Differential Roles of Signaling Pathways for Proliferation and Migration of Rat Pancreatic Stellate Cells

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Masamune, A., Kikuta, K., Satoh, M., Kume, K. and Shimosegawa, T. Differential Roles of Signaling Pathways for Proliferation and Migration of Rat Pancreatic Stellate Cells. Tohoku J. Exp. Med., 2003, 199(2), 69–84 —— Activated pancreatic stellate cells (PSCs) have recently been implicated in the pathogenesis of pancreatic fibrosis and inflammation. Accumulation of PSCs is a fundamental feature of pancreatic fibrosis, and platelet-derived growth factor (PDGF)-BB is the most potent mitogen for PSCs. But, the molecular mechanisms responsible for PDGF’s actions in PSCs are largely unknown. In hepatic stellate cells, it has been established that activation of both phosphatidylinositol (PI) 3-kinase and extracellular-signal regulated kinase (ERK) pathways is required for PDGF-BB-induced proliferation and migration. The aim of this study was to elucidate the signaling pathways mediating PDGF-BB’s actions in PSCs. PSCs were isolated from rat pancreas tissue and used in their culture-activated, myofibroblast-like phenotype. Culture-activated PSCs expressed PDGF α- and β-receptors. PDGF-BB induced autophosphorylation of its receptor, followed by the activation of PI 3-kinase, Akt, and ERK pathways. Activation of PI 3-kinase was not required for PDGF-BB-induced ERK activation. PDGF-BB induced approximately fivefold increase in proliferation and chemotaxis of PSCs. Inhibition of ERK pathway with PD98059 completely blocked proliferation, whereas PD98059 had a modest inhibitory effect on cell migration (approximately 50%). On the other hand, inhibition of PI 3-kinase pathway with wortmannin or LY294002 almost completely inhibited migration, but did not affect proliferation of PSCs. In conclusion, our results suggest that ERK pathway regulates...
Chronic pancreatitis as well as pancreatic cancer are accompanied by progressive fibrosis that is characterized by loss of functional tissue and its replacement by extracellular matrix components (Suda et al. 1990; Etemad and Whitcomb 2001; Lankisch 2001). In contrast to liver fibrosis, the molecular mechanisms of pancreatic fibrosis remain to be elucidated. In 1998, star-shaped cells in the pancreas, namely pancreatic stellate cells (PSCs), were identified and characterized (Apte et al. 1998; Bachem et al. 1998). In normal pancreas, stellate cells are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed (“activated”) from their quiescent phenotype into highly proliferative myofibroblast-like cells which express the cytoskeletal protein \(\alpha\)-smooth muscle actin, extracellular matrix components including type I collagen, cell growth factors, and inflammatory cytokines. In culture, PSCs are morphologically very similar to the hepatic stellate cells, and there is accumulating evidence that PSCs play a critical role in pancreas fibrosis (Apte et al. 1998; Bachem et al. 1998; Haber et al. 1999) as hepatic stellate cells do in liver fibrosis (Friedman 2000). In addition, PSCs may participate in the pathogenesis of acute pancreatitis (Masamune et al. 2002c). In view of their roles in pancreatic fibrosis and inflammation, it is of particular importance to elucidate the molecular mechanisms underlying their activation and cellular functions. The activation of signaling pathways such as p38 mitogen-activated protein (MAP) kinase (Masamune et al. 2003) is likely to play a central role for PSC activation as in the case of hepatic stellate cells (Mann and Smart 2002). However, the precise intracellular signaling pathways in PSCs are largely unknown.

Stellate cell proliferation and the expansion of their pool are a fundamental feature of pancreatic fibrosis (Haber et al. 1999). PDGF has been shown to be the most potent mitogen of PSCs, and is likely to be an important mediator of the increased proliferation of the cells both in vivo and in vitro (Apte et al. 1999; Luttenberger et al. 2000). PDGF is a polypeptide growth factor that exists as a disulfide-linked homodimer (PDGF-AA or -BB) or a heterodimer (PDGF-AB) of two chains, A or B (Williams 1989; Claesson-Welsh 1994). Two PDGF receptor subtypes bind the three isoforms of PDGF differentially; PDGF \(\beta\)-receptor can interact only with B-chain containing isoforms whereas PDGF \(\alpha\)-receptor can bind all three isoforms. Binding of the ligands to the receptors leads to dimerization of receptor subunits, phosphorylates itself on tyrosines (known as “autophosphorylation”), changes its cytoplasmic conformation, activates endogenous tyrosine phosphorylating activity, and initiates intracellular signaling (Williams 1989; Claesson-Welsh 1994). For downstream of PDGF receptor, there are at least two major signaling pathways, one involving phosphatidylinositol 3-kinase (PI3-kinase) and the other Ras/Raf/extracellular-signal regulated kinase (ERK) pathways (Williams 1989; Claesson-Welsh 1994). These signaling pathways have been shown to mediate several proliferation and migration in response to PDGF-BB, whereas PI3-kinase mediates cellular migration, but not proliferation of PSCs. ———— MAP kinase; phosphatidylinositol 3-kinase; platelet-derived growth factor; pancreatic stellate cells; pancreatic fibrosis

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cellular functions such as proliferation (Marra et al. 1997), and migration (Kundra et
al. 1994; Wennstrom et al. 1994) in a cell-type specific manner. In hepatic stellate cells, it
has been established that PI3-kinase contributes to the activation of ERK pathway and
that activation of both PI3-kinase and ERK pathways is necessary for PDGF-induced cell
proliferation and migration (Marra et al. 1997; Friedman 2000). However, PDGF-
induced signaling pathways in PSCs are largely unknown. On the other hand, accu-
mulation of PSCs may also result from PSC migration, but no previous studies have ad-
ressed whether PSCs are capable of migration in response to chemical gradients.

In this study, we examined the PDGF-
induced signaling pathways in PSCs. We here show that PDGF-BB activated PI3-
kinase, Akt, and ERK pathways. PSCs were capable of migration as well as proliferation
in response to PDGF-BB. Inhibition of ERK pathway decreased both proliferation and
migration of PSCs whereas inhibition of PI3-kinase blocked migration, but not prolif-
eration of PSCs.

**MATERIALS AND METHODS**

**Materials**

[γ-32P] ATP was from Amersham Biosciences UK, Ltd. (Buckinghamshire, Eng-
land). Collagenase P was from Roche Diagnostics (Mannheim, Germany). Rat recom-
binant PDGF-BB was from R & D Systems (Minneapolis, MN, USA). Rabbit poly-
clonal antibodies against PDGF α- and β-
receptors were from Santa Cruz Biotech-
nology (Santa Cruz, CA, USA). Monoclonal anti-phosphotyrosine antibody (clone 4G10),
and monoclonal antibody against the p85
subunit of PI3-kinase were obtained from
Upstate Biotechnology Inc. (Lake Placid,
NY, USA). Rabbit antibodies used for
Western blotting were from Cell Signaling
Technology Inc. (Beverly, MA, USA).

PD98059, wortmannin, and LY294002 were
from Calbiochem (La Jolla, CA, USA). All
other reagents were from Sigma-Aldrich (St.
Louis, MO, USA) unless specifically de-
scribed.

**Cell culture**

All animal procedures were performed in
accordance with the National Institutes of
Health Animal Care and Use Guidelines. Rat PSCs were prepared from the pancreas
tissues of male Wistar rats (Japan SLC Inc.,
Hamamatsu) weighting 200–300 grams as
previously described (Shinjia et al. 2002) with
modifications. Rats were anesthetized by
intraperitoneal injection of sodium pentobar-
bital, the abdomen was opened, and a can-
nula was inserted into the right jugular vein.
After perfusion with Hanks’ balanced salt
solution (without Ca2+ or Mg2+) supple-
mented with 0.5 mM ethylene glycol-bis (2-
aminoethyl)ether)-N,N,N’,N’-tetraacetic acid
at 2.5 ml/minute for 10 minutes, another per-
fusion was performed with 0.03% collagenase
P in Hanks’ balanced salt solution (with Ca2+
and Mg2+) at 2.5 ml/minute for 12 minutes.
The distended pancreas was resected, minced
with scissors, and shaken in the 0.03% col-
lagenase P solution for 10 minutes at 37°C.
Digested tissue was pipetted through narrow
orifices, filtered through a 150-μm mesh, and
centrifuged. Cells were then resuspended in
8 ml of Gey’s balanced salt solution supple-
mented with 0.3% bovine serum albumin.
The cell suspension was mixed with 9 ml of
28.7% (wt/vol) of the Nycodenz solution
(Nycomed Pharma, Oslo, Norway) in Gey’s
balanced salt solution. The Nycodenz gra-
dient was prepared by laying the cell suspen-
sion in Nycodenz underneath 6 ml of Gey’s
balanced salt solution with bovine serum
albumin in a 50-ml centrifuge tube. The gra-
dient was centrifuged at 1400×g for 20
minutes. 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 Ham's F-12 containing 10% heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), penicillin sodium, and streptomycin sulfate. All experiments were performed using cells between passages two and five. Unless specifically described, we incubated PSCs in serum-free medium for 24 hours before the addition of experimental reagents.

**Immunostaining**

Serum-starved PSCs were grown directly on glass coverslips, and immunostaining for PDGF α- and β-receptors was performed as previously described (Masamune et al. 1999) using a streptavidin-biotin-peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo). Briefly, cells were fixed with 100% methanol for 10 minutes at −20°C, and then endogenous peroxidase activity was blocked by incubation in methanol with 0.3% hydrogen peroxide for 30 minutes. After immersion in normal rabbit serum for 1 hour, the slides were incubated with rabbit polyclonal anti-PDGF-receptor antibodies at a dilution of 1:100 overnight at 4°C. The slides were incubated with biotinylated goat anti-rabbit immunoglobulin antibody for 45 minutes, followed by peroxidase-conjugated streptavidin for 30 minutes. Finally, color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto). As a control, the primary antibody was substituted with non-immune rabbit IgG.

**Immunoprecipitation**

Serum-starved PSCs were treated with the appropriate conditions, quickly placed on ice, and washed once with ice-cold phosphate-buffered saline. The monolayer was lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl at pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of each aprotinin, leupeptin, and pepstatin, 1 mM activated sodium orthovanadate [Na_3VO_4]). The samples were centrifuged at 12 000×g for 5 minutes to remove insoluble cell debris. The protein concentration in the supernatant was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Cell lysates (approximately 250 μg) were incubated with the anti-PDGF β-receptor or anti-phosphotyrosine antibodies overnight at 4°C. The immune complex was absorbed to protein A-agarose beads (Upstate Biotechnology Inc.) for 2 hours at 4°C.

**PI3-kinase assay**

PI3-kinase assay was performed using PI as a substrate after immunoprecipitation with anti-phosphotyrosine antibody as previously described (Ghosh Choudhury et al. 1991; Marra et al. 1996). The product, PI3-phosphate, was resolved by thin layer chromatography in chloroform: methanol: water: 30% ammonium hydroxide (60 : 47 : 11.3 : 2, v/v) as a solvent. After drying, the plates were autoradiographed. Unlabeled PI3-phosphate was run in parallel to determine its position.

**Western blot analysis**

Activation of ERK1/2 was assessed by Western blot analysis using antiphosphospecific ERK1/2 antibody (Thr202/Tyr204) as previously described (Masamune et al. 2002d). Briefly, cells were lysed in sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue) for 15 minutes on ice. The samples were then sonicated for 2 seconds, boiled for 5 minutes, and centrifuged at 12 000×g for 5 minutes to remove insoluble cell debris. Cellular proteins (approximately 100 μg)
were fractionated on a 10% SDS-polyacrylamide gel. They were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and the membrane was incubated overnight at 4°C with rabbit anti-phosphospecific ERK1/2 antibody (at 1 : 1000 dilution). After incubation with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 hour, proteins were visualized using an ECL kit (Amersham Biosciences UK, Ltd.). The levels of total ERK1/2, phosphospecific Akt (Ser473), and total Akt were determined in a similar manner. Negative control blots probed using non-immune IgG in place of the specific antibodies gave no signal.

**Cell proliferation assay**

Serum-starved PSCs (approximately 20–30% density) were treated with PD98059 or PI3-kinase inhibitors for 30 minutes, and then stimulated with PDGF-BB (at 25 ng/ml) for 72 hours in serum-free medium. Cell proliferation was assessed using a commercial kit (CellTiter non-radioactive cell proliferation assay; Promega, Madison, WI, USA) according to the manufacturer’s instruction. Cell viability was determined by differences in absorbance at wavelength 570 vs. 690 nm.

**Cell migration assay**

Serum-starved PSCs were trypsinized, and resuspended in serum-free medium containing 1% albumin at a concentration of 3×10⁶ cells/ml. For the assay, we used modified Boyden chambers with 8-μm-pore filters (Iwaki glass Co., Ltd., Funabashi) coated with rat-tail type I collagen. PDGF-BB (at 25 ng/ml) was added to the lower chamber, and 250 μl of cell suspension was added to the upper chamber. The chambers were then incubated at 37°C for 24 hours. At the end of the incubation, the cell suspension in the upper chamber was aspirated, and the upper part of the filter was cleaned with cotton plugs. The cells migrated to the underside of the filter were stained with Difquick (Sysmex, Kobe), and viewed at 200× magnification. Cell counts were obtained in six randomly chosen fields.

**Statistical analysis**

The results were expressed as mean± standard deviation (mean±s.d.). Lumograms and autoradiograms are representative of at least three experiments. Differences between the groups were evaluated by the two-tailed unpaired Student’s t-test.

**RESULTS**

**Activated PSCs expressed PDGF α- and β-receptors**

We first studied the expression of PDGF α- and β-receptors in PSCs by Western blotting and immunostaining. Culture-activated, serum-starved, PSCs expressed PDGF α- and β-receptors as assessed by Western blotting and immunostaining (Fig. 1). Negative control using non-immune rabbit IgG in place of the specific antibodies gave little staining (data not shown).

**PDGF-BB activated PI3-kinase**

The PDGF receptors dimerize, and become autophosphorylated on tyrosine residues upon binding to their ligands (Claesson-Welsh 1994). Indeed, PDGF-BB induced tyrosine phosphorylation of the PDGF β-receptor in a time-dependent manner (Fig. 2A). In this study, the isof orm PDGF-BB was selected for use because the previous study reported that PDGF-AA had little effect on PSC proliferation (Apte et al. 1999). To assess the direct link between activated PDGF receptor and tyrosine phosphorylation, total cellular lysates were immunoprecipitated with anti-PDGF β-receptor antibody, and immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. PDGF-BB caused phosphorylation of
Fig. 1. Expression of PDGF receptors in culture-activated PSCs. 
(A) Total cell lysates (approximately 100 μg) prepared from culture-activated, serum-starved, 
PSCs were subjected to Western blotting using antibodies against PDGF α- and β-receptors. 
(B, C) Serum-starved PSCs were grown directly on glass coverslips. Immunostaining for 
PDGF α- (panel B) and β-receptors (panel C) was performed using a streptavidin-biotin- 
peroxidase complex detection kit. (Original magnification, ×20 objective.)

p85 subunit of PI3-kinase, coprecipitating 
with the activated receptor (Fig. 2B, upper 
panel). The identity of p85 subunit of PI3- 
kinase was confirmed using anti-p85 subunit 
of PI3-kinase antibody (Fig. 2B, lower panel). 

Tyrosine phosphorylation of p85 leads to 
activation of a catalytic subunit p110 
(Claesson-Welsh 1994). To establish if 
PDGF-BB activates PI3-kinase in PSCs, we 
assessed PI3-kinase activity in anti-
Fig. 2. PDGF-BB activated PI3-kinase.
Serum-starved PSCs were treated with PDGF-BB (at 25 ng/ml) for the indicated time.
(A) Total cell lysates were immunoprecipitated with anti-PDGF β-receptor antibody. The
immunoprecipitates were separated by 7.5% SDS-polyacrylamide gel electrophoresis, and
blotted with anti-phosphotyrosine antibody. Migration of the molecular weight markers is
shown on the left.
(B) Total cell lysates were immunoprecipitated with anti-PDGF β-receptor antibody. The
immunoprecipitates were separated by 7.5% SDS-polyacrylamide gel electrophoresis, and
blotted with anti-phosphotyrosine (upper panel) or anti-p85 subunit of PI3-kinase (lower panel)
antibodies. Migration of the molecular weight markers is shown on the left. IB: immunoblot-
ting.
(C) Total cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. PI3-
kinase activity in the immunoprecipitates was determined using PI as a substrate. The
product, PI3-phosphate, was resolved by thin layer chromatography, and autoradiographed.
The arrow shows the migration of PI3-phosphate.
phosphotyrosine immunoprecipitates from PDGF-BB-stimulated PSCs. Weak activity was present in samples from untreated cells. PDGF-BB induced a significant increase in PI3-kinase activity, with peaking between 5 and 15 minutes (Fig. 2C). Similar results were obtained with anti-PDG F β-receptor immunoprecipitates from PDGF-BB-stimulated PSCs (data not shown). Collectively, these data indicated that PDGF-BB activated PI3-kinase together with phosphorylation of p85, which becomes associated with the activated PDGF receptor in vivo.

**PDGF-BB activated Akt, and ERK1/2**

The downstream effectors of PI3-kinase activation are not fully elucidated, but include protein kinase Cζ, Akt, and p70 S6 kinase (Chan et al. 1999). In particular, Akt has attracted much attention because of its role in cell survival and proliferation (Philpott et al. 1997). We first examined whether PDGF-BB activated Akt by Western blotting using anti-phosphospecific Akt antibody at Ser473 (Chan et al. 1999). Phosphorylated Akt was barely detected in unstimulated cells, but was readily apparent within 5 minutes (Fig. 3A). Akt phosphorylation was rather prolonged, and could be observed even after 24-hour-incubation.

It has been shown that PDGF-BB activated ERK pathway followed by an increased expression of c-fos in a variety of cell-types (Claesson-Welsh 1994). Activation of ERK results from its phosphorylation on serine and threonine residues by the dual specificity kinase MAP kinase kinase (Robinson and Cobb 1997). Addition of PDGF-BB

![Fig. 3. PDGF-BB induced activation of Akt and ERK1/2.](image-url)

Serum-starved PSCs were treated with PDGF-BB (at 25 ng/ml) for the indicated time. Total cell lysates (approximately 100 μg) were prepared, and separated by 10% SDS-polyacrylamide gel electrophoresis. The activation of Akt (panel A) and ERK1/2 (panel B) was determined by Western blotting using anti-phosphospecific antibodies. The levels of total Akt and total ERK1/2 were also determined.
A

PDGF (+)
Cont W LY PD
PI3-phosphate

B

PDGF (+)
Cont W LY PD
P-Akt
Total Akt
P-ERK
Total ERK

Fig. 4. PDGF-BB activated PI3-kinase and ERK pathways independently. Serum-starved PSCs were left untreated (“Cont”) or were treated with PDGF-BB (at 25 ng/ml) in the absence or presence of wortmannin (“W” at 100 nM), LY294002 (“LY” at 10 μM), or PD98059 (“PD” at 100 μM) for 5 minutes in serum-free medium. (A) Total cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine antibody, and PI3-kinase activity was determined using PI as a substrate. The radioactive spots of PI3-phosphate are shown. (B) Total cell lysates (approximately 100 μg) were prepared, and separated by 10% SDS-polyacrylamide gel electrophoresis. The levels of phosphorylated Akt, total Akt, phosphorylated ERK, and total ERK were determined by Western blotting.

to serum-starved PSCs led to phosphorylation of ERK within 5 minutes and returned to near baseline after 2 hours (Fig. 3B).

PDGF-BB activated PI3-kinase and ERK pathways independently

A possible cross-talk between ERK and PI3-kinase pathways has been suggested in hepatic stellate cells (Marra et al. 1997); incubation of hepatic stellate cells with 100 nM wortmannin, a dose which almost completely blocked PI3-kinase, resulted in approximately 50% reduction of ERK activity (Marra et al. 1997). We therefore examined whether PDGF-BB activated PI3-kinase and ERK pathways independently, or a possible cross-talk might exist between these pathways. We employed specific inhibitors of PI3-kinase, wortmannin (Ui et al. 1995) and LY294002 (Vlahos et al. 1994), as well as PD98059, a specific inhibitor of MAP kinase kinase and consequent ERK activation (Dud-
Both of wortmannin at 100 nM and LY294002 at 10 μM blocked PI3-kinase activity and phosphorylation of Akt, indicating that Akt is a downstream target of PI3-kinase in PSCs (Fig. 4). Wortmannin and LY294002 did not affect the ERK phosphorylation (Fig. 4). On the other hand, PD98059 at 100 μM inhibited PDGF-induced ERK phosphorylation, but did not affect PI3-kinase activity and phosphorylation of Akt (Fig. 4). In these experiments, incubation as long as 72 hours with 100 μM PD98059, 100 nM wortmannin or 10 μM LY294002 did not result in any changes in cell morphology and cell viability as assessed by trypan blue exclusion test (data not shown). Taken together, these results excluded a cross-talk between ERK and PI3-kinase/Akt pathway in response to PDGF-BB in PSCs.

**Molecular mechanisms of PDGF-BB-induced cell proliferation**

We then examined the role of these signaling pathways in PDGF-BB-induced proliferation. In agreement with the previous reports (Apte et al. 1999; Luttenberger et al. 2000), PDGF-BB induced approximately fivefold increase of cell proliferation in serum-free medium after 72 hours, and PD98059 inhibited PDGF-BB-induced cell proliferation in a dose-dependent manner (Fig. 5A). The inhibitory effects were significant starting at 25 μM, and at 100 μM PD98059, the stimulation of cell proliferation by PDGF-BB was virtually abolished. In contrast, both wortmannin (at 100 nM) and LY294002 (at 10 μM) did not affect PDGF-BB-induced proliferation of PSCs (Fig. 5B). Thus, activation of ERK pathway but not PI3-kinase plays an important role in PDGF-BB-induced proliferation.

**PDGF-BB induced migration of PSCs**

It has been shown that PDGF induced migration of several types of cells including
hepatic stellate cells (Marra et al. 1997), but it remains unknown whether PSCs are capable of migration. To test whether PDGF could induce PSC migration, cells were subjected to a chemotactic assay in modified Boyden chambers using PDGF-BB as a stimulus. PDGF-BB, at 25 ng/ml, induced approximately five-fold increase in chemotaxis of PSCs (Fig. 6). To examine if this increased migration was simply caused by the stimulation of random motility by PDGF (i.e., chemokinesis), we incubated PSCs with placing PDGF-BB in both chambers. When PDGF-BB was present in both chambers, the increase was only 1.5-fold (data not shown), indicating that chemotaxis accounts for the great majority of PDGF’s effect on PSC migration.

To assess the role of ERK and PI3-kinase pathways in chemotaxis in response to PDGF-BB, PSCs were treated with PDGF-BB in the presence or absence of PD98059 or inhibitors of PI3-kinase. PD98059 at 100 µM had a modest inhibitory effect on cell migration (approximately 50%) (Fig. 6). Exposure of PSCs to wortmannin or LY294002 almost completely inhibited the increase in cell migration, and their effects were more marked than those produced by PD98059. Thus, activation of PI3-kinase and ERK was required for optimal chemotaxis in response to PDGF-BB.

**DISCUSSION**

Following pancreatic injury, PSCs undergo a transformation from quiescent cells to activated myofibroblast-like cells, which actively proliferate, and produce cytokines and extracellular matrix proteins. The intracellular signaling pathways regulating activation and cellular functions of PSCs remain largely unknown. We here reported that PDGF-BB, which is the most potent mitogen of PSCs in vitro, activated ERK1/2, PI3-kinase, and Akt. PDGF-induced proliferation was blocked by the inhibitor of ERK1/2 pathway, but not by those of PI3-kinase pathway. In addition, we have shown that PSCs are capable of migration in response to PDGF-BB. PDGF-induced chemotaxis was blocked by inhibitors of these signaling pathways, indicating differential roles of these pathways in PDGF-induced cellular functions of PSCs (Fig. 7). This is in contrast to hepatic stellate cells where activation of both PI3-kinase and ERK pathways was necessary for PDGF-induced cell proliferation and migration (Marra et al. 1997).
To our knowledge, this is the first report showing cell-type specific variations in signal transduction between PSCs and hepatic stellate cells.

During tissue repair and inflammatory processes in the pancreas, PDGF is secreted by various cells including platelets, mononuclear cells, and activated macrophages (Ross 1989). Exposure of PSCs to PDGF in vivo is likely to occur in conditions of pancreatic inflammation characterized by the presence of platelets and activated macrophages (Bachem et al. 1998; Luttenberger et al. 2000). PDGF-induced effects on PSCs in vivo may be further aided by the upregulation of PDGF receptors on the surface of PSCs. In this regard, it should be noted that in a rat model of pancreatic fibrosis, immunostaining for PDGF β-receptor was found to be notably increased in association with areas of fibrosis, and the expression of the PDGF β-receptor, but not of the PDGF α-receptor, was closely associated with desmin staining, suggesting that PSCs expressed the PDGF β-receptor (Haber et al. 1999). This is in accord with the finding of this study that culture-activated PSCs expressed PDGF β-receptor. Co-distribution of PDGF with cells expressing its receptor confirms a functional role of PDGF in the development of pancreatic fibrosis. In addition, we have shown that PSCs are capable of migration in response to PDGF-BB. It is conceivable that the ability to stimulate chemotaxis plays an important role in the fulfillment of the in vivo function of PDGF in tissue repair as well as in pathological processes (Ross 1989). Accordingly, proliferation and there-
by expansion of cell population together with recruitment of PSCs by migration to the area of pancreatic injury appear to be the most relevant mechanisms of pancreatic fibrosis.

Our results are in agreement with the very recent report by Jaster et al. (2002) showing that ERK is a key mediator of mitogenic signals in rat PSCs. We have previously reported that ethanol and acetaldehyde at clinically relevant concentrations activated ERK, but failed to induce proliferation in PSCs (Masamune et al. 2002b). Thus, activation of ERK is required, but not sufficient for proliferation of PSCs. The downstream targets of ERK critical for chemotaxis are unknown but likely to include myosin light-chain kinase. Klemke et al. (1997) showed that activation of ERK by the extracellular matrix is responsible for phosphorylation of myosin light-chain kinase, which acquires a greater ability to phosphorylate myosin light chains, resulting in increased cell migration. In hepatic stellate cells, it has been shown that preincubation of the cells with drugs increasing intracellular cyclic adenosine monophosphate levels, such as pentoxifylline, leads to a remarkable reduction in the PDGF-induced ERK activation and mitogenesis (Pinzani et al. 1996). These results are in agreement with other studies showing that agents able to elevate intracellular cyclic adenosine monophosphate levels may reduce cell growth via inhibition of Raf kinase, which is an upstream activator of ERK, an action thought to occur through phosphorylation of Raf-1 by cyclic adenosine monophosphate-activated protein kinase (Graves et al. 1993; Wu et al. 1993). Signals leading to chemotaxis in other types of cells ultimately require cell-matrix interaction; binding of integrins to extracellular ligands activates focal adhesion kinase, which then generates a tyrosine phosphorylation cascade within the cell (Otey 1996). Further studies will trace the complete path from focal adhesion kinases and integrins to PI3-kinase in PSCs.

In agreement with the results of this study, PI3-kinase is known to be indispensable for PDGF-induced chemotaxis, which is mediated by PDGF β-receptor, in diverse cell types (Williams 1989; Claesson-Welsh 1994). We here showed that PDGF induced rapid activation of Akt. The downstream effectors of PI3-kinase include protein kinase Cγ and p70 S6 kinase as well as Akt (Chan et al. 1999), but it remains to be elucidated which effectors play a central role in PDGF-induced chemotaxis in PSCs. Genetic studies in Dictyostelium indicated that chemoattractant-mediated Akt activation was required for cell polarity and proper chemotaxis; Akt null cells could not polarize properly when placed in a chemotactic gradient and the cells moved slowly (Meili et al. 1999). On the other hand, the capacity of a PDGF β-receptor mutant to mediate DNA synthesis appears to be dependent on the cell type in which it is expressed (Wennstrom et al. 1994). Along this line, the effects of PI3-kinase activation in proliferation widely varied among cells examined (Bornfeldt et al. 1995; Marra et al. 1997), and activation of PI3-kinase was not required for proliferation of PSCs in response to PDGF-BB. The underlying mechanism of the discrepant observations concerning the functional role of PI3-kinase in PDGF-induced chemotaxis and proliferation remains to be elucidated. One explanation may be due to the existence of several p110 subunits that may be differentially expressed and may have distinct substrate specificities (Hu et al. 1993).

Cross-talk between the MAP kinase and PI3-kinase pathways has been demonstrated in several studies. In hepatic stellate cells, activation of PI3-kinase pathway is partially involved in ERK activation by PDGF; incubation of hepatic stellate cells with wortmannin resulted in approximately 50% reduction
of phosphorylation and activation of ERK (Marra et al. 1997). However, wortmannin and LY294002 herein did not affect the PDGF-induced ERK activation in PSCs, excluding similar cross-talk between PI3-kinase/Akt and ERK pathways in our experimental system. Again, our results showed another cell-type specific variation in signal transduction between PSCs and hepatic stellate cells. Because activation of Akt plays important role in cell survival through the transduction of anti-apoptotic signals (Philpott et al. 1997), it would be interesting to examine whether activation of PI3-kinase/Akt pathway plays a role in apoptosis of PSCs. Elucidation of the molecular mechanisms of PSC apoptosis as well as regulation of PDGF-induced PSC proliferation and migration would be of particular importance for the development of new strategies against pancreatic fibrosis and inflammation. We previously reported that troglitazone, a ligand of the peroxisome proliferator-activated receptor-γ, modulated profibrogenic and proinflammatory actions in PSCs in vitro (Masamune et al. 2002a). Very recently, Shimizu et al. (2002) have reported that troglitazone prevented the progression of pancreatic inflammatory process and fibrosis in an animal model of chronic pancreatitis, suggesting that PSCs are potential a target of anti-fibrogenic and anti-inflammatory strategies in vivo as well. Experiments along this line are under way in our laboratory.

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References


