**Objective:** Pancreatic stellate cells (PSC) are considered as the principal effector cells in pancreatic fibrosis. We studied the role of platelet-derived growth factor (PDGF) in the activation of PSC.

**Methods:** Cultured rat PSC were co-incubated with PDGF-BB (25 ng/mL) and different doses (0-40 ng/mL) of PD98059, a specific inhibitor of extracellular signal-regulated kinase (ERK). Expressions of pERK1 protein and of collagen α1(I) mRNA were measured.

**Results:** Expression of pERK1 protein was up-regulated by PDGF-BB, and was down-regulated in a dose-dependent manner by PD98059. Expression of collagen α1(I) mRNA also showed an increase with PDGF-BB and non-dose-dependent inhibition by PD98059.

**Conclusion:** Our findings suggest that PSC activation is mediated by PDGF signal pathway, and ERK1 protein plays an important role in this activation.

*Indian J Gastroenterol 2005;24:100-103*

Recent studies suggest that pancreatic stellate cells (PSC) are closely associated with the development of pancreatic fibrosis. Studies on the mechanisms of activation of PSC may explain the process of pancreatic fibrosis and help find therapeutic methods to inhibit fibrosis. It has been previously shown that extra- and intra-cellular signal pathways play a key role in activation of PSC. In this study, we looked at the activation of PSC by platelet-derived growth factor BB (PDGF-BB), and the effect of PD98059, a specific inhibitor of extracellular signal-regulated kinase (ERK), on this activation, in order to determine the role of PDGF-BB signal pathway in PSC activation.

**Methods**

**Isolation and culture of PSC**

Rat PSC were isolated by a modification of the enzyme-digestion method described previously. In brief, male Sprague-Dawley rats (Tongji Medical College, Huazhong Science and Technology University, Wuhan, China) weighing 180-200 g were killed under sodium phenobarbital (50 mg/Kg intraperitoneally) anesthesia. Pancreatic tissue was excised in sterile condition, freed cautiously from fat, minced using scissors, washed twice with D-Hank’s solution, and digested by 0.2% trypsin (Zhongshan, Beijing, China) at room temperature for 20 min. The digested tissue was filtered through a 150-mm nylon mesh. Cells were washed with D-Hank’s solution and resuspended in 9.5 mL Gey’s balanced salt solution containing 0.3% BSA. The PSC were then purified using Nycodenz (Sigma, St. Louis, USA) density gradient centrifugation and resuspended in MDEM (Gibco, Los Angeles, USA) containing 15% fetal calf serum (Hyclone, South Logan, USA), 10% heated inactivated horse serum (Sanli, Heilongjiang, China), and antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL). These cells were then cultured at 37°C in 5% CO₂ in bottles that were pre-coated with rat-tail collagen (Sigma; 5 µg/mm²). The culture medium was changed every 3 days, and cells were examined at regular intervals using phase-contrast microscopy. Once a confluent growth was achieved, the cells were harvested and re-plated in a medium containing 10% FCS at a density of 1.0×10⁶ cells. After the second passage, FCS content of culture medium was reduced to 5%. Cells within the initial three passages were used for further experiments.

**Immunohistochemistry**

Passaged cells were plated and fixed with 95% alcohol on glass coverslips coated with rat-tail collagen. Immunohistochemical staining for rabbit polyclonal anti-rat glial fibrillary acidic protein (GFAP) antibody (Santa Cruz, California, USA; diluted 1:100) was then performed, as per the manufacturer’s (Linfei, Wuhan, China) instructions.

**Treatment of PSC with PDGF-BB and PD98059**

Cultured PSC harvested in logarithmic growth phase were seeded in 6-well uncoated plastic culture plates (1×10⁶ per well). Cells were serum starved and cultured in Dulbecco minimum essential medium (DMEM). In separate wells, cells were incubated with PDGF-BB (Sigma; 25 ng/mL), without and with PD98059.
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(Promega, Madison, USA) in concentrations of 5, 10, 20 and 30 ng/mL, respectively. Cells were harvested after co-incubation at 37°C for 24 h, and subjected to Western blot for phosphorylated and activated form of ERK1 and RT-PCR for collagen α1(I) mRNA. All tests were repeated five times.

Western blotting for pERK1

Total protein was extracted from the cultured PSC. After measurement of protein concentration using Coomassie blue method and bovine serum albumin standards, 50 µg of protein was run on a 12% preparative sodium dodecylsulfate polyacrylamide gel electrophoresis under reducing conditions and transferred to a nitrocellulose membrane by wet blotting. Membranes were cut into strips, blocked in 5% dry milk in tris-buffered saline for 2 h, incubated overnight with rabbit monoclonal anti-rat pERK1 antibody or polyclonal anti-rat ERK1 antibody (Santa Cruz, CA, USA), and then with HRP-coupled secondary antibodies at 37°C for 40 min, followed by detection using a chemiluminescent substrate.

RT-PCR for collagen α1(I) mRNA

Total cellular RNA was extracted from PSC using TRIzol-reagent kit, and quantified using spectrophotometry. cDNA was prepared using 1 µg of RNA, 1 µL oligo-T (dT15) primer, 1 µL RNasin (Promega, USA), and 2.5 U M-MLV polymerase (Promega, USA) at 45°C for 60 min, followed by enzyme inactivation at 90°C for 5 min. PCR reactions were then set up using primers specific for rat collagen α1(I) [sense: 5’-TACAGCACGCTTGTGGATG-3′, and antisense: 5’-TTGGGATGGAGGGAGTTTA-3′ (expected product size = 190 bp)] and β-actin [5’-GCGTTGACATCCGTAAAGAC-3′ and 5’-TAGGAGCCAGGGCAGTAA-3′ (111 bp)]. The reaction mixtures (25 µL) contained 3 µL of cDNA, 1 µL dNTP (10 mmol/L), 2.5 µL 10×buffer, 1.5 µL MgCl2 (25 mmol/L), 2.5 U Taq DNA polymerase (Promega) and 50 pmol of each primer, and were subjected to initial denaturation at 94°C for 5 min, 30 cycles of amplification (94°C for 30 s [denaturation], 55°C for 45 s [annealing] and 72°C for 45 s [extension]), followed by final extension at 72°C for 7 min. The PCR products (10 µL) were subjected to 2% agarose gel electrophoresis for 45 min, ethylene bromide staining, photography under UV illumination, and image analysis using ULTRA-LUM software (ULTRA-LUM, Claremont, USA ). The amount of collagen α1(I) mRNA was expressed as relative intensity of the band of its PCR product compared with that of β-actin.

Statistical analysis

Results are expressed as mean (standard deviation). Data were analyzed using Kruskall-Wallis H test and analysis of variance; p values below 0.05 were considered significant.

Results

Characterization of isolated rat PSC

On microscopy, cells showed adherence to the walls from 8-12 h, assumed a stellar, angular appearance at 24 h, and became confluent at 5-7 days. These were harvested and passaged after full confluence was achieved at 10-14 days. Freshly isolated cells stained positive for GFAP (Fig. 1).

Expression of pERK1 protein

There was no difference in the expression of total ERK1 protein in different experiments; however, the expression of pERK1 protein showed a change. The pERK1/ERK1 ratio increased from 0 to 0.95 (0.51) after addition of PDGF-BB (Fig. 2). Addition of

Fig 1: Immunohistochemical staining for glial fibrillary acidic protein: cultured pancreatic stellate cells show positive reaction (several brown granules scattered in the cytoplasm) (400X)

Fig 2: Western blotting for measurement of expression of pERK1 and total ERK1 proteins. A: serum starved cells (controls) without PDGF-BB and PD98059; B: cells incubated with PDGF-BB (25 ng/mL); C-F: cells incubated with PDGF-BB (25 ng/mL) and PD98059 (5, 10, 20, 30 ng/mL, respectively)
PD98059 in concentrations of 5, 10, 20 and 30 ng/mL led to a reduction in pERK1/ERK1 ratio to 0.43 (0.38), 0.39 (0.40), 0.21 (0.25) and 0.22 (0.29), respectively. Thus, PDGF-BB treatment increased the expression of pERK1 (H=19.27, p<0.01), and this increase was inhibited by PD98059 in a dose-dependent manner, reaching a peak at 20 mg/L.

Expression of collagen α1(I) mRNA

The intensity of collagen α1(I) band relative to the β-actin mRNA increased after PDGF-BB treatment from 0.15 (0.11) to 0.55 (0.40) (Fig. 3). Addition of PD98059 in various concentrations (5, 10, 20 and 30 ng/L) led to a reduction of the ratio to 0.33 (0.34), 0.27 (0.34), 0.28 (0.29) and 0.22 (0.25), respectively. Thus, PDGF-BB treatment led to an increase in collagen α1(I) mRNA expression, which was significantly reduced with PD98059 treatment (H=16.62, p<0.01), though in a non-dose-dependent manner.

Discussion

PSC have morphologic and biological similarities with HSC. Since hepatic stellate cells (HSC) are major effector cells in hepatic fibrogenesis, it has been proposed that PSC may play a role in pancreatic fibrosis. Cytokines such as PDGF and transforming growth factor can activate and stimulate PSC to synthesize collagens and, therefore, regulate the expression of pERK1 protein and collagen α1(I) mRNA in different groups of PSC, and to further explain the influences of PDGF and ERK on PSC activation. We found that there was no change in the expression of total ERK1 protein, whereas that of pERK1 protein changed. There was almost no expression of pERK1 in primary isolated PSC; PDGF-BB treatment led to an increase in pERK1 expression, which was blocked by PD98059 in a dose-dependent manner. Similar changes were observed with PDGF-BB and PD98059 treatment in the expression of collagen α1(I) mRNA, except that the PD98059-induced inhibition occurred at lower concentrations of this compound and was non-dose-dependent.

The results are perhaps explained as follows: (a) pERK1 protein can up-regulate the expression of collagen α1(I) mRNA powerfully. At initial stage, the expression of collagen α1(I) mRNA is rapidly down-regulated following the reduction of pERK1 protein in PSC treated by PD98059. When the level of pERK1 protein is further reduced, the expression of collagen α1(I) mRNA is out of the regulation by pERK1 protein, and remains stable at lower levels; (b) PSC activation and ECM synthesis result from multi-factor stimulations. It is not only mediated by PDGF signal pathway, but also influenced by other factors such as other cytokines, oxidative stress or injury. Therefore, the mechanism for the regulation of collagen α1(I) gene in activated PSC is quite complicated.

In summary, PDGF can activate PSC and up-regulate the expression of pERK1 protein and collagen α1(I) mRNA; these effects of PDGF on PSC can be partially blocked by PD98059, a specific ERK inhibitor. This suggests that ERK1 plays an important role in the activation of PSC stimulated by PDGF-BB. Therefore, ERK1 may be a potential target to inhibit PSC activation and to prevent development of pancreatic fibrosis.

References


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References


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Image

Primary pneumatosis cystoides intestinalis with pneumoperitoneum and spontaneous resolution

Pneumatosis cystoides intestinalis (PCI) is a condition characterized by multiple gas-filled cysts in the submucosa and subserosa of the bowel wall. Patients affected can present with pneumoperitoneum due to the rupture of cysts.1

A forty-year-old man presented with vague epigastric discomfort and mild fever of 2 weeks’ duration. Abdominal radiographs revealed pneumoperitoneum. CT scan delineated multiple gas-filled cysts on the wall of the small bowel (Fig). As the patient had no features to suggest any systemic illness, a diagnosis of primary pneumatosis intestinalis affecting the small bowel presenting with pneumoperitoneum was made. He was managed conservatively. On follow up, he had no significant symptoms and serial abdominal X-rays showed resolution of pneumoperitoneum after 6 weeks.

Differentiating PCI from intestinal polyposis is sometimes difficult with endoscopy and barium studies. CT scan is the best imaging modality for confirmation of the diagnosis as well as for differentiating primary from secondary forms.2

As PCI can regress spontaneously, asymptomatic patients need no specific treatment.1 Symptomatic patients can be treated with high-flow oxygen and/or antibiotics targeting the replacement of hydrogen in the cysts produced by the bacteria implicated in causation of PCI. Surgical management is reserved for complications like obstruction and bowel infarction.1

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