images of these lipids, and Figure 2C, F, and I are the immunohistological staining patterns of the gastric mucosal markers MUC5AC[17], H(+)-K(+)-ATPaseβ[18] and claudin18[19], respectively. MUC5AC staining was specific for the surface region of the mucosa. H(+)-K(+)-ATPaseβ is a fundic gland marker. Claudin18 is expressed throughout the mucosa. The ion at *m/z* 725.5 was present in the surface of the gastric mucosa, which corresponded to the area of MUC5AC staining. The ion at *m/z* 780.5 was highly expressed in the bottom of the mucosa, which contains the fundic glands. The ion at *m/z* 782.5 was uniformly spread in the mucosa, similar to the area of claudin18 staining.

DISCUSSION

Lipids are important functional molecules in the human body. Phospholipids, which are constituents of plasma membrane, have recently been recognized to have important roles in cellular systems. For example, PC

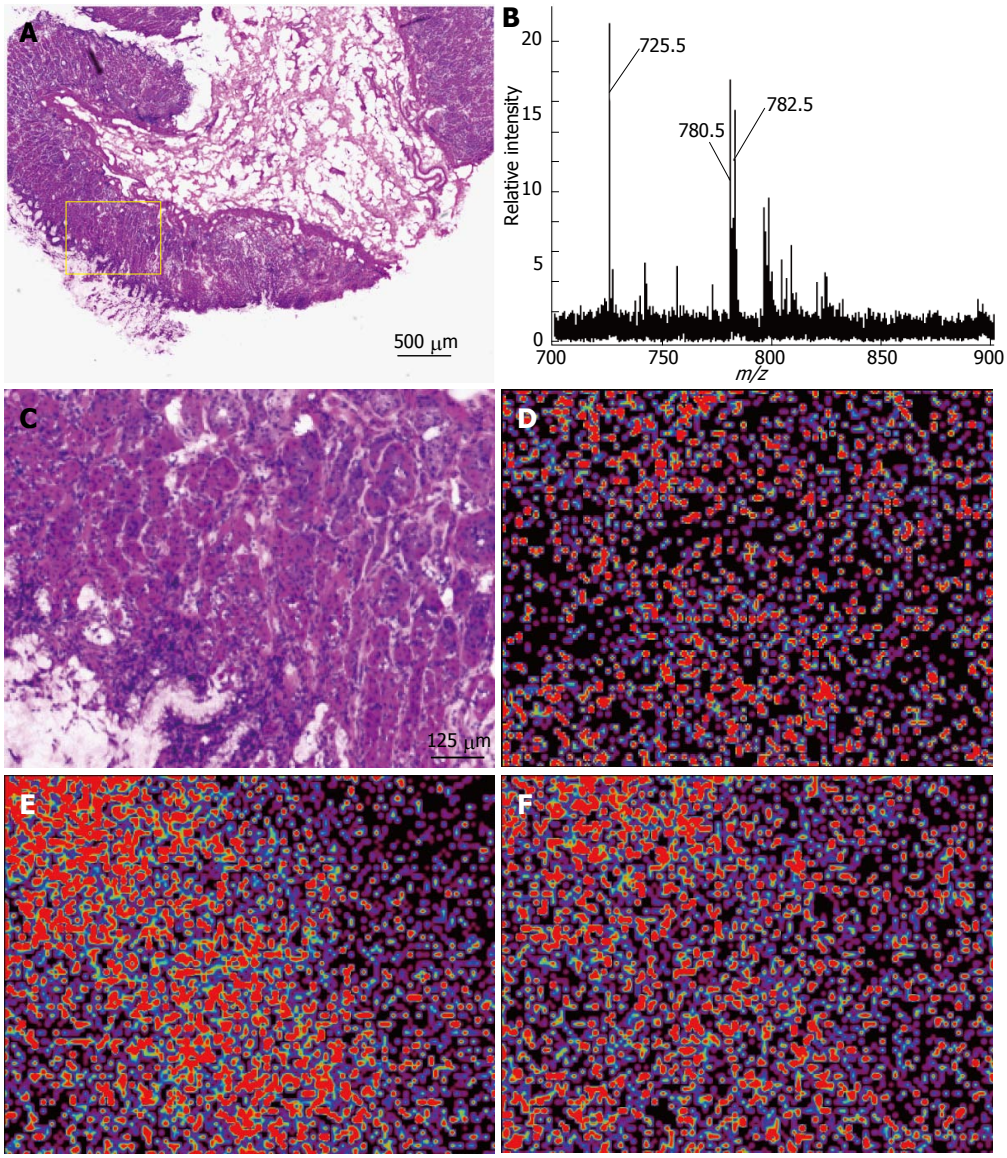

Figure 1 Imaging mass spectrometry analysis of a gastric mucosa. A: HE staining of the gastric mucosa. Inset, ROI of the imaging analysis; B: Averaged spectra obtained from five gastric mucosae; C: Magnified view of the ROI represented in the inset of (A), HE; D: The ion at *m/z* 725.5; E: The ion at *m/z* 780.5; F: The ion at *m/z* 782.5 were imaged using BioMap.

(16:0/16:0) plays an important role as a surfactant in the reduction of surface tension in the lung[20,21]. PC (16:0/18:1) has been shown to be a physiological PPARα ligand, regulating lipid metabolism and glucose homeostasis[22]. Moreover, PC (16:0/20:4) and PC (16:0/18:2) are crucial for the inactivation of Akt kinase[23]. Sphingolipids are also involved in cellular functions such as the cell cycle, apoptosis, senescence, and inflammation[24-26]. In this study, we identified three highly expressed lipid molecules, SM (d18:1/16:0), PC (16:0/18:2) and PC (16:0/18:1), in gastric mucosae (Figures 1 and 2). SM (d18:1/16:0) was mainly localized to the foveolar epithelium of the gastric mucosa (Figure 2B and C). The foveolar epithelium secretes mucus and bicarbonate ions to prevent the damaging effects by pepsin and acid. Because SM molecules are mainly distributed in the plasma membrane, they may cooperate with mucus and bicarbonate ions to protect the mucosal surface. PC (16:0/18:2) co-localized with the fundic gland marker H(+)-K(+)-ATPaseβ. Intriguingly, this observation may be related to the knowledge that Akt phosphorylation is suppressed in fundic glands under ordinary conditions (Figure 2E and F). Considering that Akt phosphorylation may increase the risk of various cancers, including gastric cancer[27,28], the presence of PC (16:0/18:2) may be involved in the sustainability of the gastric mucosa, including the prevention of malignant transformation of gastric mucosae. The role of PC (16:0/18:1) in the gastric mucosa is unknown. This PC species is an endogenous PPARα ligand, leading to the activation of target genes such as *Acox1* and *Cpt1a*; this pathway lowers triglycerides and raises HDL. However, PPARα itself is not expressed in the stomach[29]; it is abundant in the liver. Thus PC (16:0/18:1) in gastric mucosae may have a function other than as a PPARα ligand (Figure 2H and I).

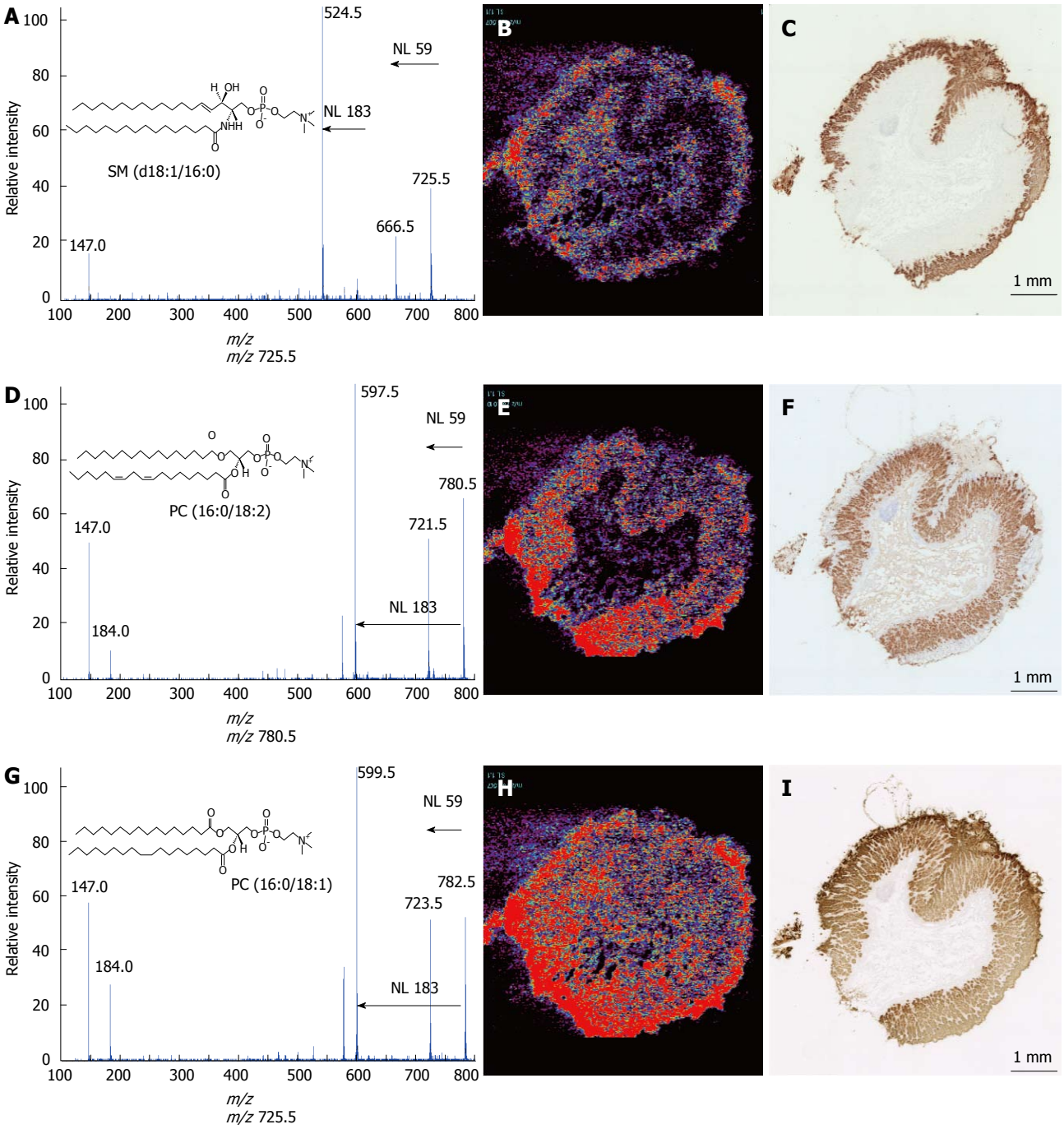

Figure 2 Ion assignment of *m/z* 725.5, 780.5, and 782.5 and immunohistochemical analyses of the gastric mucosae. MS/MS analyses were performed to identify the ions at *m/z* 725.5 (A), 780.5 (D), and 782.5 (G). The ion images of *m/z* 725.5 (B), 780.5 (E), and 782.5 (H) are shown using BioMap software. Immunohistochemical analyses were performed using the antibodies against MUC5AC (C), H(+)-K(+)-ATPaseβ (F), and claudin18 (I) in the adjacent specimens used in the imaging MS analyses. Scale bar, 1 mm. MS: Mass spectrometry.

In conclusion, this study, for the first time, clarified the lipids localized in the human gastric mucosa near the fundic glands. Because we have just reached this level of the modality, in terms of resolution and the ability to identify molecules, the information available on human tissue is currently limited. Our results will be the basis for further investigations of phosphatidylcholine and sphingomyelin species in physical and pathological conditions of the human stomach and will help the precise understanding of the nature of lipid function in the stomach.

COMMENTS

Background

Because antibodies against lipids are difficult to generate, more innovative methodologies are needed in lipid research field to analyze human disease. The authors developed the imaging mass spectrometry (MS) apparatus "iMScope"

to visualize the lipid distribution in the pathological specimen and applied this technique to the measurement of gastric mucosae.

Research frontires

iMScope can irradiate using a thinner laser than other imaging MS modalities, which enables the finest ion image of lipids in the world.

Innovations and breakthroughs

To the best of the authors' knowledge, this is the first time that lipid images of gastric mucosae were obtained.

Applications

Because the authors showed functional lipid images in gastric mucosae, these lipid distributions may reflect the significant role of lipids in the homeostasis of gastric mucosae.

Terminology

Imaging MS is a novel technique that enables us to visualize many biomolecules at one time. The apparatus of imaging MS is composed of a microscope and a mass spectrometer. In the microscopic part, the authors can determine the region of interest (ROI) within the specimen sample and then scan this ROI with the laser. Ions from the evaporated vapors are transferred to the mass spectrometric part, where their mass spectra are obtained. The scanned data are then visualized along a two-dimensional axis.

Peer-review

This report combines the imaging MS with immunohistochemistry to show the lipid spatial distribution on gastric mucosae. Imaging MS is shown to be a useful tool to survey the distribution of biomolecules in the pathological samples. This report firstly applied the iMSope to locate the lipids including both phospholipids and sphingolipid in gastric mucosa, which is helpful to better understand the lipid's function in stomach.

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