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Abstract

In studies of the pathogenesis of pancreatic fibrosis, pancreatic stellate cells (PSCs) have recently gained attention. In the present study, we established a new collagenase perfusion method through thoracic aorta cannulation to isolate PSCs, and we studied gene expression of TGF-beta1, type I collagen, and connective tissue growth factor using primary cultured PSCs. Our method facilitated PSC isolation, and by our new method, 4.3 +/- 1.2 x 10(6) PSCs were obtained from a rat. In comparing the expression of these genes with that of hepatic stellate cells (HSCs), we observed a similar pattern, although PSCs expressed type I collagen gene earlier than did HSCs. These results suggest that PSCs may play an important role in fibrosis of the pancreas, as HSCs do in liver fibrosis; in addition, PSCs may exist in a preactivated state or may be more easily activated than are HSCs. We also isolated the PSCs from a WBN/Kob rat, the spontaneous pancreatitis rat, and compared the gene expression with that from a normal rat.

KEYWORDS: pancreatic stellate cell, transforming growth factor beta, connective tissue growth factor, collagenase perfusion, WBN/Kob rat

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Original Article

Establishment of a Novel Collagenase Perfusion Method to Isolate Rat Pancreatic Stellate Cells and Investigation of Their Gene Expression of TGF-β1, Type I Collagen, and CTGF in Primary Culture or Freshly Isolated Cells

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In studies of the pathogenesis of pancreatic fibrosis, pancreatic stellate cells (PSCs) have recently gained attention. In the present study, we established a new collagenase perfusion method through thoracic aorta cannulation to isolate PSCs, and we studied gene expression of TGF-β1, type I collagen, and connective tissue growth factor using primary cultured PSCs. Our method facilitated PSC isolation, and by our new method, $4.3 \pm 1.2 \times 10^6$ PSCs were obtained from a rat. In comparing the expression of these genes with that of hepatic stellate cells (HSCs), we observed a similar pattern, although PSCs expressed type I collagen gene earlier than did HSCs. These results suggest that PSCs may play an important role in fibrosis of the pancreas, as HSCs do in liver fibrosis; in addition, PSCs may exist in a preactivated state or may be more easily activated than are HSCs. We also isolated the PSCs from a WBN/Kob rat, the spontaneous pancreatitis rat, and compared the gene expression with that from a normal rat.

Key words: pancreatic stellate cell, transforming growth factor beta, connective tissue growth factor, collagenase perfusion, WBN/Kob rat

Pancreatic fibrosis is one of the most important pathological features in chronic pancreatitis [1]. The pathogenesis of pancreatic fibrosis, however, has not been sufficiently studied compared with that of hepatic fibrosis.

In the liver, the pathogenesis of fibrosis has been well studied [2, 3]. The most important cells for liver fibrogenesis are hepatic stellate cells (HSCs). After liver injury, HSCs proliferate and synthesize the extracellular matrix (ECM). Many kinds of cytokines, including transforming growth factor (TGF)-β1, have been suggested to be involved in the activation of HSCs. It has been found that TGF-β1 stimulates the synthesis of ECM molecules and inhibits cell proliferation of HSCs *in vitro* [4].

TGF-β1 has been revealed to play an important role in chronic pancreatitis and in pancreatic fibrosis [5, 6]. Furthermore, stellate-shaped cells have been separated and cultured and are now referred to as pancreatic stellate cells (PSCs) [7-12]. Like HSCs, PSCs have droplets containing vitamin A, and long-cultured cells become myofibroblast-like in shape and are positive for α-smooth muscle actin. [8, 9]

Several studies of PSCs have suggested that they

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might play an important role in pancreatic fibrosis, as HSCs do in liver fibrosis [13, 14]. However, studies of primary-cultured PSCs have been rare, probably due to difficulties in isolating a sufficient number of cells. In this study, we developed a novel collagenase perfusion method for the pancreas, performed primary culturing of PSCs, and investigated the gene expression of TGF-β1, type I collagen, and connective tissue growth factor (CTGF).

Materials and Methods

Isolation and culture of pancreatic stellate cells (PSCs). PSCs were isolated as follows. One-year-old male Wistar rats weighing 500–700 g were anesthetized by i.p. injection of sodium pentobarbital (50 mg per kg body weight), the abdomen and chest were opened, and a cannula was inserted into the thoracic aorta. After perfusion with Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} (Invitrogen Corporation, Carlsbad, CA, USA) and containing 0.5 mM Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) at 15 ml/min for 5 min, perfusion with 0.03% collagenase (Sigma, St. Louis, MO, USA) in HBSS with Ca^{2+} and Mg^{2+} at 15 ml/min for 12 min was performed. The distended pancreas was resected and minced with scissors and shaken with 35 ml of 0.03% collagenase solution for 8 to 12 min at 37 °C. Digested tissue was pipetted through narrow orifices and filtered through a 150-μm mesh. A modified method of density gradient centrifugation [14] in the liver was then performed. Cells were washed and then resuspended in 8.0 ml of Gey's Balanced Salt Solution (GBSS) (Invitrogen) containing 0.3% bovine serum albumin (BSA). The cell suspension was mixed with 9.0 ml of 28.7% (wt/vol) of Nycodenz® (Nycomed Pharma AS, Oslo, Norway) in GBSS. The Nycodenz gradient was prepared by layering the cell suspension in Nycodenz underneath 6 ml of GBSS with BSA in a 50-ml centrifuge tube. The gradient was centrifuged for 20 min at $1,400 \times g$. Stellate cells separated into a fuzzy band just above the interface of the Nycodenz solution and the aqueous buffer. This band was harvested, and the cells were washed and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) containing 10% fetal bovine serum (FBS) and Antibiotic-Antimycotic solution (Invitrogen). The viability of isolated cells was assessed by trypan blue exclusion, and cells were counted using a haematocytometer. The cells were seeded in a density of 5×10^4 cells/cm^2 and maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The flowchart of this method is shown in Fig. 1. The culture medium was changed the day after seeding and each second day thereafter. Cells were harvested on days 2, 4, and 7.

To compare the freshly isolated PSCs of the fibrotic pancreas and those of the normal pancreas, we used 20-week-old male WBN/Kob rats, which are spontaneous pancreatitis rats [15] (purchased from Japan SLC, Shizuoka, Japan), and the isolation of PSCs was performed by the same method. PSCs from the WBN/Kob rats and normal rats were cultured for 2 h in IMDM containing 10% FBS until PSCs attached themselves to the plastic dishes; they were then washed twice with HBSS without Ca^{2+} or Mg^{2+} to remove contaminating

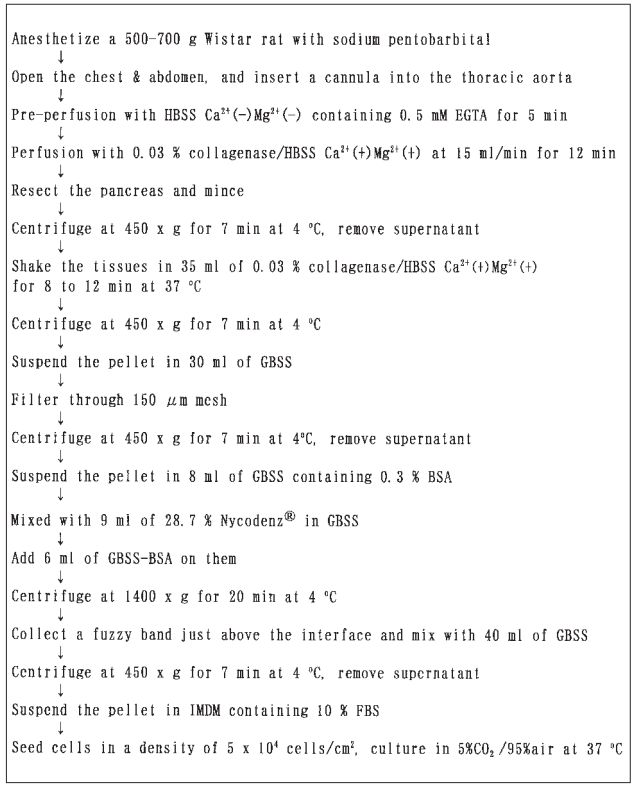

Fig. 1 The flowchart of the PSC isolation method.

Abbreviations

CTGF, connective tissue growth factor; ECM, extracellular matrix; HSC, hepatic stellate cell; PSC, pancreatic stellate cell; TGF-β, transforming growth factor beta.

non-PSCs, and TRIZOL® reagent (Invitrogen) was directly applied to extract RNAs for Northern blot analysis.

All animal experiments were performed in accordance with the Guidelines for Animal Experiments of Okayama University Medical School.

Hepatic stellate cell (HSC) culture. To compare the gene expression of cultured PSCs with HSCs, HSCs were isolated from rats weighing 500–700 g and cultured. Briefly, HSCs were purified by single-step density gradient centrifugation with Nycodenz after collagenase-pronase perfusion [14, 16]. HSCs were seeded at a density of 5×10^4 cells /cm^2 and maintained in IMDM containing 10% FBS and Antibiotic-Antimycotic solution. Cells were harvested on days 2, 4, and 7.

Microscopy and immunocytology. Using phase-contrast microscopy, cultured cells were identified based on their stellate-like morphology.

For the immunocytology, PSCs were cultured for 7 days in Lab-Tek tissue culture chamber/slides (Miles Scientific, Napervill, IL, USA). Glass slides were fixed for 30 min in methanol. Thereafter, glass coverslips were air-dried and stored at 4 ˚C until cells were stained. Briefly, after being incubated in methanol with 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity and rinsed with phosphate-buffered saline (PBS) to block nonspecific reaction with 10% normal goat serum for 20 min, glass slides were incubated with the primary antibodies (mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody, DAKO JAPAN, Kyoto, Japan, or rabbit polyclonal anti-desmin antibody (Chemicon International, Temecula, CA, USA) at working dilutions (1:25 or 1:10, respectively) at 4 ˚C overnight. After rinsing 3 times with PBS, ENVISION/HRP (DAB) Kit (DAKO JAPAN) was used as the second antibodies and for color development.

RNA extraction and Northern blot analysis. Total RNA was extracted from cultured PSC or HSC samples by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction [17] using TRIZOL® reagent according to the manufacturer's instructions. Northern blot analysis of TGF-β1, Type I collagen, CTGF, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed as follows. Total cellular RNA (10 μg each for primary cultured PSCs or HSCs, 3.3 μg each for PSCs from WBN/Kob and normal rats) was resolved by agarose/formaldehyde gel electrophoresis, as described previously [18], and transferred to Hybond-XL membrane (Amersham Biosciences, Buckinghamshire, England) and baked at 80 ˚C for 3 h. Ethidium bromide staining of the agarose gel was used to verify equal loading and blotting of total RNA. Hybridization probes labeled with [α-^{32}P] dATP (Amersham Biosciences) were asymmetrically amplified using the Strip-EZ™ PCR probe synthesis kit (Ambion, Inc., Austin, TX, USA) according to the manufacturer's instruction manual. Template plasmids for TGF-β1, Type I collagen, CTGF, and GAPDH were prepared as described previously [19]. For cultured PSCs and HSCs, α-SMA gene expression was also examined. Template plasmid for α-SMA was prepared as described previously [20]. Prehybridizations were performed in ULTRAhyb™ hybridization buffer (Ambion, Inc.) at 42 ˚C for 3 h. Hybridizations were performed in the same hybridization buffer supplemented with 0.5×10^6 cpm/ml labeled cDNA at 42 ˚C overnight. The membrane was washed in 2 × SSC, 0.1% SDS at room temperature for 15 min twice, then in 0.1 × SSC, 0.1% SDS at 68 ˚C for 45 min twice and exposed to Kodak X-OMAT AR™ films (Eastman Kodak Co., Rochester, NY, USA) at −80 ˚C with an intensifying screen for times ranging from overnight to several days. To re-use blotting membrane, hybridized probes were removed using Strip-EZ™ PCR probe removable kit (Ambion, Inc.) according to the manufacturer's instruction manual.

Results

Isolation of PSCs. Photographs of rat thoracic aorta and pancreas re- and post-collagenase perfusion are shown in Fig. 2.

After thoracic aorta cannulation, the pancreas was successfully perfused and remarkably distended after collagenase perfusion, as shown in Fig. 2 C. After density gradient centrifugation, $4.3 \pm 1.2 \times 10^6$ PSCs were obtained from each rat. Purity and viability were > 85% and > 90%, respectively. Yield, purity, and viability of WBN/Kob rats' PSCs were similar. Yield, purity, and viability of HSCs were 1.7×10^7, 70%, and 76%, respectively.

Phase-contrast microscopy and immunocytology of PSCs. Phase-contrast microscopy of PSCs 4 h after seeding are shown in Fig. 3A and 3B. Stellate-shaped cells with droplets in the cytoplasm are present. PSCs after 2 days of culturing became

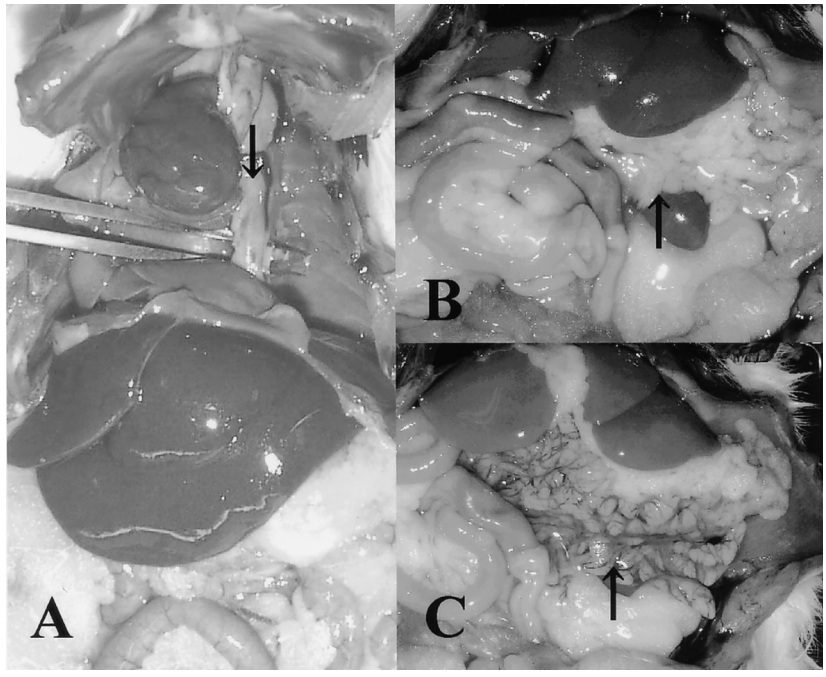

Fig. 2 Photographs of rat thoracic aorta and pancreas pre- and post-collagenase perfusion. (**A**) Photograph of a rat thoracic aorta. The arrow indicates the cannula insertion point. (**B**) Photograph of a rat pancreas before collagenase perfusion. The arrow indicates a normal pancreas. (**C**) Photograph of a rat pancreas after collagenase perfusion. The arrow indicates a distended pancreas.

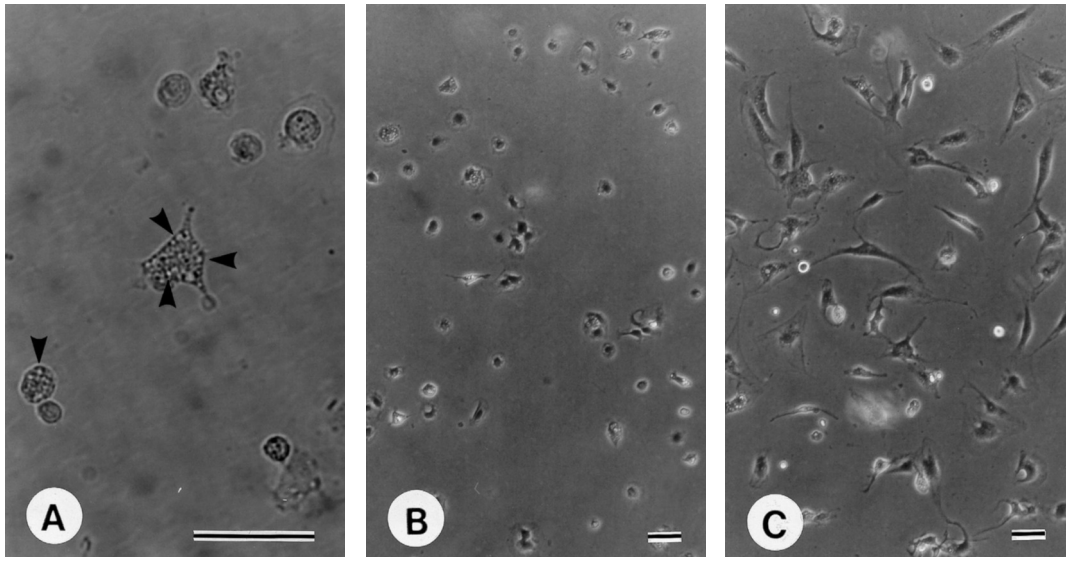

Fig. 3 Phase-contrast microscopy of cultured PSCs. (**A** and **B**) PSCs 4 h after seed. Arrowheads indicate lipid droplets. (**C**) PSCs after 2 days of culturing. Bars indicate 50 μm.

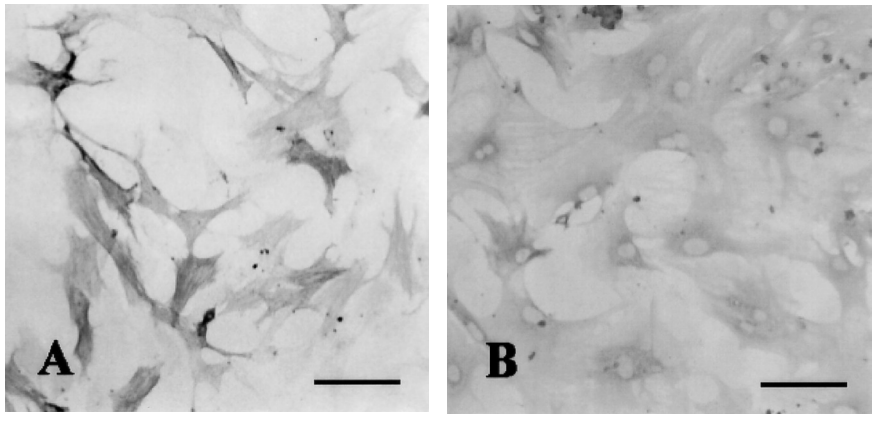

Fig. 4 Immunocytology of PSCs after 7 days of culturing. Cells are positive for α-smooth muscle actin (**A**) and desmin (**B**) in the cytoplasm. Bars indicate 50 μm.

spindle-shaped, as shown in Fig. 3 C. Immunocytology of PSCs after 7 days of culturing is shown in Fig. 4A and 4B. Cells express both α-smooth muscle actin and desmin in the cytoplasm. These results were compatible with those of previous reports [8, 9].

Gene expression of TGF-β1, Type I collagen, α-SMA, and CTGF in primary culture. The results of Northern blot analysis of cultured HSCs and PSCs are shown in Fig. 5. We examined gene expression in primary cultured HSCs and PSCs from days 2, 4, and 7.

TGF-β1 signals of both HSCs and PSCs were continuously expressed from day 2 to day 7.

On days 4 and 7, α-SMA gene of PSCs were expressed, which reflected the activation of cultured PSCs and was compatible with the immunocytology.

In contrast, type I collagen signals were increasingly expressed from day 2 to day 7. Furthermore, type I collagen gene expression of PSCs appeared earlier and was stronger than that of HSCs. On day 2, type I collagen expression of PSCs was already visible.

To determine whether PSCs do express the CTGF gene, we performed Northern blot analysis of cultured PSCs from day 2 to day 7. As shown in Fig. 5, Both HSCs and PSCs showed a gradual increase in expression of the CTGF gene. Like type I collagen, CTGF gene expression of PSCs appeared earlier and was stronger than that of HSCs. On day 2, CTGF expression of PSCs was already visible.

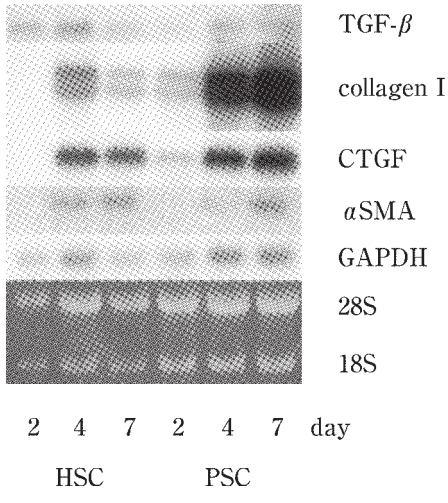

Fig. 5 Expression kinetics of TGF-β1, type I collagen, α-SMA, and CTGF genes in primary cultured HSCs and PSCs. TGF-β1, type I collagen, α-SMA, CTGF, and GAPDH mRNAs were determined by Northern blot analysis. RNAs were obtained at 2, 4, and 7 days of culturing. The results of 2 experiments are shown for PSCs. 18S and 28S show ribosomal RNAs.

Comparison of gene expression of freshly isolated PSCs from fibrotic and normal pancreatic tissue. Total RNA amounts from PSCs of one normal rat or from one WBN/Kob rat were approximately 3.3–3.5 μg. Therefore, 3.3 μg each of total RNA was resolved by agarose/formaldehyde gel electrophoresis.

The results of Northern blot analysis of TGF-β1, Type I collagen, and CTGF of the freshly isolated PSCs of the WBN/Kob rat and normal rat are shown in Fig.

6. TGF-β1 signals from PSCs of both the WBN/Kob and normal rat were expressed equivalently. Regarding CTGF, the gene expression of both normal rat PSCs and WBN/Kob PSCs were weak. In contrast, the type I collagen signal of WBN/Kob's PSCs was more intense than that of normal PSCs.

Discussion

Fibrosis is one of the major pathological aspects of both the liver and pancreas [1–3]. In the liver, HSCs play a major role in fibrosis, and many related studies have been performed. Recently, PSCs have also received attention as an important player in pancreatic fibrosis. Several isolation methods for PSCs have been reported [7–9] (Table 1). As the pancreas is a small organ, it is difficult to separate many PSCs, and most studies have utilized several passaged cells, which are transformed and fully activated cells [7, 10–12]. Of course, the results from these studies have been useful, but if a method for obtaining sufficient number of PSCs were established, many additional experiments could be planned.

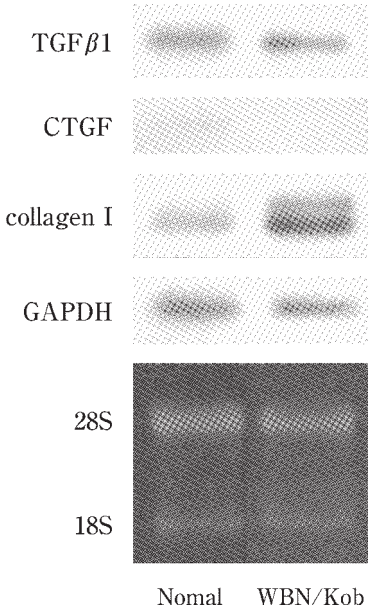

Fig. 6 Comparison of the gene expressions of the freshly isolated PSCs of the normal and fibrotic pancreas. TGF-β1, type I collagen, CTGF, and GAPDH mRNAs were determined by Northern blot analysis. RNAs were obtained from freshly isolated PSCs of a normal rat and those of a WBN/Kob rat. 18S and 28S show ribosomal RNAs.

Bachem *et al.* have described a common bile duct cannulation method that allows one to get many PSCs [8]. As the rat common bile duct is very thin, using this method requires experience and skill. In the present study, we developed a novel collagenase perfusion method through thoracic aorta cannulation. We were able to use a thicker cannula than for common bile duct cannulation, which made it easy to cannulate. Our method also allowed us to obtain sufficient numbers of cells to investigate. There are many researchers studying liver fibrosis using HSCs, and we believe use of our method is as easy as that for HSC isolation, which would allow these researchers to study the field of pancreas fibrosis easily. Therefore, the most important finding of the present work is the establishment of an easy and sufficient method for PSC isolation.

TGF-β1 is a key cytokine of liver fibrosis [2, 3], and CTGF has a TGF-β1 responsive element in its promoter sequence [21], CTGF is thought to be a downstream effector of TGF-β1, and it may coordinate the action of TGF-β1. Mori *et al.* showed that TGF-β caused the induction of skin fibrosis and CTGF was necessary for the maintenance of persistent fibrosis [22]. Furthermore, di Mola *et al.* showed overexpression of CTGF, TGF-β1, and type I collagen in cases of human chronic pancreatitis [23]. Therefore, we examined the

Table I Reported Isolstion methods of pancreatic stellate cells

Authors	Saotome T. *et al.*	Bachem MG. *et al.*	Apte MV. *et al.*	Shinji T. *et al.*
Year	1997	1998	1998	2002
Reference No.	(7)	(8)	(9)	
Animal	human	rat	rat	rat
Yield	unknown	$1.5-8\times10^6$/a rat	2.0×10^5/g pancreas	$4.3\pm1.2\times10^6$/a rat

gene expression of TGF-β1, CTGF, and type I collagen of cultured or freshly isolated PSCs.

In our case, cultured PSCs expressed TGF-β1, type I collagen, and CTGF genes, all of which HSCs express in liver fibrosis [2, 3, 24]. The expression patterns of these genes in primary cultured PSCs were similar to those of primary cultured HSCs. There were, however, some differences between cultured PSCs and HSCs. Separated PSCs attached to culture dishes and showed a stellate shape at 2 h after seeding, which was earlier than for HSCs (data not shown). Apparent gene expression of type I collagen was seen at day 2. These results may indicate that PSCs and HSCs play similar roles in fibrosis. PSCs, however, might exist in a preactivated state or be easily activated compared with HSCs.

We also compared the gene expression of PSCs from normal rat pancreas with that from fibrotic rat pancreas. PSCs from the WBN/Kob rat showed a strong expression of type I collagen. This result was reasonable and supports the usefulness of our new method. CTGF expression of PSCs from the WBN/Kob rat was weak, a result that differed from the culture-activated CTGF expression of PSCs (Figs. 5 and 6). By Nycodenz gradient centrifugation, the fully activated PSCs may not be included, and the obtained PSCs will include different activation stages of PSCs. Therefore, freshly isolated PSCs from fibrotic pancreas and culture-activated PSCs cannot be compared directly. The reason why WBN/Kob male rats experience spontaneous pancreatic fibrosis is still unknown. Our new method may help to solve this problem, as further studies can be carried out using the new PSC isolation method.

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