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FORUM REVIEW ARTICLE

The Fibrosis of Chronic Pancreatitis: New Insights into the Role of Pancreatic Stellate Cells

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Abstract

Significance: Prominent fibrosis is a major histological feature of chronic pancreatitis, a progressive necroinflammatory condition of the pancreas, most commonly associated with alcohol abuse. Patients with this disease often develop exocrine and endocrine insufficiency characterized by maldigestion and diabetes. Up until just over a decade ago, there was little understanding of the pathogenesis of pancreatic fibrosis in chronic pancreatitis. ***Recent Advances:*** In recent times, significant progress has been made in this area, mostly due to the identification, isolation, and characterization of the cells, namely pancreatic stellate cells (PSCs) that are now established as key players in pancreatic fibrogenesis. In health, PSCs maintain normal tissue architecture via regulation of the synthesis and degradation of extracellular matrix (ECM) proteins. During pancreatic injury, PSCs transform into an activated phenotype that secretes excessive amounts of the ECM proteins that comprise fibrous tissue. ***Critical Issues:*** This Review summarizes current knowledge and critical aspects of PSC biology which have been increasingly well characterized over the past few years, particularly with respect to the response of PSCs to factors that stimulate or inhibit their activation and the intracellular signaling pathways governing these processes. Based on this knowledge, several therapeutic strategies have been examined in experimental models of pancreatic fibrosis, demonstrating that pancreatic fibrosis is a potentially reversible condition, at least in early stages. ***Future Directions:*** These will involve translation of the laboratory findings into effective clinical approaches to prevent/inhibit PSC activation so as to prevent, retard, or reverse the fibrotic process in pancreatitis. *Antioxid. Redox Signal.* 15, 2711–2722.

Introduction

THE PROCESS OF FIBROGENESIS is well recognized as an essential part of the tissue healing process, which usually leads to restoration of tissue structure and function (24). However, when this physiological process goes awry, the result is abnormal/pathological fibrosis, with significant adverse effects on the architecture and function of affected organs/tissues. An understanding of the cellular and molecular mechanisms that regulate normal and abnormal fibrogenesis is an essential step towards the development of relevant therapeutic approaches to prevent/retard the fibrotic process in any given organ system.

Abnormal deposition of fibrous tissue is a characteristic histological feature of two major diseases of the pancreas: i) chronic pancreatitis, a progressive necroinflammatory condition of the pancreas that often results in exocrine and endocrine insufficiency and is most commonly caused by alcohol abuse in Western society (Fig. 1) and ii) pancreatic cancer (38). [Note: This review will not cover the role of PSCs in pancreatic cancer, due to space limitations]. For a long time, pancreatic fibrosis was considered to be a mere epiphenomenon of chronic injury, and up until 1998, little attention had been paid to elucidating the mechanisms responsible for this process. However, there has been rapid progress in this field over the past 12 years and it is now clear that, far from being a passive response to injury, pancreatic fibrosis is an active process that is potentially reversible, at least in the early stages. Much of the rapid advancement in knowledge in this field can be attributed to the development of methodologies to isolate and culture the cells that are now known to be key players in pancreatic fibrogenesis, namely the pancreatic stellate cells (PSCs) (7, 13). This ability to culture PSCs provided a much needed *in vitro* tool enabling researchers to examine PSC biology in health as well as in disease states.

PSC Biology in Health

The first report of the possible existence of pancreatic stellate cells was published over 20 years ago by Watari (97). Taking his cue from knowledge in the field of hepatic fibrosis

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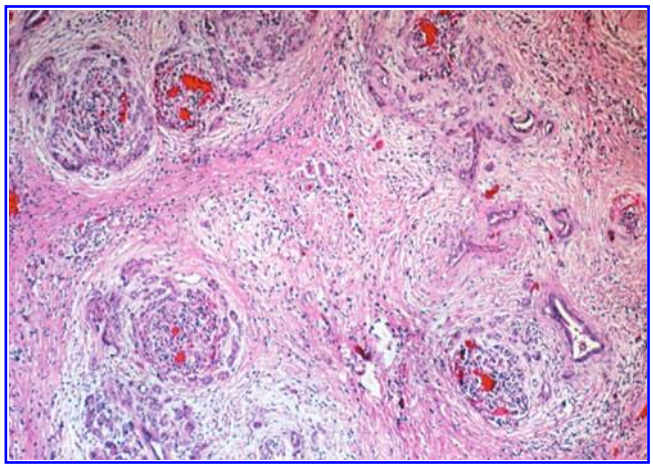

FIG. 1. Human chronic pancreatitis. Hematoxylin and eosin stained sections of human alcoholic chronic pancreatitis depicting abundant fibrosis surrounding atrophic acinar cells and distorted pancreatic ducts. [Reprinted with permission from American Gastroenterological Association and John Wiley and Sons: (95a). (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

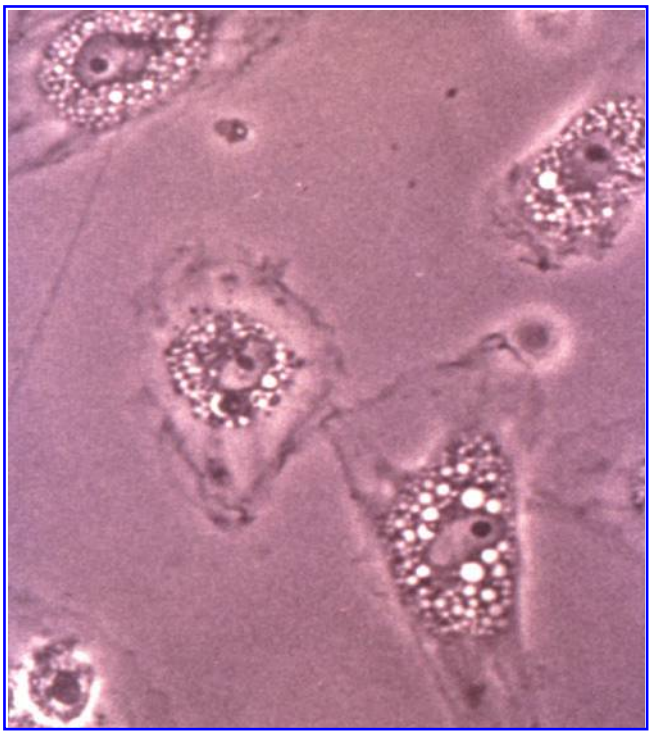

FIG. 2. Phase contrast micrograph of pancreatic stellate cells in early culture. The figure shows PSCs within 48 h of isolation and culture on plastic, exhibiting a polygonal shape with abundant lipid droplets in the cytoplasm surrounding the nucleus. [Reprinted with permission from BMJ Group: (7).] (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

and the proven role of vitamin A storing cells (also variously termed Ito cells or hepatic stellate cells) in liver fibrogenesis (19), Watari used fluorescence and electron microscopic techniques to examine the pancreas of vitamin A-loaded mice. He reported the presence of star-shaped cells which, when exposed to UV light at 328 nm, emitted a rapidly fading blue-green fluorescence characteristic of vitamin A (this autofluorescence most likely emanates from retinyl esters, the storage form of vitamin A). A few years later, Ikejiri demonstrated positive, but sparsely distributed vitamin A fluorescence in normal pancreatic sections from both rat and human pancreas (28). However, little progress was made in this area for the better part of a decade after Ikejiri's publication, due to either a loss of research interest or more likely, due to the lack of a method to successfully isolate and culture PSCs. This situation was altered with the publication of an article by Apte *et al.* in 1998 (7), describing the isolation, culture, and characterization of PSCs from normal rat pancreas. Soon after this article was published, a publication by Bachem and colleagues reported isolation of human pancreatic stellate cells from diseased pancreas (chronic pancreatitis tissue) (13). These important advances provided a much needed *in vitro* tool for in depth studies of the morphology and function of PSCs.

Pancreatic stellate cells can be isolated from normal pancreas (from rodents and humans) by density gradient centrifugation (7, 95), a method that takes advantage of the fact that PSCs have vitamin A-containing lipid droplets in their cytoplasm (as noted earlier) which give the cells a certain density and enables them to be separated from the other major cell type, acinar cells. Phase contrast micrographs of PSCs in early culture (<48 hours) reveal polygonal cells with abundant lipid droplets surrounding a central nucleus (Fig. 2) (7). When subjected to UV light at 328 nm, the cells display the transient blue-green fluorescence characteristic of vitamin A. The identity of PSCs in culture can be confirmed by immunostaining studies for the stellate cell selective markers desmin (Fig. 3A), glial fibrillary acidic protein (GFAP), nestin, nerve growth factor, and neural cell adhesion molecule (7). Using the above PSC selective markers, it has been possible to study the location of these cells in whole pancreatic sections. [*Note: The neural markers noted above are expressed in PSCs and neuronal cells but not in fibroblasts; hence the term used for these markers is 'stellate cell selective markers' and not 'stellate cell specific' markers*]. Histological and immunohistochemical studies of pancreatic sections have demonstrated that PSCs are located in close proximity to the basal aspect of pancreatic acinar cells. In sections immunostained for the marker desmin (a cytoskeletal protein), PSCs can be seen as cells with a central cell body and long cytoplasmic projections extending along the base of adjacent acinar cells (Fig. 3B). In health, PSCs exist in their quiescent phenotype and exhibit the presence of abundant vitamin A-containing lipid droplets in their cytoplasm. Electron microscopy reveals a prominent rough endoplasmic reticulum, collagen fibrils, and vacuoles (lipid droplets) surrounding a central nucleus. It is estimated that PSCs form 4%–7% of all parenchymal cells in the normal pancreas (7, 13).

Numerous biological characteristics of PSCs have now been described. These include:

i. The presence, in quiescent PSCs, of cytoplasmic vitamin A lipid droplets (as noted earlier) and the loss of these lipid droplets when the cells are activated (PSC activation is

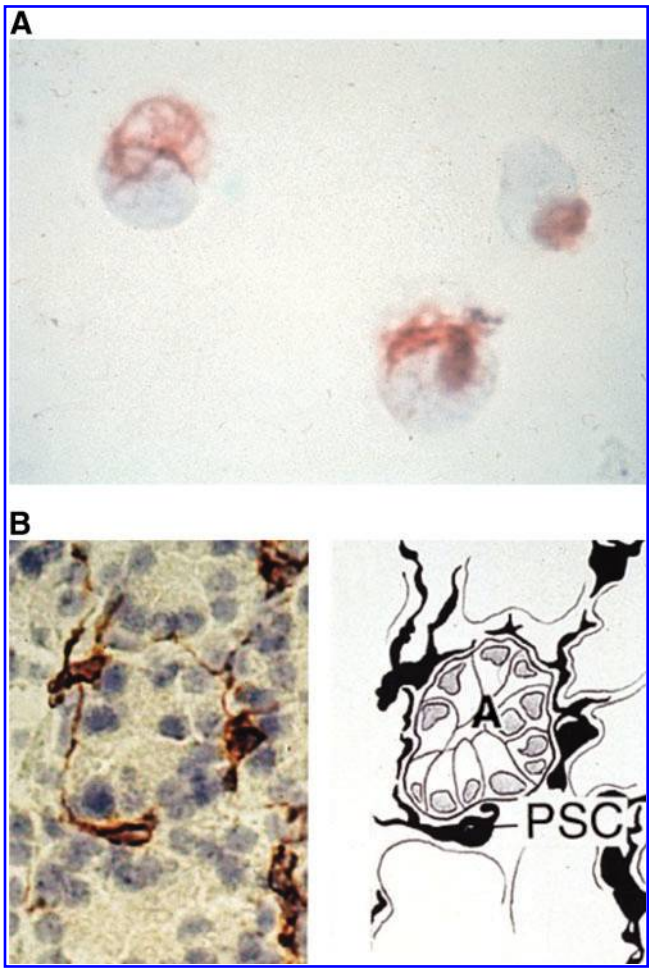

FIG. 3. (A) Immunostaining for desmin in freshly isolated pancreatic stellate cells and (B) desmin positive PSCs in rat pancreas. (A) The figure depicts freshly isolated PSCs showing brown cytoplasmic staining for the PSC selective marker desmin, next to the nucleus. **(B)** The *left panel* depicts a frozen section of the pancreas immunostained for desmin, while the *right panel* shows a corresponding line diagram. An acinus (A) made up of individual acinar cells is surrounded by brown stained, desmin positive stellate cells (PSC) with a central cell body and long cytplasmic projections extending along the basolateral aspect of acinar cells. [Reprinted with permission from BMJ Group: (7).] (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

discussed in detail later in this review). The specific mechanisms responsible for the accumulation (and also the loss) of vitamin A in the cells are not completely understood. Interestingly, a recent study by Kim *et al.* (37) suggests that albumin (a protein that is endogenously expressed in PSCs and is co-localized with vitamin A in the lipid droplets) may play a role in the formation of these lipid droplets. The authors report that transfection of activated PSCs (that had lost their vitamin A lipid droplets), with expression plasmids for albumin induced the re-appearance of vitamin A lipid droplets in PSC cytoplasm (as assessed by UV autofluorescence) and also made the cells resistant to the activating effects of transforming growth factor beta (TGFβ). More recently, the authors have reported that albumin is a downstream effector of the nuclear receptor peroxisome proliferator activated receptor γ (PPARγ) which is known to inhibit PSC activation (discussed later) (36);

ii. The ability of PSCs to proliferate (8, 76);

iii. The ability to migrate (75);

iv. The capacity to synthesise and secrete the extracellular matrix proteins that make up fibrous tissue (collagen, laminin, fibronectin) (7, 13);

v. The capacity to synthesise and secrete matrix degrading enzymes (matrix metalloproteinases, MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs) (74). The fact that PSCs not only synthesise but also degrade ECM proteins suggests that, in health, PSCs play an important role in the maintenance of normal architecture of the pancreas by regulating ECM turnover;

vi. The ability to phagocytose necrotic acinar cells. Shimizu *et al.* (78) have reported that PSCs act as phagocytic cells in an animal model of acute pancreatitis induced by bile-duct ligation and also in the WBN/Kob model of spontaneous chronic pancreatitis. In addition, they have shown that cultured PSCs internalize apoptotic neutrophils as well as necrotic acinar cells. Interestingly, engulfment of necrotic acinar cells by PSCs appears to induce necrotic death of the PSCs themselves. The authors speculate that PSCs may exhibit a locally protective immune function to inhibit disease progression in early pancreatic injury. The concept of an immune role for PSCs is supported by a study in the liver demonstrating that hepatic stellate cells (HSCs) can act as antigen presenting cells—they can process lipid antigens and present them to natural killer cells via CD1d and can also process protein antigens and present them to CD4 and CD8 positive T cells. (90, 98);

vii. The potential for progenitor cell function. Mato *et al.* (56) isolated and expanded pancreatic cells from lactating rats by mitoxantrone (a drug that acts through multidrug transporter systems) selection. They report that the mitoxantrone-resistant cells that survived exhibited a PSC phenotype (fibroblast-like with vitamin A lipid droplets), expressed the stem cell marker ABCG2 transporter (ATP binding cassette G2 transporter) and were able to secrete insulin after cell differentiation. However, whether such a selected 'drug resistant' population is representative of normal PSCs remains to be fully examined.

Role of Pancreatic Stellate Cells in Fibrogenesis

Evidence accumulated over recent years using both *in vivo* and *in vitro* approaches (discussed in detail below) clearly indicates that PSCs become activated during pancreatic injury, a process during which the cells lose their vitamin A stores and transform into a myofibroblast-like phenotype, which synthesizes and secretes excess amounts of fibrous tissue and can be identified by positive staining for the activation marker alpha smooth muscle actin (αSMA). Left unchecked, persistent PSC activation can eventually result in the development of overt fibrosis. Characteristics of quiescent and activated PSCs are detailed in Table 1.

In vivo *studies*

These studies have involved examination of pancreatic tissue from patients with chronic pancreatitis (predominantly alcohol-induced) and from animal models of acute and chronic pancreatitis.

Human studies. With regard to studies with human chronic pancreatitis sections (17, 25), immunohistochemical and histological analyses have established that:

i. Collagen is the predominant extracellular matrix protein in areas of fibrosis, as indicated by the Sirius red stain for collagen;

ii. Areas that stain positive for collagen (by Sirius red) also exhibit positive immunostaining for αSMA, suggesting the presence of activated pancreatic stellate cells in the fibrotic areas;

iii. αSMA-positive cells in fibrotic areas are the only cells that exhibit positive staining for messenger RNA for collagen (as assessed by *in situ* hybridization) indicating that activated PSCs are the principal source of the collagen deposited in fibrotic pancreas (Fig. 4);

iv. Pancreatic acinar cells adjacent to areas of fibrosis exhibit strongly positive staining for transforming growth factor ß (TGFß, a known profibrogenic growth factor for stellate cells), while such staining is absent in acinar cells remote from bands of fibrosis. These observations suggest that TGFß secreted by pancreatic acinar cells may have a paracrine effect on PSCs, leading to increased collagen synthesis by the cells. TGFß staining is also apparent in spindle shaped cells in the fibrotic bands.

v. Fibrotic areas of the pancreas exhibit significant staining for 4-hydroxynonenal (4HNE, a lipid peroxidation product), indicating increased oxidant stress in the vicinity of activated PSCs. This is an important observation given the *in vitro* findings that PSCs respond to oxidant stress by activation (vide infra).

vi. Expression of the receptor for platelet-derived growth factor (PDGF) is significantly increased (at both mRNA and protein levels) in areas of fibrosis in chronic pancreatitis. In view of the fact that PDGF is a potent mitogenic and chemotactic factor for stellate cells, increased PDGF receptor expression on the PSCs may be one of the mechanisms responsible for the increased numbers of PSCs observed in fibrotic areas. Interestingly, one of the stellate cell selective markers, NGF, has been reported to be overexpressed in human chronic pancreatitis (20). Since NGF is expressed by neuronal cells as well as PSCs, it is possible that proliferating PSCs in fibrotic areas may contribute to the observed increase in NGF staining in this disease.

Animal models. The human studies described above strongly support the concept that activated PSCs are associated with pancreatic fibrosis, but are limited by their cross-sectional, point-in-time nature. Thus, in order to gain an insight into the chronological events that occur during the development of fibrosis in chronic pancreatitis, researchers have turned to experimental models. Several rat models of pancreatic fibrosis have been reported in the literature to date, but only some have been used to assess the specific role of PSCs in fibrogenesis. These include : i) trinitrobenzene sulfonic acid (TNBS) injection into the pancreatic duct (25); ii) intravenous injection of an organotin compound dibutyltin chloride (DBTC) (18); iii) spontaneous chronic pancreatitis in WBN/Kob rats (70, 79); iv) severe hyperstimulation obstructive pancreatitis (SHOP), involving intraperitoneal (IP) injections of supramaximal doses of caerulein (a synthetic analogue of cholecystokinin, a major pancreatic secretagogue) + bile-pancreatic duct ligation (65); v) repeated IP injections of a superoxide dismutase inhibitor (57); vi) intragastric high dose alcohol administration + repeated

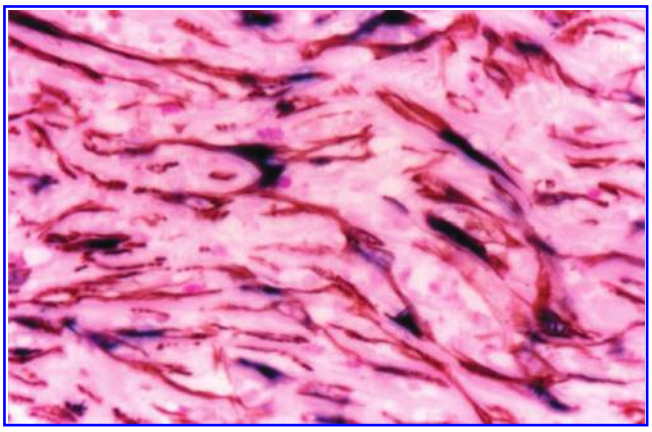

FIG. 4. Dual staining of a human chronic pancreatitis section for α smooth muscle actin (αSMA) and mRNA for collagen. The figure depicts a high power view (X400) of a pancreatic section immunostained for the PSC activation marker αSMA and for collagen mRNA using *in situ* hybridization. The *brown staining* for αSMA is co-localized with the *blue staining* for collagen mRNA, indicating that the αSMA positive cells are the source of collagen in fibrotic areas. [Reprinted with permission from Elsevier: (10).] (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Table 1. Characteristics of Quiescent and Activated Pancreatic Stellate Cell Phenotypes

	Quiescent PSCs	*Activated PSCs*
Vitamin A lipid droplets	Present	Absent
α Smooth muscle actin	Absent	Present
Proliferation	Limited	Increased
Migration	Limited	Increased
Extracellular matrix production	Limited	Increased
Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix proteinases (TIMPs)	Complement of MMPs and TIMPs to maintain normal ECM turnover	Change in types of MMPs and TIMPs to facilitate ECM deposition
Production of cytokines	Limited	Increased (PDGF, TGFβ, CTGF, IL1, IL6, IL15)
Capacity for phagocytosis	Absent	Present

caerulein injections (86, 88); vii) chronic alcohol administration (liquid diet) with repeated cyclosporin and caerulein injections (23), and viii) chronic alcohol administration with repeated endotoxin (LPS, lipopolysaccharide) injections (96). Mouse models of pancreatic fibrosis that have examined the role of PSCs include : i) transgenic mice overexpressing TGFβ (92) or the EGF receptor ligand heparin binding epidermal growth factor-like growth factor (HB-EGF) (15) and ii) repetitive pancreatic injury induced by repeated injections of supramaximal caerulein (66). Another potentially useful mouse model that has been recently described is the transgenic mouse overexpressing IL-1β (44). These mice develop chronic pancreatitis as evidenced by fibrosis and atrophy/loss of acinar cells. However, while the authors note activation of PSCs in this model, the role of PSCs per se in the development of chronic pancreatitis was not examined.

There is no doubt that all the above models represent useful tools to assess pathophysiological processes during pancreatic injury. However, with all but one of these models, pancreatic injury is produced by relatively nonphysiological methods (*e.g.*, injections of toxin into the pancreatic duct, administration of supraphysiological levels of caerulein, or bile duct ligation). Therefore, the direct clinical relevance of these models may be questioned. In contrast, the rat model of chronic pancreatitis produced by chronic alcohol administration and repeated endotoxin exposure (96) is based on a well-recognized clinical phenomenon, that is, raised circulating endotoxin levels in alcoholics secondary to increased gut mucosal permeability (16, 72). Thus, this model represents the most physiologically relevant model of chronic alcoholic pancreatitis described to date.

The above studies have examined the effects on PSCs over the time-course of injury, commonly using techniques such as histology, immunohistochemistry, and *in situ* hybridization. The general consensus is that PSCs are activated early in the course of the injury, possibly due to paracrine effects of factors (such as cytokines and reactive oxygen species) produced by injured acinar cells and/or inflammatory cells during the acute phase of the injury. It is also evident that activated PSCs are the predominant source of collagen in fibrotic areas (confirming the results observed in human studies). Increased numbers of activated PSCs (as determined by αSMA staining) in areas of fibrosis have also been demonstrated in the above animal models (Fig. 5). Interestingly, in the TNBS model (25), receptor expression for the mitogen PDGF was found to be increased in fibrotic areas, providing a possible mechanism for the observed proliferation of PSCs (this finding concurs well with that described by Casini *et al.* (17) in human chronic pancreatitis).

More recently, evidence of pancreatic oxidant stress as assessed by measurement of the lipid peroxidation product malondialdehyde, has been reported in the TNBS model (41). This finding again supports the observed increase in oxidant stress in human chronic pancreatitis noted earlier, and is relevant to PSC function in view of the known activating effects of oxidant stress on PSCs (detailed later). Upregulation of TGFβ mRNA expression has been reported in the caerulein-induced pancreatic fibrosis model by Ulmasov and colleagues (89). Supporting these findings are results from another recent study in the same model, reporting increased expression of two TGFβ regulated genes, SM22α and Cygb/STAP, in activated PSCs in fibrotic areas of the pancreas (101). These findings are consistent with the now well established profibrogenic effects of TGFβ on PSCs.

Source of activated PSCs in the fibrotic pancreas—Evidence from animal models. There has been growing interest in the question of the source of activated PSCs in the injured pancreas. Researchers have sought to determine whether the production of fibrosis during injury is entirely attributable to the resident population of PSCs which becomes activated during the injury process, or whether cells from other sources such as the bone marrow may contribute to the fibrotic process by migrating to the pancreas in response to chemotactic signals from the injured organ. In this regard, two recent studies have provided some interesting data (43, 82). The first study used a gender mismatch and chimeric approach whereby green fluorescent protein (GFP)-labeled bone marrow-derived cells (BMDC) obtained from GFP transgenic male donor mice were transplanted into lethally irradiated wild-type female mice (43). The recipient mice were then subjected to repeated injections of caerulein to produce chronic pancreatitis. Using fluorescence microscopy and *in situ* hybridization for the Y chromosome, the authors reported the presence of GFP and Y chromosome positive cells in the pancreas that also expressed the PSC marker desmin. However, these cells constituted a relatively small proportion (5%) of the total PSC population, and their numbers remained constant over the whole duration (45 weeks) of the study, raising questions about the significance of bone marrow-derived PSCs in the fibrotic process.

More recently, Sparmann and colleagues (82) have used a similar GFP-labeled BMDC transplant approach in a model of acute pancreatitis induced by the chemical toxin dibutyltin chloride (DBTC). The authors reported that GFP-labeled PSCs

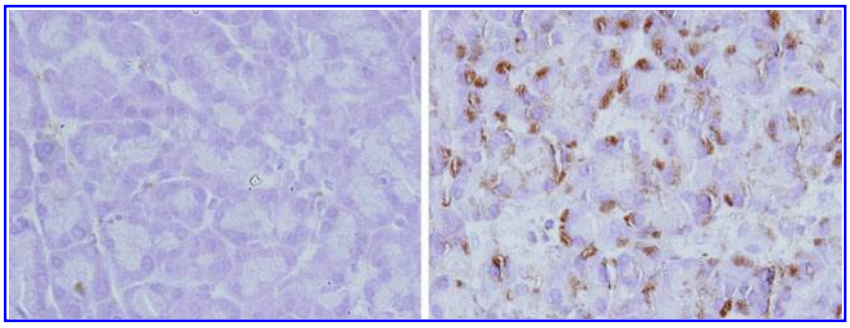

FIG. 5. Immunostaining for α smooth muscle actin (αSMA) in rat pancreas. The figure depicts a chronic pancreatitis section from an alcohol-fed endotoxin challenged rat showing abundant brown αSMA staining (*right panel*), with no positive αSMA staining in the normal pancreas of a control diet fed, endotoxin challenged rat (*left panel*). [Reprinted with permission from Elsevier: (96).] (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

formed approximately 7% of PSCs in the early phase (within 1 week) of DBTC-induced injury. This number increased to 18% at 4 weeks when the acute pancreatitis had resolved and the gland was restored to normal, leading the authors to speculate that bone marrow-derived progenitor cells represented a source of renewable stellate cells in the pancreas which may play a role in the tissue healing process. The findings of the above studies support the concept that a small proportion of the PSC population in the pancreas may be bone marrow derived, but additional studies with different models of pancreatic fibrosis are needed before the contribution of bone marrow-derived PSCs can be fully elucidated.

In vitro *studies*

As noted earlier, the development of a method to isolate and culture PSCs from rat pancreas, was a long awaited first step to characterizing the mechanisms responsible for activation of PSCs during pancreatic injury. The rat PSC isolation method has now been successfully adapted for both mouse and human pancreas, thereby significantly expanding the scope of studies on PSC biology.

Cultured PSCs have now been used for studies designed to identify the molecules/factors and the cellular pathways mediating PSC activation during pancreatic injury. In general, the selection of putative activating factors for examination has been based upon the knowledge that during the process of tissue injury, PSCs are likely to be exposed to : i) growth factors and cytokines including TGFβ, PDGF, tumor necrosis factor α, and interleukins (known to be upregulated during pancreatic damage (68, 93)); ii) oxidant stress (known to occur during both acute and chronic pancreatitis (9, 17, 69, 87)); iii) alcohol and its metabolites acetaldehyde and fatty acid ethyl esters (FAEEs) [given the well-known role of alcohol in pancreatitis (100)], as well as endotoxin [given the known association of alcohol abuse and endotoxinemia (72) and the correlation of circulating endotoxin levels with severity of pancreatitis (1, 99)]; iv) increased pancreatic pressure due to the 'compartment syndrome' of chronic pancreatitis (29, 42).

In addition to the above factors, there has been an ever growing list of factors (reported to be overexpressed in chronic pancreatitis) that have been examined in recent years for their effects on PSC function. These include the hemostatic protein fibrinogen (49), the endothelial cell-derived vasoconstrictor endothelin-1 (32), cyclooxygenase 2 (COX-2, the inducible form of the rate limiting enzyme that converts arachidonic acid to prostaglandin) (4), galectin-1 (a beta-galactoside binding lectin) (52), and hyperglycemia (67) (given that diabetes is a known complication of chronic pancreatitis).

PSC activation in response to exposure to the above potential activating factors *in vitro* has been assessed using at least one or more of a number of parameters such as cell proliferation, αSMA expression, ECM protein synthesis, matrix degradation via the production of matrix metalloproteinases, loss of vitamin A stores, cell migration, cytokine release, and contractility.

A discussion of each of the above factors in detail is beyond the scope of this review, but the overall findings can be summarized as follows:

i. PDGF is a potent mitogenic and chemotactic factor for PSCs (8, 31, 75).

ii. TGFβ and its downstream effector connective tissue growth factor (CTGF) are potent fibrogenic cytokines inducing the synthesis and secretion of collagen, fibronectin, and laminin by PSCs (8, 76). TGFβ also increases the production of matrix metalloproteinase 2 (MMP2) by PSCs (74). Since MMP2 degrades basement membrane collagen, it is hypothesized that the consequent loss of normal collagen (matrix) facilitates the deposition of abnormal (fibrillar) collagen, thereby promoting fibrosis.

iii. The proinflammatory cytokines TNFα, monocyte chemotactic protein (MCP-1), and interleukins IL1, IL6, and IL13 induce PSC activation as indicated by increased proliferation, αSMA expression, and/or collagen synthesis (62, 63). Importantly, in addition to responding to exogenous cytokines via paracrine pathways, PSCs have been shown to produce endogenous inflammatory mediators such as TGFβ, MCP-1, IL1, IL8, and RANTES (Regulated on Activation Normal T Cell Expressed and Secreted), all of which are capable of activating the cells via autocrine pathways (2, 62, 77). Furthermore, the production of these cytokines can be stimulated by exogenous compounds such as ethanol, acetaldehyde, TGFβ, and CTGF (34, 62). There is also evidence of autocrine loops between certain cytokines in PSCs. Using cultured rat PSCs, Aoki *et al.* have reported that IL-1β and IL6 produced by PSCs can each stimulate the autocrine secretion of TGFβ by the cells, and vice versa (3, 5). In contrast to the stimulatory effects of autocrine IL1-β and IL6 on autocrine TGFβ production in rat PSCs, a recent study has reported that IL13 (a Th2 lymphokine) suppresses TGFβ secretion by rat PSCs, although it induces PSC proliferation (80). The authors have shown that rat PSCs do not produce IL13 but express the receptor system for IL13. However, the relevance of these observations (including the IL13-induced suppression of TGFβ secretion by PSCs) to human pancreatic fibrosis is difficult to assess since the authors were unable to demonstrate the presence of the IL13 receptor system in human pancreatitis specimens. Thus, the role of IL13 in human chronic pancreatitis is yet to be fully clarified. Furthermore, it is likely that any suppressive effects of IL13 on autocrine TGFβ secretion by human PSCs will be negated by the strong inductive effects of the other cytokines (that are well established as major players in human pancreatitis) on autocrine TGFβ production by PSCs. In general, the ability of PSCs to be activated via autocrine pathways suggests that once activated, PSCs are capable of perpetuation of activation even in the absence of the initial trigger factors (Fig. 6). This phenomenon may represent one of the mechanisms responsible for progression of chronic pancreatitis despite the cessation of the initial insult, for example, alcohol (see below) and/or acute flare.

iv. Exposure to a pro-oxidant complex such as iron sulfate/ascorbic acid or hydrogen peroxide (which increases oxidant stress within PSCs) leads to their activation as indicated by increased αSMA expression and collagen synthesis (10, 35). Importantly, this oxidant stress-induced activation is prevented by the antioxidant α-tocopherol (vitamin E) (10). In addition to responding to exogenous reactive oxygen species/oxidant stress, PSCs have been shown to generate reactive oxygen species (ROS) within the cell (11, 55). Masamune *et al.* (55) have demonstrated that PSCs express NADPH oxidase (an enzyme that is primarily found in phagocytic cells such as neutrophils and macrophages) to generate intracellular ROS, which, in turn, mediate activation of PSCs.

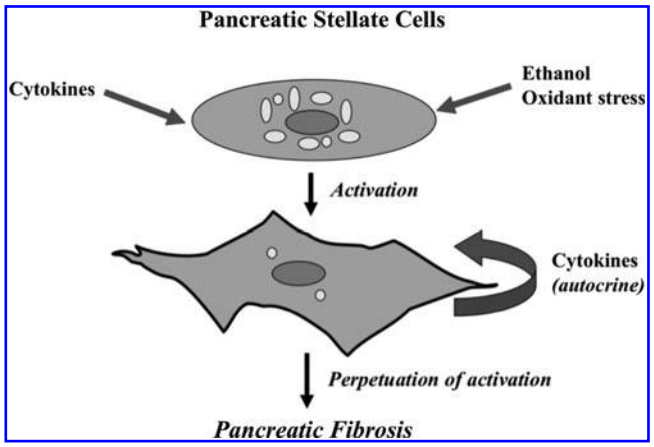

FIG. 6. Perpetuation of PSC activation. A diagrammatic representation of the postulated pathway for a perpetually activated state for PSCs. Pancreatic stellate cells are activated via paracrine pathways by exogenous factors such as cytokines, oxidant stress, ethanol, and its metabolites. Activated PSCs synthesize and secrete endogenous cytokines that influence PSC function via autocrine pathways. It is possible that this autocrine loop in activated PSCs perpetuates the activated state of the cell, even in the absence of the initial trigger factors, leading to excessive ECM production and eventually causing pancreatic fibrosis.

v. Ethanol itself has been reported to directly activate PSCs as indicated by increased expression of the activation marker αSMA and increased synthesis of extracellular matrix proteins (10, 51). This activation is most likely due to the oxidation of ethanol to acetaldehyde (via the enzyme alcohol dehydrogenase (ADH) which has been shown to be active in PSCs), and the subsequent generation of oxidant stress within the cell (10, 39). Interestingly, ethanol has been shown to upregulate PDGF-induced NADPH oxidase activity within PSCs (26), further strengthening the concept that ROS generated within PSCs play a role in PSC activation.

It is also important to note that ethanol can cause activation of PSCs from their quiescent phase and does not require the cells to be pre-activated to exert its stimulatory effects (10). This is a critical observation suggesting that, *in vivo,* PSC activation may occur early during chronic alcohol intake even in the absence of necroinflammation. Perpetuation of this activation may occur during ethanol-induced necroinflammatory episodes leading to the development of fibrosis. Furthermore, ethanol inhibits apoptosis of PSCs induced by serum starvation as assessed by standard apoptosis markers, such as Annexin V staining, TUNEL staining, and caspase 3 and 9 activities (94). These findings suggest that ethanol intake may facilitate survival of PSCs *in vivo.* A recent study has shown that the endotoxin lipopolysaccharide (LPS) can also inhibit PSC apoptosis while, at the same time, stimulating PSC proliferation (94). Notably, the inhibitory effect of LPS on PSC apoptosis is further enhanced in the presence of ethanol (94). These findings support the concept that alcohol and endotoxin exert synergistic effects on PSCs which promote cell activation and survival, thereby promoting pancreatic fibrosis.

In contrast to the activating effects of the oxidative ethanol metabolite acetaldehyde on PSCs, the nonoxidative ethanol metabolites (FAEEs) have not been shown to affect PSC proliferation or activation, although one of these esters, palmitic acid ethyl ester, has been reported to stimulate specific signalling molecules within PSCs (see below) (47).

An intriguing aspect of the story related to ethanol and PSCs is the possible link between ethanol and cytoplasmic vitamin A (retinol). The most consistent feature of PSC activation is loss of the vitamin A-containing lipid droplets from the cytoplasm; conversely, exposure of activated PSCs to exogenous vitamin A or to its metabolites all transretinoic acid (ATRA) and 9-cis retinoic acid (9-RA) leads to induction of quiescence in the cells (60). Since PSCs have the capacity to metabolize both ethanol and retinol and since these compounds are metabolized by similar classes of enzymes (*i.e.,* alcohol dehydrogenases and aldehyde dehydrogenases), there is a possibility that ethanol exerts its activating effect on PSCs by acting as a competitive inhibitor of retinol metabolism by PSCs. Evidence for such competitive inhibition has been reported in hepatic stellate cells (64). Although direct evidence is not yet available in PSCs, McCarroll *et al.* (60) have shown that retinol is unable to exert its full inhibitory effect on PSC activation in the presence of ethanol (60). These findings support the concept that competitive inhibition of retinol metabolism may also occur within PSCs in the presence of ethanol.

vi. As mentioned earlier, pancreatic tissue pressure is elevated in chronic pancreatitis compared to normal pancreas (29, 42). Asaumi *et al.* (12) examined the effects of externally applied pressure on cultured PSCs using a sealed pressure loading apparatus into which culture flasks bearing PSCs were placed. Using helium gas, the pressure in the chamber was raised to 80 mmHg, and PSC morphology and gene expression of αSMA, procollagen α1 and TGFβ1 were assessed. The authors reported that PSCs were activated in response to pressure and, notably, this activation was mediated via intracellular ROS, since the effect of pressure on PSCs could be prevented in the presence of anti-oxidants such as N-acetyl cysteine (NAC) and epigallocatechin gallate (a green tea polyphenol).

vii. Fibrinogen, COX-2, galectin 1, hyperglycemia, and endothelin 1 have all been reported to induce PSC activation as assessed by one or more parameters, including proliferation, migration, collagen production, αSMA expression, and cytokine expression (4, 32, 49, 52, 67).

Signaling Pathways in PSCs

In view of the observed responsiveness of PSCs to numerous exogenous and endogenous stimuli, it is not surprising that there has been considerable research activity over the past few years to delineate the intracellular signaling events/pathways that ultimately regulate PSC behaviour, particularly cell functions that are critical to the fibrotic process, including proliferation, synthesis, and degradation of extracellular matrix, migration, and apoptosis. The main aim of these studies has been to identify and characterize specific signaling molecules which could be targeted to prevent or reverse PSC activation and thereby prevent or retard the fibrotic process.

One of the major pathways that regulates cell functions such as protein synthesis, cell differentiation, and cell division is the mitogen-activated protein kinase (MAPK) pathway

(40). Consequently, this pathway has been the focus of attention of numerous studies on PSCs. It is now established that the effects of ethanol, acetaldehyde, and oxidant stress on PSCs are mediated by activation of all three classes of the MAPK pathway, namely extracellular signal regulated kinase (ERK1/2), p38 kinase, and c-jun amino terminal kinase (JNK) (30, 51, 59), as well as the nuclear transcription factor AP-1 (51). Masamune *et al.* (51) have recently reported that the nonoxidative metabolite of ethanol, palmitic acid ethyl ester (PAEE), also activates the same pathways in human PSCs. Ethanol and acetaldehyde have also been found to activate two signaling molecules upstream of the MAPK cascade, phosphatidylinositol 3 kinase (PI3K) and protein kinase C (PKC) (6).

The recently observed synergistic effects of ethanol and endotoxin *in vivo* have prompted studies on the LPS signaling pathway in PSCs. It has now been shown that PSCs express the LPS receptor TLR4 (Toll-like receptor 4) as well as the associated molecules CD14 and MD2 (50, 94). Notably, exposure of PSCs to LPS *in vitro* leads to upregulation of TLR4 expression (94). Other Toll-like receptors, TLR2, 3, and 5 have also recently been reported in PSCs, and exposure of the cells to relevant TLR ligands have been shown to activate the transcription factor NFkB (50). This finding is of interest because NFkB is known to induce anti-apoptotic proteins such as IAPs (inhibitor of apoptosis proteins) (14) and may provide an explanation for the LPS-induced inhibition of PSC apoptosis observed *in vitro*.

With respect to growth factors, several pathways have been identified as regulators of PDGF-induced PSC proliferation, including ERK and JAK/STAT (Janus activated kinases/Signal induced activation of transcription) (31), while PDGF-induced PSC migration has been shown to be a function of the PI3K pathway (58). It is important to note that cross-talk exists between PI3K and ERK in PSCs, so that modulation of one pathway is usually associated with a change in the function of the other (53, 58). Another signaling molecule that has been reported to influence PSC migration is Indian hedgehog (IHH) (81). This is a member of the hedgehog peptide family that participates in several events such as pancreas development, patterning, and differentiation. PSCs express smoothened (Smo) and patched-1 (Ptch1) proteins that are essential components of the hedgehog receptor system. The ligand IHH induces chemotactic as well as chemokinetic migration of PSCs, and this is accompanied by relocation of the transcriptional factor Gli-1 to the nucleus. The IHH-induced increase in PSC migration is associated with localization of membrane type I–matrix metalloproteinase (MT1-MMP) to the surface of PSCs, where it presumably aids basement membrane degradation so as to facilitate cell movement. The profibrogenic growth factor TGFβ has been shown to exert its effect on PSCs via the intracellular signaling mediators SMAD2 and 3 (71). This cytokine is also known to increase its own mRNA expression in PSCs in an autocrine manner, a process regulated by the ERK pathway (71).

Since expression of αSMA is a marker of PSC activation, the regulation of the actin cytoskeleton and cell morphology have lately been the subject of some study. The small GTP protein Rho and its downstream effector Rho kinase have been shown to regulate the actin cytoskeleton, stress fiber formation, and alteration of cell shape during the PSC activation process (45).

In addition to the signaling pathways regulating the effects of PSC activators, those related to factors that inhibit/prevent PSC activation have also been studied. The induction of quiescence in PSCs by retinol and its metabolites ATRA and 9-cisRA) is reported to be mediated via inhibition of MAPK (ERK, JNK and p38 kinase) signaling (60). Curcumin, a polyphenol compound found in turmeric, has been reported to decrease PDGF-induced PSC proliferation via inhibition of the ERK pathway (54). Curcumin also inhibits cytokine-induced PSC activation by inhibiting the MAPK pathway and preventing AP-1 activation in the cells. Recently, peroxisome proliferator-activated receptor γ (PPARγ, a ligand-activated transcription factor which controls cellular growth and differentiation) has also been implicated as an important factor in induction of PSC quiescence (46, 48) since its ligands such as troglitazone inhibit PDGF-induced and culture-induced activation of PSCs (79).

Reversal of Pancreatic Fibrosis

The rapid advances over the past decade in our understanding of PSC biology in health and disease and of the fibrogenic process in the pancreas have provided an evidence-based rationale for testing the efficacy of antifibrotic strategies *in vivo*, although it must be acknowledged that these are mostly limited to studies with experimental models of pancreatic fibrosis. Space constraints preclude a detailed description of the results of each of these studies. Briefly, the therapeutic approaches used to date include i) Antioxidants: vitamin E (22) (the subclass tocotrienol has been shown to induce PSC death via apoptosis and autophagy (91)), oxypurinol, and allopurinol, both xanthine oxidase inhibitors (73, 85), ellagic acid, a plant-derived polyphenol with antioxidant, anti-inflammatory, and anti-fibrosis activities (84), and salvianolic acid, a herbal medicine with free radical scavenging properties (41); ii) TGFβ suppression: using TGFβ neutralizing antibodies (61), a herbal medicine Saiko-keishi-to (83) or a plant alkaloid halofuginone that inhibits downstream Smad3 phosphorylation (101); iii) TNFα inhibition: using a TNFα antibody (27), soluble TNFα receptors or an inhibitor of TNFα production pentoxifylline (73); iv) anti-inflammatory agents: protease inhibitors such as camostat mesilate that inhibit proinflammatory cytokine production by monocytes (21), and the synthetic carboxamide derivative IS-741 which suppresses macrophage infiltration into the pancreas, with a consequent decrease in *in vivo* PSC activation (33); and v) modulation of signaling molecules using the PPARγ ligand troglitazone (79)).

Most recently, in the model of alcoholic chronic pancreatitis produced by repeated endotoxin challenge in alcohol-fed rats, it has been convincingly demonstrated that withdrawal of alcohol from the diet after established early pancreatitis, can lead to complete reversal of pancreatic fibrosis (Fig. 7) (94). This effect is likely due to the fact that the alcohol-induced inhibition of PSC apoptosis (described earlier) is removed in the absence of alcohol, thereby enabling the loss of activated PSCs through cell death and halting the fibrogenic process. These findings provide, for the first time, strong experimental evidence to support the concept that abstinence from alcohol is likely to inhibit progression of disease in patients with alcoholic pancreatitis.

In conclusion, the central role of pancreatic stellate cells in the pathogenesis of pancreatic fibrosis is now well

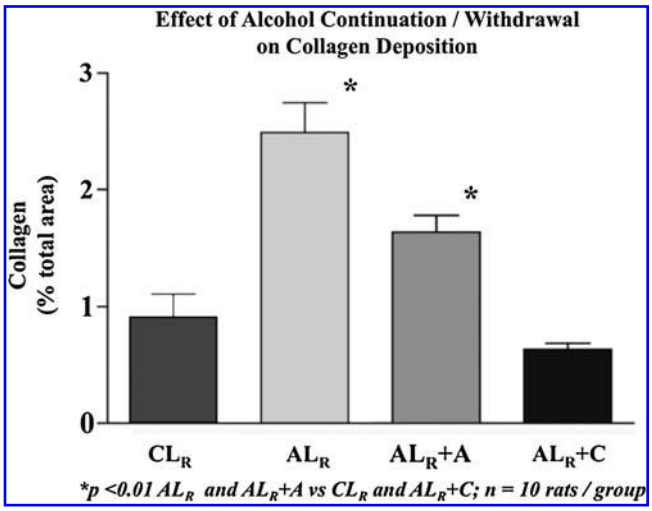

FIG. 7. Reversal of collagen deposition after established pancreatitis. The graph depicts results of morphometric analysis of collagen staining (using Sirius Red) in pancreatic sections from four groups of animals : a) CL_R: control diet fed rats challenged with three repeated doses of lipopolysaccharide (endotoxin); b) AL_R: alcohol-fed rats challenged with three repeated doses of lipopolysaccharide (endotoxin); c) $AL_R + A$ alcohol-diet fed rats challenged with three repeated doses of lipopolysaccharide (endotoxin), then continued on alcohol for 3 weeks; d) $AL_R + C$ alcohol-diet fed rats challenged with three repeated doses of lipopolysaccharide (endotoxin), with withdrawal of alcohol for 3 weeks. AL_R animals had significantly increased collagen staining compared to CL_R animals. Continuation of alcohol feeding after established pancreatitis ($AL_R + A$) resulted in a persistent increase in collagen staining. Withdrawal of alcohol after established pancreatitis resulted in a significant decrease in collagen staining to control (CLr) levels. $p < 0.01$ AL_R and $AL_R + A$ vs CL_R and $AL_R + C$; $n = 10$ rats/group.

established. The cellular functions of these cells, their interactions with other pancreatic cells and the intracellular pathways that regulate specific responses in both health and disease are becoming increasingly clear. The challenge now is to direct research efforts towards translation of the pre-clinical findings into effective therapeutic strategies to interrupt the PSC activation process, thereby enabling prevention/retardation of the fibrotic process in pancreatitis.

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Abbreviations Used

4HNE = 4-hydroxynonenal
ABCG2 transporter = ATP binding cassette G2 transporter
BMDC = bone marrow-derived cells
COX2 = cylclooxygenase 2
DBTC = dibutyltin chloride
ECM = extracellular matrix
EGF = epidermal growth factor
GFP = green fluorescent protein
HB-EGF = heparin binding epidermal growth factor-like growth factor
IL = interleukin
LPS = lipopolysaccharide
NGF = nerve growth factor
PPARγ = peroxisome proliferator activated receptor γ
PSC = pancreatic stellate cell
TGFß = transforming growth factor ß
TIMP = tissue inhibitor of metalloproteinases
TLR = Toll-like receptor
TNBS = trinitrobenzene sulfonic acid
UV = ultraviolet

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