Platelet-Derived Growth Factors Stimulate Proliferation and Extracellular Matrix Synthesis of Pancreatic Stellate Cells: Implications in Pathogenesis of Pancreas Fibrosis

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SUMMARY: At present, the cell-cell interactions and molecular mechanisms of pancreas fibrogenesis are largely unknown. The purpose of this study was to investigate paracrine stimulatory loops between platelets and pancreatic stellate cells (PSC). Human PSC were obtained by outgrowth from fibrotic human pancreas. Native platelet lysate (nPL) and transiently acidified platelet lysate (aPL) were added to cultured PSC (passage 4 to 7) in the absence of serum. The synthesis of collagen types I and III and c-fibronectin (cFN) was demonstrated on protein (immunofluorescence and quantitative immunoassay) and mRNA (Northern blot) level. Using sections of human pancreas with acute pancreatitis, platelet aggregates in capillaries were demonstrated by transmission electron microscopy. nPL, and to an even greater extent aPL, significantly increased the synthesis of collagen types I and III and of c-FN (120 μl/ml aPL increased collagen type I concentration in PSC supernatants by 1.99 ± 0.17 times and c-FN of 2.49 ± 0.28 times, mean ± SD, $n = 3$). nPL and aPL also significantly stimulated cell proliferation (increased bromodeoxyuridine (BrdU) incorporation by 6.4 ± 0.78 times and 10 ± 0.29 times, respectively). By preincubating aPL with transforming growth factor β (TGFβ)- and platelet-derived growth factor (PDGF)-neutralizing antibodies and the TGFβ-latency associated peptide, respectively, TGFβ1 was identified as the main mediator stimulating matrix synthesis and PDGF as the responsible mitogen. Our data demonstrate that platelets contain fibrogenic mediators that stimulate proliferation (PDGF) and matrix synthesis (TGFβ1) of cultured PSC. We suggest that platelets and PSC cooperate in the development of pancreas fibrosis. (*Lab Invest 2000, 80:47–55*).

*C*hronic pancreatitis is characterized by the presence of chronic inflammatory lesions, by the destruction of exocrine parenchyma, and by fibrosis (Kennedy et al, 1987; Uscanga et al, 1987; Sarles et al, 1990; Di Magno et al, 1993). At present, the pathobiochemical and molecular mechanisms resulting in pancreas fibrosis are controversial and largely unknown. Activation of fibroblasts and "fibroblast-like" cells was reported to be a common observation in acute and chronic pancreatitis (Elsässer et al, 1986, 1989; Uscanga et al, 1987). It was suggested that periacinar fibroblast-like cells might play a role in fibrogenesis by synthesizing significant amounts of extracellular matrix, in particular collagens (Gress et al 1994; Kato et al, 1996; Saotome et al, 1997). Recently the presence of retinoid-containing pancreatic stellate cells (PSC) was demonstrated in rats and humans (Apte et al, 1998; Bachem et al, 1998). We have cultured and characterized the cells and have shown that PSC synthesize the same extracellular matrix components that are found in pancreas fibrosis, namely collagen types I and III and fibronectin (Bachem et al, 1998). Because PSC are similar in their morphology and biochemistry to hepatic stellate cells (Bachem et al, 1998), PSC are suspected to play the same role in pancreas fibrogenesis as hepatic stellate cells do in liver fibrosis.

Elsässer and coworkers (Elsässer et al, 1986, 1989) have shown that in the cerulein-induced pancreatitis model, proliferation of acinar and centroacinar cells is associated with an accelerated mitotic activity of fibroblasts that is followed by a stimulated synthesis and deposition of collagen. During regeneration from cerulein-induced pancreatitis, the expression of transforming growth factor β (TGFβ) is enhanced in acinar and "stromal cells" of rat pancreas (Gress et al, 1994). Interestingly, TGFβ-protein increase was detected 24 hours earlier as the increase in TGFβ1 mRNA expression (Gress et al, 1994), suggesting that platelets might be the source of the early TGFβ release in cerulein-induced pancreatitis. Following acinar cell injury, platelets aggregate in areas of inflammation (Bockman, 1988; Bockman et al, 1986). Platelets represent the major storage site for TGFβ (Assoian et al,

1983, 1986) and contain several other growth factors, such as platelet-derived growth factor (PDGF) (Antoniades et al, 1979; Heldin et al, 1981) and epidermal growth factor/transforming growth factor α (Oka and Orth, 1983), which all represent fibrogenic and/or mitogenic mediators. In this study we investigated the effect of platelet lysate on human PSC in cell-proliferation and extracellular matrix synthesis. To define the role of activated TGFβ1, we compared the effects of native platelet lysates (nPL) with those of transiently acidified platelet lysates (aPL). During ultrasonification of platelets, TGFβ1 is released in a latent form from alpha-granules and can be activated by transient incubation at pH 2 (Miyazono et al, 1988). Transiently acidified platelet lysate increased the synthesis of c–fibronectin (cFN) and collagen types I and III in cultured human PSC and enhanced their proliferation. Using neutralizing antibodies to TGFβ1 and PDGF, we identified TGFβ1 as the main stimulator of matrix synthesis and PDGF as the mitogen responsible for stimulating PSC proliferation.

Results

Demonstration of Platelet Aggregates in Acute Pancreatitis

Figure 1 shows a capillary lumen within pancreatic tissue from a female patient suffering from acute biliary pancreatitis. Within the capillary lumen, an aggregation of platelets is found. The majority of platelets contain typical electron-dense granules, others are already degranulated.
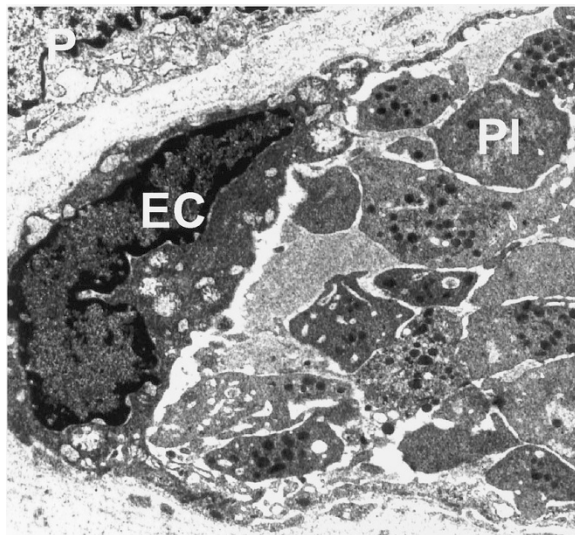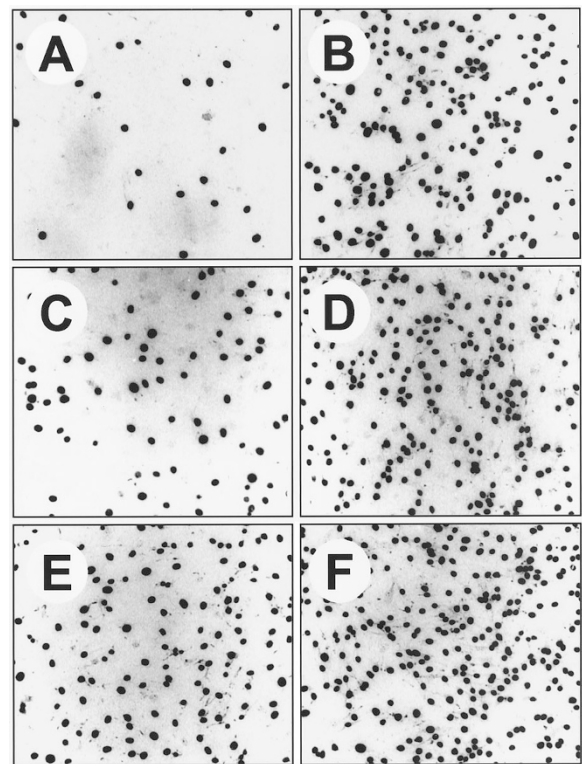

Figure 1.

Electron micrograph of a capillary lumen containing an aggregation of platelets. Pancreatic tissue was surgically resected from a woman with acute biliary pancreatitis. The tissue was fixed for 1 hour in a mixture of 2.5% glutaraldehyde and 2.5% formaldehyde. Standard procedures were used for dehydration and embedding. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a transmission electron microscopy. *Pl*, platelet; *EC*, endothelial cell; *P*, pericyte. Original magnification ×4500.

### Effect of Platelet Lysate on Pancreatic Stellate Cell Proliferation

To investigate the effect of platelet lysate on proliferation of cultured PSC, BrdU-incorporation was measured during a labeling period of 16 hours. Optimal BrdU concentration and labeling time was determined in earlier experiments. Incorporated BrdU was immunostained (Fig. 2) and measured by time-resolved fluorescence-immunoassay (Fig. 3b). DNA was measured 6 days after addition of PL to cultured PSC (Fig. 3a). As shown in Figure 2, addition of platelet lysate to PSC cultured in the presence of 0.5% fetal calf serum resulted in an increased number of stained cell nuclei. In control PSC (Fig. 2A), a fraction of 0.09 incorporated BrdU; in the presence of 10% fetal calf serum (Fig. 2B) a fraction of 0.68 was stained; addition of 120 μl aPL (Fig. 2F) resulted in an increased BrdU incorporation to a fraction of 0.98. The effects of aPL (Fig. 2, D and F) were more pronounced than nPL (Fig. 2, C and E).

By 144 hours after addition of PL, DNA content per culture increased from 0.68 μg/well (control) to 1.13 μg/well in cultures receiving 120 μl nPL, and to 1.97 μg/well in cultures receiving 120 μl aPL (Fig. 3a). Furthermore, BrdU immunoassay demonstrated that

Figure 2.

Effect of platelet lysate on bromodeoxyuridine (BrdU) incorporation (immunoperoxidase staining). Human pancreatic stellate cells (PSC) were cultured in the presence of 0.5% fetal calf serum (FCS). Four hours after addition of platelet lysates (30 and 120 μl/ml medium) BrdU (final concentration 10^{-5} M) was added. Cultures were stopped 16 hours later. BrdU incorporation was visualized by immunoperoxidase staining using diaminobenzidine (DAB). (A) Control culture receiving 0.5% FCS; (B) PSC receiving 10% FCS; (C) addition of 30 μl/ml native platelet lysate (nPL); (D) addition of 30 μl/ml transiently acidified platelet lysate (aPL); (E) addition of 120 μl/ml nPL; (F) addition of 120 μl/ml aPL.
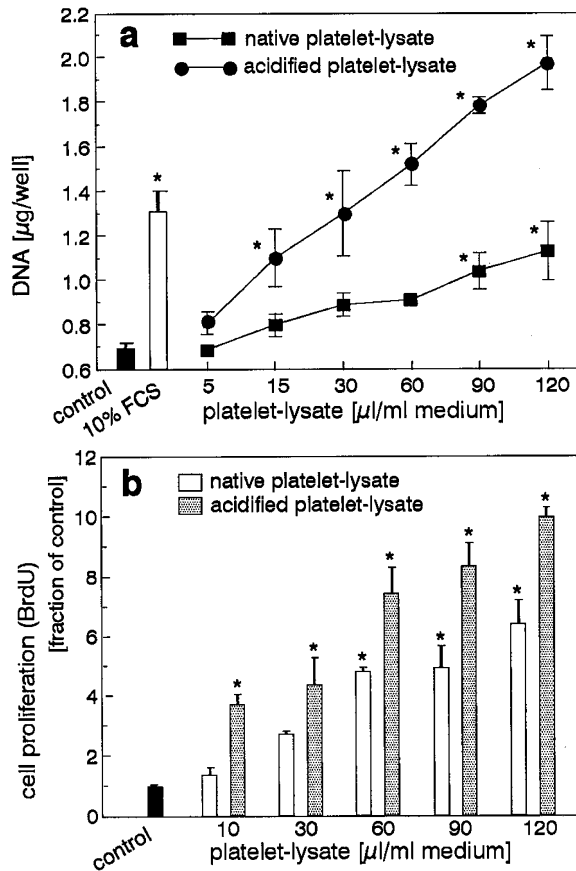
Figure 3.

Effect of platelet lysate on proliferation of cultured human PSC. (a) Measurement of DNA. Human PSC were seeded at 10^5 cells/cm^2 and cultured in the presence of 0.5% FCS. Seventy-two hours after addition of native and transiently acidified platelet lysates (5, 15, 30, 60, 90, and 120 μl/ml medium) the medium was changed and cell cultures were stimulated for further 72 hours. Cultures were stopped and DNA was measured by fluorometry using bisbenzimide. Mean values ± SD of three independent experiments, each with $n = 3$. (b) Measurement of BrdU incorporation. Human PSC were cultured in the presence of 0.5% FCS. Four hours after addition of nPL and aPL (10, 30, 60, 90, and 120 μl/ml medium) BrdU (final concentration 10^{-5} M) was added. Cultures were stopped 16 hours later. BrdU incorporation was measured quantitatively as described in the Methods section. Mean values ± SD of three independent experiments, each with $n = 3$.

nPL, and even more notably aPL, dose-dependently stimulated PSC proliferation (Fig. 3b). nPL (120 μl/ml) enhanced BrdU incorporation 6.4-fold and aPL (120 μl/ml) enhanced BrdU incorporation 10-fold (Fig. 3b) over control levels (Fig. 3b).

Effect of Platelet Lysate on the Synthesis of Collagen and Fibronectin by Cultured Pancreatic Stellate Cells

To demonstrate the effects of PL on cell-associated collagens and fibronectin, cultured human PSC were incubated for 36 hours with nPL and aPL, respectively. Thereafter, cultures were fixed and immunostaining was performed. As shown in Figure 4, intensive staining patterns of collagen type I (Fig. 4, C and E) and fibronectin (Fig. 4, D and F) were observed in cultures receiving 120 μl/ml nPL (Fig. 4, C and D) and aPL (Fig. 4, E and F). Collagen type I immunoreaction was

detected predominantly intracellularly with the highest intensity in the perinuclear region. PSC stimulated with PL appeared larger (Fig. 4, C and E) compared with control cells (Fig. 4A) receiving 0.5% fetal calf serum. Fibronectin (Figs. 4, B, D, and F) was located intracellularly and in extracellular fibrillar structures that varied in density. As shown in Figure 5, the steady state levels of collagen type I-mRNA and fibronectin-mRNA were only slightly increased by nPL and aPL in secondary cultured human PSC.

The stimulating effects of PL on the synthesis of extracellular matrix were also demonstrated by measuring the concentration of procollagen-peptides I and III and cFN in cell culture supernatants. As shown in Figure 6a, addition of PL to cultured PSC caused a dose-dependent increase in cFN concentration. Fibronectin concentrations were expressed per μg DNA to exclude the effect of PL on cell number. At all concentrations tested, aPL stimulated cFN synthesis to a higher degree than nPL (an increase of 1.6 ± 0.3 versus 2.49 ± 0.28 (Fig. 6a). To increase the collagen proline hydroxylation, ascorbic acid (100 μg/ml medium) was added to the culture medium in experiments demonstrating the effects of PL on collagen synthesis. Calculations of procollagen-I and -III-peptide concentrations on their corresponding DNA showed that aPL, but not nPL, increased collagen synthesis significantly (Fig. 6, b and c).

To identify the fibrogenic mediators that stimulate proliferation and matrix synthesis, aPL was preincubated for 30 minutes with the TGFβ-latency associated peptide (LAP) and neutralizing antibodies to PDGF and TGFβ1, respectively, before the lysate was added to cultured PSC. Neutralizing anti-PDGF(AB) significantly reduced the stimulatory effect of PL on BrdU-incorporation (Fig. 7a), whereas anti-TGFβ1 and LAP significantly reduced the cFN synthesis stimulating activity of PL (Fig. 7b).

Discussion

In this study we demonstrate for the first time that native and – in particular – transiently acidified platelet lysate increased the synthesis of cFN and collagen types I and III in cultured human PSC. In addition, platelet lysate enhanced proliferation of these cells. By preincubating the platelet lysates with neutralizing antibodies to TGFβ1 and PDGF, we identified TGFβ1 as the main mediator matrix synthesis-stimulating and PDGF as the most important mitogen in PL.

The role of platelets as a source of an early TGFβ-release in acute pancreatitis was suggested by an earlier study by our group demonstrating that the TGF-β protein increased earlier than TGFβ1 mRNA in pancreas tissue of rats with cerulein pancreatitis (Gress et al, 1994). In this model, TGFβ1 gene expression paralleled collagen type I gene expression and after the initial release from platelets, TGFβ1 was produced by inflammatory cells, stromal cells, and acinar cells (Gress et al, 1994). Furthermore, recently obtained data suggest that initially released TGFβ1 could induce its own de novo synthesis in PSC,
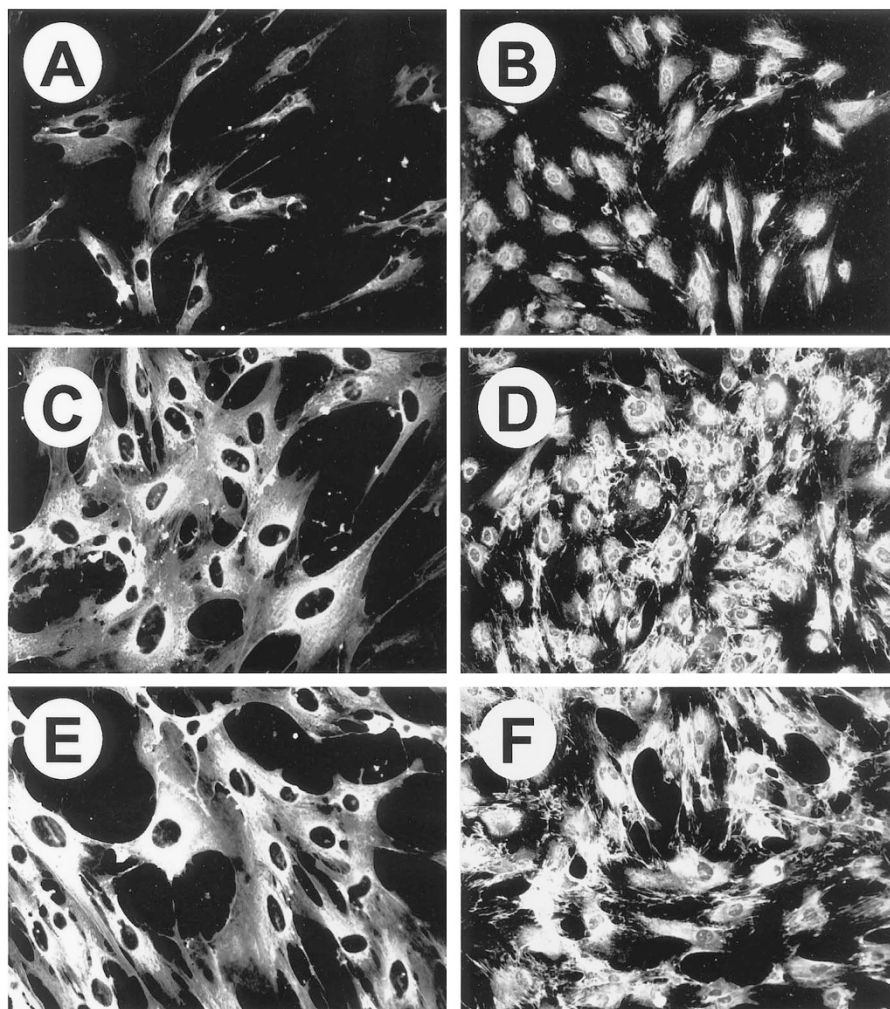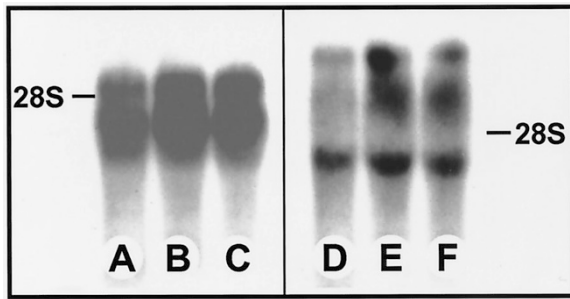
Figure 4.
Fluorescence micrographs showing the immunoreactivity of collagen type I and fibronectin in cultured human PSC stimulated with platelet lysate. Cultured human PSC (4th passage) grown on glass coverslips were stimulated for 36 hours with 120 μl/ml nPL and aPL. Thereafter cultures were washed, fixed in acetone, and immunostained. (A, C, and E) collagen type I; (B, D, and F) fibronectin; (A and B) control 0.5% FCS; (C and D) 120 μl/ml nPL; (E and F) 120 μl/ml aPL. To compare staining intensities, all micrographs were taken using the same exposure time. (A, C, and E) original magnification ×400; (B, D, and F) original magnification ×200.

leading to a self-perpetuation of pancreas fibrogenesis by autocrine stimulatory loops (Bachem et al, 1999).

The central role of TGFβ in experimental and human fibrogenesis is documented by several studies (for review see Gress et al, 1998). TGFβ neutralizing antibodies injected into rats during regeneration from cerulein-pancreatitis significantly reduced collagen types I and III protein and mRNA compared with control (without anti-TGFβ1) (Menke et al, 1997). Furthermore, anti-TGFβ1 inhibited the rise of the steady state levels of TGFβ1 mRNA and TGFβ2 mRNA the second day after the cerulein infusion (Menke et al, 1997), suggesting that the early TGFβ1 release by platelets induces the synthesis of TGFβ. In summary, the data obtained in experimental models convincingly identified TGFβ as the most prominent fibrogenic mediator in pancreas. In vivo latent TGFβ is activated predominantly by plasmin (Lyons et al, 1990). In vitro transient acidification represents the most effective procedure to activate latent TGFβ (Miyazono et al,

1988). Acidified platelet lysate, containing active TGFβ1 in combination with other growth factors stored in platelets, stimulated matrix synthesis and PSC proliferation. Preincubation of the acidified platelet lysate with neutralizing antibodies to TGFβ1 and the TGFβ-LAP significantly reduced cFN synthesis.

In addition, TGFβ platelets contain in their alpha-granules numerous proteins, including adhesion factors, plasma proteins, coagulation factors, protease inhibitors (Davey and Luscher, 1968; Levine and Wohl, 1976; Nachman and Harpel, 1976; Koutts et al, 1978; Zucker et al, 1979; Plow and Collen, 1981), and the growth factors PDGF (Antoniades et al, 1979; Heldin et al, 1981), EGF/TGFα (Oka and Orth, 1983), and IGF (Karey and Sirbasku, 1989; Karey et al, 1989). All three isoforms of PDGF, the AB heterodimer and the AA and BB homodimers, have been isolated from human platelets. In addition to its mitogenic potency, PDGF has been shown to stimulate collagen (Butt et al, 1995) and fibronectin synthesis (Bachem et al, 1998) and to act as a potent chemoattractant for mesenchymal

Figure 5.
Northern blot hybridization of collagen α1(I) and fibronectin. NPL and aPL were added to secondary cultured human PSC. Cultures were stopped after 10 hours and RNA was isolated. RNA (30 μg/lane) was separated by agarose gel electrophoresis and transferred to Hybond-N membranes. Ethidium bromide staining of the agarose gels was used to verify equal loading and blotting of total RNA (data not shown). Hybridizations were performed by standard procedures using 0.5×10^6 cpm/ml labeled cDNA. Exposure to X-ray films was at $-70°$ C for 7 to 10 days. Lanes A, B, and C, collagen type I; lanes D, E, and F, fibronectin; lanes A and D, control; lanes B and E, 100 μl/ml aPL; lanes C and F, 100 μg/ml nPL.
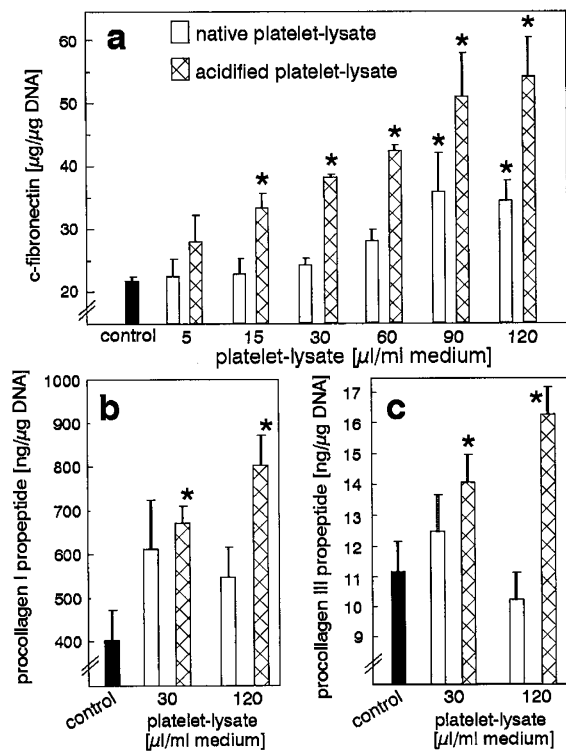
Figure 6.
Effect of platelet lysate on extracellular matrix synthesis of cultured pancreatic stellate cells. NPL and aPL were added to secondary cultured PSC grown in Dulbecco's modification of Eagle's medium with 0.5% FCS. After 48 hours cultures were stopped and fibronectin (a), procollagen I propeptide (b), and procollagen III propeptide (c) were measured in PSC supernatants; DNA was measured in the cell layer. In (a) values are expressed as means ± SD of three independent experiments, each performed in triplicate culture wells; in (b) and (c) values are expressed as means ± SD of two independent experiments, each performed in triplicate culture wells. *, denotes a statistically significant difference ($p < 0.05$) compared with control.

(Seppa et al, 1982) and inflammatory cells (Deuel et al, 1982). Because preincubation of the acidified platelet lysate with neutralizing antibodies to PDGF signifi-

cantly reduced the mitogenic activity by 60%, we conclude that PDGF is at least responsible for more than half the mitogenic activity.

TGFβ acts predominantly antiproliferatively on a variety of cell types including endothelial (Haimark et al, 1986) and pancreatic acinar cells (Logsdon et al, 1992), but also proliferative effects have been reported, for example, on osteoblasts (Centrella et al, 1987). Regarding fibroblasts and vascular smooth muscle cells, TGFβ can inhibit as well as stimulate cell growth in identical cell types (Assoian and Sporn, 1986). This bifunctional effect is probably due to the interaction with other growth factors, as proposed by Roberts et al (Roberts et al, 1985). In addition, several in vitro studies verified that TGFβ stimulates cell proliferation by acting in coordination with PDGF, TGFα/EGF, and IGFs (Assoian et al, 1984; O'Keefe et al, 1988). However, our data, which showed that preincubation of acidified platelet lysate with neutralizing antibodies to TGFβ1 and the TGFβ-latency associated peptide, respectively, did not reduce the mitogenic activity, indicate that TGFβ1 is not involved in PL-induced PSC proliferation.

Modification of other growth factors by acidification may be conceivable, possibly leading to activation of a latent mitogen or destruction of a growth-inhibitory factor. IGF1 is another candidate mitogen in PL that is also complexed to binding proteins in serum. By incubation at an acidic pH, IGF might dissociate from their binding proteins. The IGF-binding proteins (IGF-BP1, IGF-BP2, and IGF-BP3) regulate the half-life of IGF in the blood and modulate IGF function (for review see Rechler and Nisley, 1990).

Materials and Methods

Materials were purchased from the following sources: rabbit anti-human collagen type I and biotin labeled goat anti-human collagen type III from Chemicon International (Temecula, California); rabbit anti-fibronectin from Behring Diagnostics (Marburg, Germany); biotin labeled anti-rabbit, biotin labeled anti-mouse, HRP labeled anti-rabbit, biotin labeled anti-goat, HRP labeled anti-mouse, fluorescein-conjugated strep-tavidin, and HRP-conjugated streptavidin, mouse anti-BrdU from Dako Diagnostika (Hamburg, Germany); streptavidin-Red 613 from Gibco BRL (Eggenstein, Germany); type I Procollagen PICP [125I] radioimmunoassay and type III Procollagen PIIINP [125I] radioimmunoassay from Orion Diagnostica (Espoo, Finland); the cDNA probes for human collagen α1 I, collagen α1 III, and fibronectin were from American Type Culture Collection (No. 61322, 61234, and 61038; Rockville, Maryland); mouse-anti-α-smooth-muscle actin, BrdU, and the High Pure RNA Extraction Kit from Boehringer-Mannheim Biochemica (Mannheim, Germany); TSA Indirect from NEN Life Science Products (Boston, Massachusetts; bisbenzimide, calf thymus DNA, yeast t-RNA, diethyl-endtriaminepentaacetic (DTPA), monoclonal anti-fibronectin, and β-aminopropionitrile from Sigma Chemie (Deisenhofen, Germany); ascorbic acid (Merck, Darmstadt, Germany); enhancement solution and
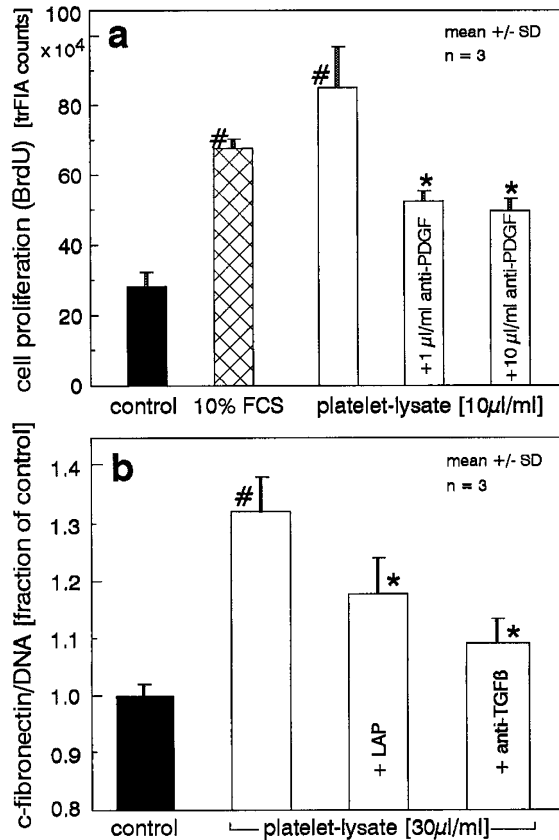
Figure 7.

Identification of PDGF as the responsible mitogen and TGFβ1 as the matrix-synthesis-stimulating activity of platelet lysate. (a) Effect of anti-PDGF on platelet lysate-induced proliferation of cultured PSC. aPL was preincubated for 30 minutes with neutralizing anti-PDGF (1 μl/ml and 10 μl/ml) before lysates were added to secondary cultured PSC grown in Dulbecco's modification of Eagle's medium with 0.5% FCS. Four hours after addition of platelet lysates (10 μl/ml medium), BrdU (final concentration 10^{-5} M) was added. Cultures were stopped 16 hours later. BrdU incorporation was measured quantitatively as described in the Methods section. Values are expressed as means $\pm$ SD of one representative experiment, each performed in triplicate culture wells. #, denotes a statistically significant ($p < 0.05$) difference to control; *, denotes a significant ($p < 0.05$) difference to platelet lysate without anti-PDGF. (b) Effect of anti-TGFβ1 and TGFβ1-LAP platelet lysate-induced cFN synthesis of cultured PSC. aPL was preincubated for 30 minutes with neutralizing anti-TGFβ1 (1 μl/ml and 10 μl/ml) before lysates were added to secondary cultured PSC grown in Dulbecco's modification of Eagle's medium with 0.5% FCS. After 24 hours cultures were stopped and cFN was measured in PSC supernatants; DNA was measured in the cell layer. Values are expressed as means $\pm$ SD of one representative experiment, each performed in triplicate culture wells. #, denotes a statistically significant ($p < 0.05$) difference compared with control; *, denotes a significant ($p < 0.05$) difference to platelet lysate without preincubation with LAP and anti-TGFβ1.

europium-conjugated streptavidin from Wallac Oy (Turku, Finland); and neutralizing anti-human TGFβ1, TGFβ-latency-associated peptide, and neutralizing anti PDGF from R and D Systems (Minneapolis, Minnesota). The 18S rRNA probe was generously provided by Dr. T.M. Gress (University Ulm, Germany). The 96-well microtiter-plates (Maxi Sorp) were from Nunc GmbH (Wiesbaden, Germany), and cell culture plates and flasks were from Falcon (Becton Dickinson, Heidelberg, Germany). Hybond-N membranes were purchased from Amersham-Buchler (Braunschweig, Germany).

Preparation of the Platelet Lysate

Frozen outdated human platelet concentrates (obtained from the Blutspendezentrale of the German Red Cross in Ulm, Germany) were thawed, lysed by sonication, and centrifuged at 4° C for 20 minutes at 3000 $\times g$. Thereafter, clotting cascade was initialized by addition of $CaCl_2$ (15 mmol/l) to the supernatant. Fibrin clots were removed and the supernatant was dialyzed extensively (cut-off Mr 3500) against Dulbeccos modification of Eagle's medium at 4° C (3 $\times$ 12 hours, 100-fold volume). One aliquot was transiently acidified with 1 N HCl (pH 2, 30 minutes) and thereafter neutralized using 1 N NaOH. Native (nPL) and the transiently acidified platelet lysate (aPL) were sterilized by passing through a 0.2-μm filter and added to cell cultures immediately.

Isolation and Culture of Pancreatic Stellate Cells

Human pancreatic stellate cells were isolated and cultured as described previously (Bachem et al, 1998). Cells were cultured in DMEM/Ham's F12, (1:1, v/v) with antibiotics and 10% fetal calf serum. All experiments were performed using passaged cells (3rd to 7th passage). The purity of the cultures was assessed by phase-contrast microscopy and positive staining of vimentin and α-smooth muscle actin.

To measure cFN- and collagen-synthesis in cell culture supernatants, PSC were grown in 24-well plates. L(+)ascorbic acid (100 μg/ml) and β-aminopropionitrile (64 μg/ml) were added to inhibit collagen crosslinking and to increase proline hydroxylation and collagen secretion. To perform immunofluorescence microscopy, cells were grown on glass coverslips in 6-well plates (10 cm^2/well). To isolate mRNA, cells were grown in flasks (75 cm^2). During stimulation with PL, fetal calf serum was reduced to 0.5%. Cell culture supernatants were removed 48 hours after addition of PL and stored at $-80°$ C until quantitative measurement of cFN, collagen type I, and procollagen I peptide (PICP).

Determination of Extracellular Matrix Synthesis

cFN was measured by time resolved fluorescence immunoassay as described (Bachem et al, 1998, 1999). All measurements (standards, controls, and samples) were obtained in duplicate. Variation in the duplicate measurements was usually between 0.5% and 5%, and did not exceed 8%.

Procollagen-I peptide and procollagen III peptide were measured using commercially available radioimmunoassays according the manufacturers instructions. All measurements were made in duplicate.

Immunofluorescence Microscopy of Collagen Type I and Fibronectin

Glass coverslips with cultured hPSC were washed in PBS to remove medium proteins, fixed for 30 minutes in $-20°$ C acetone, and blocked with TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0.5% RIA grade albumin, pH 7.4) for 45 minutes. For collagen type I, the staining

sequence was rabbit-anti-human-collagen I (1:50), biotin-anti-rabbit (1:100), streptavidin-HRP (1:100), biotin-TSA-reagent (1:40), and streptavidin-FITC (1:100). The staining sequence for fibronectin was primary antibody (rabbit-anti-fibronectin, 1:50), secondary antibody (biotin-anti-rabbit, 1:100), and streptavidin-FITC (1:100). For α-smooth muscle actin, the staining sequence was primary antibody (mouse anti-α-smooth muscle actin, 1:50), secondary antibody (HRP-anti–mouse, 1:50), biotin-TSA-reagent (1:40), and streptavidin-FITC (1:100). Each incubation step was followed by rinsing (3 × 5 minutes with PBS). Staining was observed using epifluorescence microscopy (Carl Zeiss, Oberkochen, Germany) and photographs were taken using Ektachrome 400 film (Kodak). To compare different staining intensities, exposure time was always the same. Nonspecific staining was controlled by including rabbit- or mouse-non-immune serum instead of specific primary antibody.

Electron Microscopy

Pancreatic tissue was surgically resected from 10 patients with acute pancreatitis. The tissue was fixed for 1 hour in a mixture of 2.5% glutaraldehyde and 2.5% formaldehyde, in a cacodylate buffer. Standard procedures were used for dehydration and embedding. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (Zeiss EM 10).

Determination of Cell Proliferation

BrdU-incorporation was quantified by time-resolved fluorescence of a europium-chelate (Bachem et al, 1995). Briefly, cells were labeled for 16 hours with BrdU (5×10^{-5} M). Thereafter, cell cultures were washed twice with TNT (TNT: 0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4), fixed using ethanol/acetic acid (95/5, v/v), and thereafter incubated for 20 minutes at 4° C with 0.05 M HCl. After another washing step, DNA was cleaved by incubation for 45 minutes at 80° C with formamide/trisodium-citrate (88 mg trisodium-citrate in 38 ml formamide). After 2 washing steps, non-specific binding was blocked by incubation with serum (diluted 1:1 with TNT buffer) followed by 3 washing steps. Thereafter, primary antibody (mouse-anti-BrdU IgG diluted 1:500 in TNB) was added and incubated with gentle shaking for 2 hours at 22° C. After 3 further washing steps, secondary antibody (biotin-labeled anti-mouse IgG, diluted 1:500 in TNB) was added and incubated for another 60 minutes; thereafter DTPA (Diethylentriaminepantaacetic acid, 20 μmol in TNB, pH 7.4) was added and incubated for 15 minutes at room temperature. After 3 additional washing steps europium-labeled Streptavidin (diluted in TNB 1:1000) was added and incubated for 1 hour. After 5 washing steps, enhancement solution (150 μl/well) was added, and incubated for 45 minutes at 22° C. Finally time-resolved fluorescence of the europium-chelate was counted in a 100-μl aliquot

using a Delfia Victor 1420 Multilabel Counter (Wallac Oy).

BrdU incorporation was also visualized by immunostaining. Briefly, cells were cultured in 6-well plates, stimulated, and fixed after labeling with BrdU as described above. The first steps in the staining procedure were performed in the same way as described for quantitative BrdU determination. The secondary antibody was HRP-conjugated rabbit-anti-mouse IgG (diluted 1:100 in TNB). After incubation for 120 minutes with the secondary antibody followed by 3 washing steps, the nuclei that had incorporated BrdU were stained using 3,3′-diaminobenzidine tetrahydrochloride as substrate and hydrogen peroxide 0.3% with nickel- and cobalt-chloride as intensifier. DNA was quantified as previously described (Labarca and Paigen, 1980) by fluorometry using bisbenzimide and calf thymus DNA as a standard.

RNA Isolation and Northern Blot

PSC cultures were stopped 10 hours after stimulation with PL to extract total RNA using the High Pure RNA Isolation Kit. For Northern blot analysis, 30 μg of total RNA was separated by gel electrophoresis in 1% agarose and 2.2 mol/l formaldehyde and transferred by capillary elution to Hybond-N membranes. Ethidium bromide staining of the agarose gels and hybridization with an 18S ribosomal RNA probe were used to verify equal loading and blotting of total RNA. Purified cDNA probes were labeled with [32P]dCTP. Prehybridizations (4 to 6 hours) and hybridizations (14 hours) were carried out in 6 × standard saline citrate (1 x SSC = 150 mM NaCl, 15 mM sodium citrate), 5 × Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml yeast t-RNA, 50 μg/ml sonicated human placenta DNA, 10 μg/ml polyU-homopolymer, and 50% formamide at 42° C. The hybridization buffer was supplemented with 0.5×10^6 cpm/ml labeled cDNA. The membranes were washed several times with decreasing concentrations of SSC + 0.1% SDS, the final high-stringency wash was done in 0.2 x SSC. Exposure to X-ray films was done at −70° C for 7 to 10 days.

Statistical Analysis

All quantitative measurements were obtained in duplicate. For quantitative measurement of cFN, DNA, and BrdU incorporation, results are presented as means ± SD of three independent experiments, each condition performed with 3 cultures. Procollagen-I-peptide and procollagen-III-peptide results are presented as means ± SD of two independent experiments ($n = 6$). An ANOVA-test was employed to compare different groups.

Conclusion

We concluded that factors released from platelets stimulate cell growth and the synthesis of extracellular matrix in cultured human PSC. In this complex pattern of growth factors and other bioactive peptides, TGFβ1

seems to represent the major matrix synthesis stimulating activity and PDGF the most responsible mitogen. Our results confirm the hypothesis that platelets, aggregating in injured sites of the pancreas, release growth factors that initiate and propagate fibrosis in chronic pancreatitis.

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