Lipopolysaccharide-Activated Macrophages Stimulate the Synthesis of Collagen Type I and C-Fibronectin in Cultured Pancreatic Stellate Cells

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We have recently identified and characterized pancreatic stellate cells (PSC) in rats and humans (Gastroenterology 1998, 15:421–435). PSC are suggested to represent the main cellular source of extracellular matrix in chronic pancreatitis. Now we describe a paracrine stimulatory loop between human macrophages and PSC (rat and human) that results in an increased extracellular matrix synthesis. Native and transiently acidified supernatants of cultured macrophages were added to cultured PSC in the presence of 0.1% fetal calf serum. Native supernatants of lipopolysaccharide-activated macrophages stimulated the synthesis of collagen type I 1.38 ± 0.09 -fold of control and c-fibronectin 1.89 ± 0.18 -fold of control. Transiently acidified supernatants stimulated collagen type I and c-fibronectin 2.10 ± 0.2 -fold and 2.80 ± 0.05 -fold of control, respectively. Northern blot demonstrated an increased expression of the collagen-I(α 1)-mRNA and fibronectin-mRNA in PSC 10 hours after addition of the acidified macrophage supernatants. Cell proliferation measured by bromodeoxyuridine incorporation was not influenced by the macrophage supernatants. Unstimulated macrophages released $1.97 \text{ pg TGF}\beta 1/\mu\text{g}$ of DNA over 24 hours and lipopolysaccharide-activated macrophages released $6.61 \text{ pg TGF}\beta 1/\mu\text{g}$ of DNA over 24 hours. These data together with the results that, in particular, transiently acidified macrophage supernatants increased matrix synthesis, identify TGF β as the responsible mediator. In conclusion, our data demonstrate a paracrine stimulation of matrix synthesis of pancreatic stellate cells via TGF β 1 released by activated macrophages. We suggest that macrophages might play a pivotal role in the development of pancreas fibrosis. (*Am J Pathol* 1999, 155:1749–1758)

Chronic pancreatitis is characterized by the presence of chronic inflammatory lesions, the destruction of exocrine parenchyma, and fibrosis.¹ Until now the pathobiochemical and molecular mechanisms resulting in pancreas fibrosis have been controversial and largely unknown. Fibroblast and fibroblast-like cell activation was reported to be a common observation in acute and chronic pancreatitis.^{2–6} It has been suggested that periacinar fibroblast-like cells might play a role in fibrogenesis by synthesizing significant amounts of extracellular matrix (ECM), in particular collagens.^{7,8} Studies from our group have shown that in the cerulein-induced pancreatitis model, proliferation of acinar and centroacinar cells is associated with an increase in mitotic activity of fibroblasts, which is followed by a stimulated synthesis and deposition of collagen.^{5,9} In an earlier study, we illustrated an enhanced expression of transforming growth factor β (TGF β) during regeneration from cerulein-induced pancreatitis in acinar cells and stromal cells of the rat pancreas, which was followed by stimulated matrix synthesis.¹⁰ Furthermore, we identified and characterized the matrix-producing cell type responsible for pancreas fibrosis, the pancreatic stellate cell (PSC) in mice, rats and humans.^{11,12} Phenotypic transformation of PSCs is characterized by a disappearance of fat droplets and retinyl-esters, development of a prominent endoplasmic reticulum, enhanced expression of smooth muscle α -actin, and increased synthesis of collagen types I and III and fibronectin.¹¹

Pancreas fibrosis is commonly associated with chronic inflammation.⁴ In chronic pancreatitis and in pancreas carcinoma, infiltrating mononuclear cells¹³ might potentiate fibrogenesis by the release of cytokines stimulating stromal cells¹⁴ and by their cytotoxic effects.¹⁵ The decreased intestinal motility during pancreatitis causes a bacterial translocation to the peritoneal fluid, lymph, blood, liver, and pancreas.¹⁶ Bacterial lipopolysaccharides (LPS) and the LPS-binding protein form a complex that binds to the surface receptor CD14 of the invaded monocytes, triggering the cells to become activated.^{17,18}

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LPS activation of monocytes/macrophages and aggregating platelets in areas of inflammation release several polypeptide growth factors including TGF β , TGF α /EGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF).^{19–22} It is suggested that these factors are responsible for stimulating ECM synthesis and proliferation of PSC.

Because macrophages are located close to PSC,¹¹ their capacity to release TGF β is of particular interest. Recent investigations revealed that TGF β is a multifunctional regulator of cell growth and differentiation.²³ TGF β stimulates the synthesis of ECM components and inhibits matrix degradation.²⁴ TGF β is secreted as a latent form associated with a latency-associated peptid. Latent TGF β -binding protein (LTBP) is covalently bound to the latency-associated peptid and forms a high-molecular-weight complex termed latent TGF β complex.²⁵ LTBP is assumed to play a strategic role in the assembly, secretion, and activation of latent TGF β .²⁶ In chronic pancreatitis, the TGF β precursor was detected mainly in mononuclear cells located in fibrotic areas.²⁷ LTBP is also predominantly present in mononuclear cells and in the ECM around them. The presence of both pro-TGF β and LTBP in monocytes and/or macrophages in areas of fibrosis strongly suggests that this cytokine is involved in the pathophysiology of chronic pancreatitis.²⁷

In the present study, we demonstrate that transiently acidified supernatants of LPS stimulated human monocyte-derived macrophages contain TGF β and stimulated the synthesis of fibronectin and collagen type I in cultured PSCs. These findings suggest that activated macrophages play a critical role in pancreas fibrogenesis by stimulating matrix synthesis of PSCs in a paracrine way.

Materials and Methods

Reagents

Materials were purchased from the following sources: Ficoll-Paque from Pharmacia, Biotech (Uppsala, Sweden); biotin labeled CD14(My4) and CD3-ECD from Coulter Immunotech (Hamburg, Germany); rabbit anti-human collagen type I and biotin-labeled goat anti-human collagen type III from Chemicon (Temecula, CA); rabbit anti-fibronectin from Behring Diagnostics (Marburg, Germany); biotin labeled anti-rabbit, biotin labeled anti-mouse, HRP anti-rabbit, biotin labeled anti-goat, HRP anti-mouse, fluorescein-conjugated streptavidin, HRP-conjugated streptavidin, mouse anti-BrdU, anti-CD19-PECy5, and anti-CD14-PE from DAKO (Hamburg, Germany); streptavidin-Red 613 from Gibco BRL (Eggenstein, Germany); mouse anti- α -smooth-muscle actin, bromodeoxyuridine, and the High Pure RNA Extraction Kit from Boehringer Mannheim (Mannheim, Germany); TSA Indirect from NEN Life Science Products (Boston, MA); fluorescein-conjugated *Escherichia coli* and propidium iodide (Orpegen, Heidelberg, Germany); trypan blue, bisbenzimidazole, calf thymus DNA, yeast t-RNA, diethylendiaminepentaacetic (DTPA), monoclonal anti-fibronectin, and β -aminopropionitrile from Sigma (Deisen-

hofen, Germany); ascorbic acid from Merck (Darmstadt, Germany); enhancement solution and europium-conjugated streptavidin from Wallac Oy (Turku, Finland); and TGF β 1-sR-II/Fc chimera and biotinylated anti-human TGF β 1 from R&D Systems (Minneapolis, MN). The 18S rRNA probe was generously provided by Dr. T. M. Gress (University Ulm, Germany). Ninety-six-well microtiter plates (Maxi Sorp) were from Nunc GmbH (Wiesbaden, Germany), cell culture plates and flasks were from Falcon (Becton Dickinson, Heidelberg, Germany) and petriPerm was from *In Vitro* Systems + Services (Osterode, Germany). Hybond-N membranes were purchased from Amersham-Buchler (Braunschweig, Germany).

Monocyte Isolation and Culture

Peripheral blood mononuclear cells were isolated from buffy coats of different donors by Ficoll-Paque gradient centrifugation. The mononuclear fraction was washed in PBS and then resuspended in RPMI 1640 containing 1% L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 10% fetal calf serum (FCS). Aliquots of the cell suspension (5×10^6 mononuclear cells/ml) were allowed to adhere in 75 cm² tissue culture flasks in 37°C in a humidified 5% CO₂ environment. For immunofluorescence staining cells were seeded on glass coverslips (1 cm²) in 6-well plates. To harvest cells for flow cytometric analysis, cells were seeded on petriPerm. After 2 hours the non-adherent cells were removed and fresh medium was added. Medium was changed each third day and cells were cultured for up to 14 days.

Production of Conditioned Media

Cultured macrophages in 75-cm² flasks (10–14 days after seeding) were washed with RPMI 1640. Thereafter 10 ml fresh RPMI per flask (with antibiotics, without FCS) was added and conditioned for 24 hours in the absence and presence of 8 μ g/ml LPS. Conditioned media were removed under sterile conditions, centrifuged (800 rpm, 5 minutes, 4°C) to remove cell debris, and stored at –80°C. The media were dialyzed for 36 hours against 100 vol distilled water at 4°C in tubings (ZelluTrans, Roth, Karlsruhe, Germany) with 3.5-kD molecular cutoff. Thereafter media were concentrated 10-fold by lyophilization and sterilized by passing through a 0.22- μ m pore size filter (Millex-GS, Millipore). To activate latent TGF β 1, aliquots of the media were acidified with 1 mol/L HCl for 10 minutes, then neutralized by adding 1.2 N NaOH/0.5 mol/L Hepes.

Pancreatic Stellate Cell Isolation and Culture

Human pancreatic stellate cells were isolated by outgrowth, using explant techniques from histologically fibrotic areas of the pancreas surgically resected from patients with chronic pancreatitis. Small tissue blocks were cut (0.5–1 mm³) and seeded in 10-cm² uncoated culture wells (6 per plate, 3–5 pieces/well) in the presence of 10 to 20% FCS in a 1:1 (v:v) mixture of Dulbec-

co's modified Eagle's medium (DMEM) with Ham's F12 medium. L-glutamine (2 mmol/L), penicillin/streptomycin, and amphotericin were freshly added. Tissue blocks were cultured at 37°C in a 5% CO₂-air humidified atmosphere. Eighteen hours after seeding, culture medium was changed and 24 hours later the small tissue blocks were transferred to new culture plates. The pancreatic stellate cells grew out in high number and purity from the tissue blocks 1 to 3 days later. The small tissue blocks were removed after 2 to 3 weeks.

To obtain a higher number of cells with the inactivated resting fat storing phenotype, the cells were isolated by density gradient centrifugation from the pancreas of untreated male Wistar rats as described.¹¹ Briefly, after the animals were anesthetized with pentobarbital, the abdomen was opened, the common bile duct was ligated, and a cannula was inserted into the biliopancreatic duct. The rats were exsanguinated and collagenase-containing Eagle's medium (1 mg/5 ml) was instilled intraductally. The distended pancreas was removed and shaken in an Erlenmeyer flask (37°C, 15 minutes.). After this first digestion the pancreas was minced, followed by a second digestion with collagenase (1.75 mg/5 ml, 45 minutes.). Dispersion was accomplished by up-and-down suction through cannulas with decreasing diameters. After dissociation the acini and cells were filtered through a 250- μ m nylon cloth and centrifuged after layering the filtrate on top of a dextran-Eagle-HEPES density gradient. Once centrifuged, cells were collected from the top of the gradient, washed twice, resuspended in Tris-buffered saline, and transferred on top of a Iodixanol (OptiPrep) density gradient. After another centrifugation, cells were collected from the top of the gradient, washed, and suspended in DMEM with 10% FCS, antibiotics, amphotericin, and L-glutamine. Thereafter cells were seeded in a density of 4×10^4 cells/cm².

Cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. The medium consisted of DMEM/Ham's F12 (1:1, v:v) with 10% FCS, 2% L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 1% amphotericin. Medium was changed 3 times a week. After reaching confluency, cells were subcultured by trypsinization using a 0.025% trypsin solution containing 0.01% EDTA in phosphate-buffered saline. Once the cell numbers were counted, they were again suspended in a complete medium and seeded with a density of $3\text{--}5 \times 10^4$ cells/cm². To increase the cell number to obtain sufficient amounts of RNA, cells were seeded in 75-cm² culture flasks (25 ml medium/flask).

Stimulation of Pancreatic Stellate Cells with Media Conditioned by Macrophages

Experiments were performed using PSC between passage 3–8. After passage PSC were cultured for 2 to 3 days in DMEM/HAM's F12 (1/1, v/v) in the presence of 10% FCS. Twelve hours before addition of macrophage supernatants, medium was changed to DMEM/HAM's F12 with 0.1% FCS. To study cell proliferation and matrix synthesis PSC were cultured in 24-well plates (2 cm²/

well, 1 ml medium). To study matrix synthesis, native and transiently acidified macrophage supernatants (40 and 160 μ l/ml) were added and cultures were stopped 24 hours later. To study cell proliferation BrdU (final concentration 0.5×10^{-5} M) was added 6 hours after addition of 40 μ l/ml native and transiently acidified macrophage supernatants. Cultures were stopped 18 hours later. To perform immunofluorescence microscopy of collagen and fibronectin, PSC were seeded on 1-cm² glass coverslips in 6-well plates and incubated with 40 μ l/ml transiently acidified macrophage supernatant. Cultures were stopped 38 hours later. To isolate mRNA, PSCs were grown in 75 cm² flasks containing 10 ml medium. To subconfluent quiescent cells (cultured for 12 hours in the presence of 0.1% FCS) 40 μ l/ml transiently acidified macrophage supernatant was added and cultures were stopped 10 hours later.

In all experiments the baseline (negative) control represents PSC cultured in DMEM/HAM's F12 with 0.1% FCS.

Immunofluorescence Microscopy and Flow Cytometric Analysis

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 for 45 minutes. For collagen type I the staining sequence was primary antibody (rabbit-anti-human-collagen I, 1:100), second antibody (HRP-anti-rabbit, 1:100), biotin-TSA-reagent (1:40) and streptavidin-FITC (1:100). For collagen type III the staining sequence was primary antibody (biotin labeled goat-anti-human-collagen III, 1:100), HRP-streptavidin (1:100), biotin-TSA-reagent (1:40) and streptavidin-FITC (1:100). The staining sequence for fibronectin was primary antibody (rabbit-anti-fibronectin, 1:100), second 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), second antibody (HRP-anti-mouse, 1:50), biotin-TSA-reagent (1:40) and streptavidin-FITC (1:100). Cells were viewed by epifluorescence microscopy (Carl Zeiss, Oberkochen, Germany) with appropriate filter sets.

To demonstrate phagocytosis macrophages were incubated with 3×10^7 opsonized FITC-conjugated *E. coli* for 6 hours at 37°C, washed with PBS, incubated first with biotin-anti-CD14 (1:33) and then with streptavidin-Red 613 (1:100). Thereafter, cells were fixed for 30 minutes in 4% formaldehyde. DNA was counterstained with bisbenzimidazole. Viability of cultured macrophages was assessed by trypan blue exclusion.

To analyze the cells by flow cytometry, macrophages were grown in petriPerm for 10 to 14 days and harvested by scraping. Thereafter cells were washed with PBS, resuspended, and analyzed for expression of CD14, CD3, and CD19 using an EPICS XL flow cytometer (Coulter Immunotech, Hamburg, Germany). Viable cells were detected by propidium iodide exclusion.

Quantitative Determination of Extracellular Matrix Synthesis

To measure collagen type I cells were cultured in DMEM/HAM's F12 with 0.1% FCS in the presence of ascorbic acid (100 $\mu\text{g/ml}$) and β -aminopropionitrile (100 $\mu\text{g/ml}$). By time-resolved fluorescence-immunoassay collagen type I was measured in culture supernatants 24 hours after stimulation. Briefly, 100 μl cell culture supernatant (diluted 1:4 with 0.05 mol/L NaHCO_3 , pH 9.1) were transferred to 96-well microtiter plates (Nunc-Maxi Sorp) and incubated overnight at 4°C. After 3 washing steps (wash buffer: Tris 0.05 mol/L, NaCl 0.15 mol/L, Tween 20 0.05%, pH 7.5) plates were blocked during 2 hours with assay buffer (Tris 0.05 mol/L, NaCl 0.15 mol/L, dry milk powder 5%, pH 7.5). Thereafter, the plates were incubated for 3 hours with a polyclonal rabbit-anti-human-collagen type I (diluted 1:500 in assay buffer). After washing 3 times the plates were incubated for 2 hours with the second antibody (biotin-labeled anti-rabbit IgG diluted 1:1000 in assay buffer). Thereafter, an Europium-labeled streptavidin (diluted 1:1000 in assay buffer) was added and incubated for 1 hour. After additional 3 washing steps, 100 μl enhancement solution was added for 30 minutes at room temperature and thereafter time-resolved fluorescence of the Europium chelate was measured using a Victor 1420 Multilabel Counter (Fa. Wallac, Turku, Finland). All measurements were done in duplicate.

To measure c-fibronectin, time-resolved fluorescence immunoassay was used. Briefly, 96-well microtiter plates were coated 3 hours at room temperature with gelatin (10 $\mu\text{g/ml}$) in coating buffer (0.05 mol/L NaHCO_3 , pH 9.1) and thereafter blocked overnight at 4°C using assay buffer (Tris 0.05 mol/L, NaCl 0.15 mol/L, RIA grade albumin 0.5%, pH 7.7). Standards (100 μl , 5000–19 ng/ml) and culture supernatants diluted in assay buffer were added and incubated overnight at room temperature. Thereafter, the plates were incubated for 1 hour with a monoclonal mouse-anti-c-fibronectin diluted 1:1000 in assay buffer. After washing 3 times the plates were incubated for 1 hour with the second antibody (biotin-labeled anti-mouse IgG diluted 1:1000 in assay buffer) followed by 3 washing steps. Thereafter, an Europium-labeled streptavidin (diluted 1:1000 in assay buffer) was added and incubated for 1 hour. After 3 more washing steps, 100 μl enhancement solution was added for 30 minutes at room temperature and thereafter time-resolved fluorescence of the Europium chelate was measured using a Victor 1420 Multilabel Counter. All measurements were done in duplicate.

Quantitative Determination of TGF β

TGF β 1 was measured by time-resolved fluorescence immunoassay. Ninety-six-well microtiter plates were coated overnight at room temperature with TGF β 1-sR-II/Fc Chimera (0.2 $\mu\text{g/ml}$) diluted in coating buffer (0.05 mol/L NaHCO_3 , pH 9.2). After 3 washing steps (wash buffer: PBS, Tween 20 0.05%, pH 7.4), plates were blocked during 2 hours at room temperature with blocking buffer

(PBS, Tween 20 5%, sucrose 5%, NaN_3 0.05%) and again washed 3 times. One-hundred-microliter standards (5 ng/ml – 156 pg/ml) diluted in DMEM with 0.1% RIA grade albumin and culture supernatants diluted in diluent (PBS, milk powder 0.5%, Tween 20 0.05%) were added and incubated for 2 hours at room temperature. After 3 washing steps, plates were incubated with biotin anti-human TGF β 1 diluted 1:250 in diluent (20 mmol/L Trizma-base, 150 mmol/L NaCl, 0.1% RIA grade albumin) for 2 hours, followed by 3 washing steps. Thereafter, an Europium-labeled streptavidin (diluted 1:1000 in diluent) was added and incubated for 1 hour. After additional 5 washing steps, 100 μl of enhancement solution were added for 30 minutes at room temperature and thereafter time-resolved fluorescence of the Europium chelate was measured using a Victor 1420 Multilabel Counter. All measurements were done in duplicate.

Measurement of DNA

DNA was quantified as previously described²⁸ by fluorometry using bisbenzimidazole and calf thymus DNA as a standard. Fluorescence (Ex. 350 nm, Em. 450 nm) was measured with a Victor 1420 Multilabel Counter.

Measurement of Cell Proliferation

Bromodeoxyuridine (BrdU) incorporation was quantified by time-resolved fluorescence of a Europium chelate.²⁹ Briefly, cells were labeled for 18 hours with BrdU (5×10^{-5} M). Thereafter, cell cultures were washed twice with TNT (0.1 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.5% RIA grade albumin, pH 7.4), fixed using ethanol/acetic acid (95/5, v/v), and then incubated for 20 minutes at 4°C with 0.05 mol/L 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, nonspecific binding was blocked by incubation with FCS (diluted 1:1 with TNB: 0.1 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.05% Tween 20, pH 7.4) followed by 3 washing steps. Thereafter, first 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 more washing steps, a second antibody (biotin-labeled anti-mouse IgG, diluted 1:500 in TNB) was added and incubated for another 60 minutes; thereafter DTPA (diethylenetriaminepentaacetic 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 $\mu\text{l/well}$) 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).

RNA Isolation and Northern Blot

PSC cultures were stopped 10 hours after stimulation with macrophage supernatants 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 [^{32}P]dCTP. Prehybridizations (4–6 hours) and hybridizations (14 hours) were carried out in 6 $\times$ standard saline citrate (1 $\times$ SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate), 5 $\times$ Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 $\mu\text{g}/\text{ml}$ yeast t-RNA, 50 $\mu\text{g}/\text{ml}$ sonicated human placenta DNA, 10 $\mu\text{g}/\text{ml}$ 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 $\times$ SSC. Exposure to X-ray films was done at -70°C for 7 to 10 days.

Statistics

Quantitative measurements of c-fibronectin, collagen type I, DNA, BrdU incorporation, and TGF β 1 were done in duplicate. Results for fibronectin, DNA, and BrdU incorporation are presented as mean $\pm$ SD of at least three independent experiments. To measure collagen type I and TGF β 1, two independent experiments were performed. Each condition in the different experiments was tested using three or six cultures (three or six wells). Analysis of variance was used to compare different data groups.

Results

Mononuclear Cell Isolation, Culture, and Macrophage Characterization

Mononuclear cells were isolated from buffy coats, washed, counted, resuspended in complete medium (5×10^6 cells/ml), and seeded in culture flasks, glass coverslips, and petriPerm with a density of 6.6×10^4 cells/cm 2 . After 2 hours the nonadherent cells were removed during a medium change. During the next 2 weeks in culture monocytes differentiated to macrophages, showing characteristic variations in morphology ranging from spindle-shaped cells to large spread cells with many lamellipods (Figure 1A). Immunofluorescence staining (Figure 1B) demonstrated that >95% of the monocyte-derived macrophages expressed CD14 after 14 days in culture (Figure 1B). Furthermore, during an incubation period of 6 hours, 50 to 70% of the cells showed phagocytic activity toward fluorescein-labeled *E. coli* (Figure 1B). Viability of cells which had been cultured for 2 weeks was above 95%. Flow cytometric analysis of cells cultured for 10 to 14 days in petriPerm showed about 75% CD14-positive cells (2/3 weakly and 1/3 strongly positivity), 19% CD3-positive cells, and 2% CD19-positive cells.

Figure 1. **A:** Phase contrast image of cultured macrophages. Peripheral blood mononuclear cells were isolated from buffy coats by gradient centrifugation. Cells were seeded on glass coverslips, and after 2 hours the non-adherent cells were removed by washing. Medium was changed each third day. Phase contrast micrography was performed with living cells that had been cultured for 10 days (original magnification, $\times 400$). **B:** Fluorescence micrograph of cultured macrophages. CD14 immunostaining and FITC-*E. coli* phagocytosis of macrophages derived from peripheral blood mononuclear cells during culture for 2 weeks. Before fixation cells were incubated with FITC-labeled *E. coli* for 6 hours (original magnification, $\times 400$).

Culture and Characterization of PSC

PSC that were isolated by density gradient centrifugation from rat pancreas showed the fat-storing phenotype with numerous fat droplets located in the perinuclear region of the cells (Figure 2A). Within 4 to 8 days in primary culture, the number and the size of the fat droplets decreased, and the cells developed long cytoplasmic extensions (Figure 2B) and became weakly positive for α -smooth muscle actin. After passage the fat droplets almost completely disappeared and >90% of the cells expressed α -smooth muscle actin (Figure 2C). The morphology of the passaged cells was stellate-like with long cytoplasmic extensions or spindle-shaped. Human PSC, which were obtained by outgrowth from small tissue blocks of fibrotic pancreas, were mostly stellate-like (a few were also spindle-shaped) without fat droplets in their cytoplasm. Already in primary culture these cells expressed α -smooth muscle actin and vimentin (data not shown).

Figure 2. Cultured pancreatic stellate cells. **A:** Oil red O staining of a primary cultured rat pancreatic stellate cell. PSC were isolated by density gradient centrifugation from the pancreas of untreated male Wistar rats and seeded in a density of 4×10^4 cells/cm² on glass coverslips. Eighteen hours after seeding, cells were stained using oil red O and light microscopy was performed. The cell shows a triangular shape and numerous red-stained fat droplets (original magnification, $\times 400$). **B:** Phase contrast microscopy of primary cultured rat PSC. PSC were isolated and cultured as described in **A**. Forty-eight hours after seeding, phase contrast microscopy was performed. Most cells are spindle-shaped and contain 6 to 20 perinuclear fat droplets (original magnification, $\times 200$). **C:** Fluorescence micrograph of secondary cultured human PSC. PSC were obtained by outgrowth from small tissue blocks of fibrotic pancreas. After reaching confluency, cells were passaged and seeded on glass coverslips. Three days later, cells were fixed with acetone and indirect immunofluorescence staining was performed using anti- α -sm-actin (original magnification, $\times 200$).

Effect of Macrophage-Conditioned Media on Matrix Synthesis of Pancreatic Stellate Cells

To demonstrate paracrine stimulation of pancreatic stellate cell proliferation and matrix synthesis by soluble mediators produced by macrophages, macrophage-conditioned media were added to cultured human and rat PSC. The media had been conditioned for 24 hours by unstimulated and LPS-stimulated macrophages in the absence of FCS. Immunofluorescence staining of cell-associated collagen types I and III and fibronectin, Northern blot analyses of collagen type I and fibronectin, and quantitative immunoassay for collagen type I and c-fibronectin in cell culture supernatants were used to ex-

amine the effects of macrophage supernatants on ECM synthesis of PSC. The results obtained by the different methods were corresponding. In particular, transiently acidified media from LPS-activated macrophages significantly up-regulated ECM synthesis of cultured PSC.

To demonstrate the effects of macrophage supernatants on cell-associated collagens and fibronectin, secondary cultured human PSC grown on glass coverslips were incubated with 40 μ l transiently acidified macrophage supernatant per milliliter of medium for 38 hours. Thereafter, cultures were fixed and immunostaining was performed. As shown in Figure 3, intensive staining patterns of collagen type I (Figure 3C), collagen type III (Figure 3F), and fibronectin (Figure 3I) were observed in cultures receiving supernatants of LPS-activated macrophages. Collagens were detected predominantly intracellularly. Fibronectin was located predominantly extracellularly as fibrils, varying in staining intensity and in density. There was no significant difference in staining patterns between untreated cells (Figure 3, A, D, and G) and PSC incubated with conditioned media of unstimulated macrophages (Figure 3, B, E, and H).

To demonstrate the effects of macrophage supernatants on the steady state levels of collagen type I mRNA and fibronectin mRNA, secondary cultured human PSC were grown in 75-cm² flasks containing 10 ml medium. To subconfluent quiescent cells grown for 12 hours without FCS, 400 μ l of 10-fold concentrated transiently acidified macrophage supernatant was added in the presence of 0.1% FCS. Ten hours later cultures were stopped and mRNA was isolated. Unstimulated human PSC were used as a control. As shown in Figure 4, unstimulated macrophage supernatant and, to a higher extent, supernatant from LPS-activated macrophages increased the steady state levels of collagen type I-mRNA and fibronectin-mRNA. The increase in collagen type I-mRNA is more pronounced than the increase of fibronectin mRNA.

To demonstrate the effects of macrophage supernatants on c-fibronectin synthesis and secretion, c-fibronectin concentration was additionally measured by immunoassay in human and rat PSC supernatants. Collagen type I concentration was measured in human PSC supernatants only. Native and transiently acidified macrophage supernatants (40 and 160 μ l/ml medium) were added to quiescent PSC; FCS had been reduced from 10% to 0.1% 12 hours before. The cultures were stopped 24 hours later. Collagen type I- and c-fibronectin synthesis is expressed on the basis of DNA. Native and, even more pronounced, transiently acidified supernatants of LPS-activated macrophages significantly accelerated fibronectin synthesis compared to unstimulated PSC (Figure 5). Supernatants of unstimulated macrophages increased fibronectin synthesis only in rat PSC (Figure 5B). The fibronectin synthesis-stimulating activity of macrophage supernatants was less pronounced in the presence of 0.5% FCS compared to 0.1% FCS (1.76 ± 0.07 -fold vs. 2.80 ± 0.05 -fold of control).

To measure collagen type I concentration in supernatants of cultured PSC, ascorbic acid (100 μ g/ml) and β -aminopropionitrile (100 μ g/ml) were added during the experiments to prevent collagen cross-linking and to in-

Figure 3. Fluorescence micrographs showing the immunoreactivity of collagen types I and III and fibronectin in cultured human PSC stimulated with macrophage supernatants. Secondary cultured human PSC grown on glass coverslips were stimulated for 38 hours with 40 μ l transiently acidified macrophage supernatant per ml medium (DMEM/Ham's F12 with 0.1% FCS). Thereafter cultures were washed, fixed in acetone and immunostaining was performed. **A-C:** collagen type I; **D-F:** collagen type III; **G-I:** fibronectin. **A, D, G:** control (without macrophage supernatant); **B, E, H:** PSC stimulated with supernatant of macrophages; **C, F, I:** PSC stimulated with supernatant of LPS-activated macrophages. Intensive staining patterns were observed in cultures receiving supernatants of LPS activated macrophages. Collagens were detected predominantly intracellularly. Fibronectin was located predominantly extracellularly as fibrils, varying in staining intensity and in density. There was no significant difference in staining patterns between untreated cells (**A, D, G**) and PSC incubated with conditioned media of unstimulated macrophages (**B, E, H**). Original magnification, $\times 400$.

crease proline hydroxylation. As shown in Figure 6, transiently acidified supernatants of LPS-activated macrophages significantly increased collagen type I synthesis compared to the control cells. No significant stimulation of collagen type I synthesis was observed using native supernatant.

TGF β 1 Concentration in Macrophage Supernatants

To demonstrate that TGF β 1 represents the fibrogenic mediator produced by activated macrophages, TGF β 1 concentration was measured in supernatants of unstimulated and LPS stimulated macrophages by time-resolved fluorescence immunoassay. As expected, LPS activated macrophages produced 3.4-fold more TGF β (6.61 pg/ μ g DNA) compared to unstimulated macrophages (1.97 pg/ μ g DNA) in 24 hours.

Effect of Macrophage Supernatants on PSC Proliferation

To investigate the effect of macrophage supernatants on proliferation of cultured PSC, BrdU incorporation was measured during a labeling period of 18 hours. In preceding experiments optimal BrdU concentration and labeling time had been determined. Incorporated BrdU was quantitated using time-resolved fluorescence immu-

noassay. To determine an inhibitory effect of macrophage supernatants on PSC, proliferation cells were cultured in the presence of 10% FCS and to determine a stimulatory effect PSC were cultured in the presence of 0.1% FCS. Neither native nor acidified supernatants of unstimulated and LPS-stimulated macrophages showed any inhibitory or stimulatory effect on PSC proliferation.

Discussion

This is the first report demonstrating paracrine stimulation of pancreatic stellate cells via soluble mediators produced by mononuclear cells, in particular activated macrophages. The major findings in this study are: supernatants of cultured mononuclear cells stimulate the synthesis and secretion of collagen type I and c-fibronectin in cultured human and rat PSC; transient acidification of these supernatants (whereby TGF β is activated) accelerated the matrix synthesis-stimulating effect; and addition of LPS to cultured mononuclear cells increased TGF β 1 synthesis and resulted in a higher ECM-stimulating activity of the supernatants. The enhanced stimulating activity after LPS treatment clearly points to the pivotal role of the macrophage population for TGF β production.

Chronic pancreatitis is histologically characterized by infiltration of leukocytes, duct alterations, and extended fibrosis.⁴ Leukocytes release cytokines and growth factors, eg, interleukin-1 (IL-1), IL-6, tumor necrosis factor α

Figure 4. Northern blot hybridization of collagen $\alpha 1(I)$, fibronectin, and the 18S ribosomal RNA. Transiently acidified macrophage supernatants were added to secondary cultured human PSC. Ten hours later, cultures were stopped and RNA was isolated. RNA (30 μ g/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. Hybridizations were performed by standard procedures using 0.5×10^6 cpm/ml labeled cDNA. Exposure to X-ray films was done at -70°C for 7 to 10 days. **Lane 1:** RNA isolated from unstimulated PSC (control); **Lane 2:** RNA isolated from PSC stimulated with supernatant of macrophages; **Lane 3:** RNA isolated from PSC stimulated with supernatant of LPS-activated macrophages.

(TNF- α), and TGF β , which are suggested to induce proliferation of mesenchymal cells and accelerated deposition of the ECM.^{20,23,30–32} Emmerich et al¹³ described a three- to fourfold increased number of infiltrating macrophages in chronic pancreatitis and in pancreas carcinoma. The paracrine role of macrophages during wound repair *in vivo* has been demonstrated in studies that show that fibrosis is suppressed if monocyte infiltration is blocked.²⁰ Activated macrophages release a number of cytokines including TGF β ,²⁰ which is suggested to represent one of the most prominent fibrogenic mediators.³³ In liver TGF β stimulates hepatic stellate cell transformation³⁰ and the synthesis of collagens,³¹ proteoglycans,³² and fibronectin.³⁰ In early stages of pancreas fibrosis high numbers of mononuclear cells staining positively for

Figure 5. Effect of macrophage supernatants on fibronectin synthesis of cultured pancreatic stellate cells. Native and acidified macrophage supernatants (40 and 160 μ l/ml medium) were added to secondary cultured PSC (**A**, human PSC; **B**, rat PSC). After 24 hours, cultures were stopped and fibronectin was measured in PSC supernatants; DNA was measured in the cell layer. Values are expressed as means $\pm$ SD of three independent experiments, each condition performed in triplicate culture wells. * $P < 0.05$.

both pro-TGF β and latent TGF β -binding protein (LTBP) were demonstrated.²⁷ TGF β is secreted by almost all cell types in a latent, biologically inactive complex. One particular function of LTBP is to target the latent TGF β complex to the cell surface where activation occurs.³⁴

Our data confirm earlier reports that activated (ie, LPS-treated) macrophages secrete TGF β .²⁰ Macrophages activated by LPS secrete TGF β threefold compared to unstimulated cells. Interestingly, the TGF $\beta 1$ mRNA steady state concentrations investigated by semiquantitative RT-PCR (data not shown) were similar in unstimulated compared to LPS-stimulated macrophages. These data are consistent with earlier reports on the regulation of TGF β gene expression and secretion of TGF β .²⁰

Recently, we have shown on protein and mRNA level that serum stimulated the synthesis of collagen types I and III and fibronectin in cultured hPSC.¹¹ It has been suggested that aggregating platelets release the matrix synthesis-stimulating mediators. However, now we present data demonstrating that macrophage supernatants, in particular supernatants from LPS-activated macrophages, increased the steady-state mRNA levels of fibronectin and collagen type I in cultured hPSC. Further-

Figure 6. Effect of macrophage supernatants on the synthesis of collagen type I in cultured human pancreatic stellate cells. Native and acidified macrophage supernatants (40 and 160 μ l/ml medium) were added to pancreatic stellate cells cultured in DMEM/Hams F12 in the presence of 0.1% FCS, ascorbic acid (100 μ g/ml) and β -aminopropionitrile (100 μ g/ml). After 24 hours cultures were stopped and collagen type I was measured in PSC supernatants; DNA was measured in the cell layer. Values are expressed as means $\pm$ SD of three independent experiments, each condition performed in triplicate culture wells. * P < 0.05.

more, prominent staining patterns of collagen types I and III and fibronectin were found intracellularly and extracellularly in hPSC after stimulation with conditioned medium from LPS-activated macrophages. Transient acidification of the macrophage supernatants (before addition to cultured PSC) increased collagen type I and fibronectin concentration in PSC supernatants compared to native supernatants (see Figures 5 and 6). This observation indicates that TGF β might represent the responsible fibrogenic mediator in macrophage supernatants. In experiments with PSC cultured in the presence of 0.5% FCS (instead of 0.1% FCS) the stimulatory effect of the macrophage supernatants on fibronectin synthesis was still significant but less pronounced (1.7-fold versus threefold). This result is in line with previous data demonstrating that among several polypeptide growth factors present in serum (bFGF, PDGF, insulin-like growth factors, TGF β , TNF α , TGF α) only TGF β 1 was able to stimulate fibronectin synthesis in the presence of higher serum concentrations.¹¹ The importance of TGF β in experimental and human pancreas fibrogenesis is documented by several studies.³³ In rat cerulein pancreatitis TGF β 1 gene expression parallels collagen type I gene expression.¹⁰ Interestingly, TGF β 1 protein increased earlier than TGF β mRNA in pancreas tissue, suggesting that platelets might be the source of the early TGF β 1 protein increase.¹⁰ Thereafter TGF β 1 is released by inflammatory cells, stromal cells, and acinar cells.¹⁰ To inhibit the TGF β effects during regeneration from cerulein pancreatitis in rats, Menke et al³⁵ injected TGF β 1-neutralizing antibodies and could show by this approach that collagen types I and III protein and mRNA decreased significantly compared to control (without anti-TGF β 1). Furthermore, anti-TGF β 1 inhibited the usually observed rise of the steady state levels of TGF β 1 mRNA and TGF β 2 mRNA at the second day after cerulein infusion,³⁵ suggesting that the early TGF β 1 release by platelets induces the TGF β synthesis. In sum-

mary, the data obtained in experimental models convincingly identified TGF β as the most prominent fibrogenic mediator in pancreas.

In addition to the paracrine stimulation of ECM synthesis (eg, via TGF β), macrophages modulate matrix turnover by producing the 92-kd gelatinase matrix metalloproteinase-9 and the interstitial collagenase matrix metalloproteinase-1.^{36–38} Because TGF β reduces matrix metalloproteinase synthesis,³⁹ net matrix accumulation is accelerated by an increased synthesis and a decreased matrix degradation.

In conclusion, our data indicate that activated macrophages might cooperate with pancreatic stellate cells in the development of pancreas fibrosis by secreting fibrogenic mediators (in particular TGF β), thus stimulating ECM synthesis in a paracrine way. Future studies will analyze the role of metalloproteinases in cell-cell interactions of PSC and inflammatory cells.

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