Protease-Activated Receptor-2-Mediated Proliferation and Collagen Production of Rat Pancreatic Stellate Cells

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ABSTRACT
Activated pancreatic stellate cells (PSCs) play a pivotal role in the pathogenesis of pancreatic inflammation and fibrosis. Trypsin and tryptase, which are agonists for protease-activated receptor-2 (PAR-2), are involved in the pathogenesis of pancreatitis. Here, we examined whether PSCs expressed PAR-2 and its agonists affect the cell functions of PSCs. PSCs were isolated from rat pancreas tissue. Expression of PAR-2 was examined by Western blotting and reverse transcription-polymerase chain reaction. Trypsin, activating peptide (SLIGRL-NH₂, corresponding to the PAR-2 tethered ligand), and tryptase were tested for their ability to affect proliferation, chemokine production, and collagen synthesis in culture-activated PSCs. Activation of mitogen-activated protein (MAP) kinases was assessed by Western blotting using antiphosphospecific antibodies. The effect of PAR-2 agonists on the activation of freshly isolated PSCs in culture was also examined. PAR-2 expression was observed in culture-activated PSCs, whereas it was undetectable in freshly isolated PSCs. PAR-2 agonists activated activator protein-1 and MAP kinases (extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAP kinase) but not nuclear factor κB. PAR-2 agonists induced proliferation of PSCs through the activation of extracellular signal-regulated kinase. PAR-2 agonists increased collagen synthesis through the activation of c-Jun N-terminal kinase and p38 MAP kinase. PAR-2 agonists did not induce the production of monocyte chemoattractant protein-1 and cytokine-induced neutrophil chemoattractant-1 or initiate the transformation of freshly isolated PSCs in culture. Taken together, our results suggest a role of PAR-2 in the sustenance of pancreatic fibrosis through the increased proliferation and collagen production in PSCs.

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 that express the cytoskeletal protein α-smooth muscle actin (α-SMA) and produce type I collagen and other extracellular matrix components. There is accumulating evidence that activated PSCs play a pivotal role in the development of pancreatic fibrosis. (Apte et al., 1998; Bachem et al., 1998; Haber et al., 1999; Masamune et al., 2002a). It has also been suggested that PSCs may participate in the pathogenesis of acute pancreatitis (Haber et al., 1999; Masamune et al., 2002c). The molecular mechanisms responsible for PSC activation remain to be elucidated, but the activation of signaling pathways such as p38 mitogen-activated protein (MAP) kinase (Masamune et al., 2003c), Rho-Rho kinase pathway (Masamune et al., 2003b), and c-Jun N-terminal kinase (JNK) (Masamune et al., 2004) is likely to play a role. However, intracellular signaling pathways in PSCs remain largely unknown.

Protease-activated receptor-2 (PAR-2) is a G protein-coupled receptor for trypsin and mast cell tryptase (Nystedt et al., 1994). Trypsin and tryptase cleave within the extracellular N terminus of PAR-2 at \( ^{35}SKGR \downarrow SLIGRL^{42} \), yielding a

ABBREVIATIONS: PSC, pancreatic stellate cell; α-SMA, α-smooth muscle actin; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; PAR, protease-activated receptor; IL, interleukin; TGF, transforming growth factor; NF-κB, nuclear factor κB; AP-1, activator protein-1; IκB, inhibitor of nuclear factor κB; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyl]butadiene; SP600125, anthera [1,9-cd]-pyrazole-6 (2H)-one; SP600125, anthera [1,9-cd]-pyrazole-6 (2H)-one; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; BrdU, 5-bromo-2′-deoxyuridine; MCP-1, monocyte chemoattractant protein-1; CINC-1, cytokine-induced neutrophil chemoattractant-1; GFAP, glial fibrillary acidic protein; ERK, extracellular signal-regulated kinase.
tethered ligand (SLIGRL for rat PAR-2) that binds to the second extracellular loop, followed by the activation of the receptor (Nystedt et al., 1994). Synthetic peptides corresponding to this tethered ligand domain selectively activate PAR-2 without proteolysis. Previous studies have shown that PAR-2 is widely expressed in human tissues with especially high levels in the pancreas, liver, kidney, small intestine, and colon (Nystedt et al., 1994; Bohm et al., 1996). High expression of PAR-2 in the pancreas is particularly interesting because trypsin is prematurely autoactivated within the inflamed pancreas and is believed to contribute to the development of pancreatitis (Hofbauer et al., 1998). Therefore, it is likely that trypsin cleaves and activates PAR-2 during pancreatitis. Trypsin may also trigger PAR-2 in the pancreas during inflammation, when mast cells are present (Zimnoch et al., 2002). Recent studies have suggested the role of PAR-2 in the pathogenesis of several inflammatory diseases such as intestinal inflammation (Cenac et al., 2002) and arthritis (Ferrrell et al., 2003). However, PAR-2 expression and possible regulation of cell functions in PSCs are unknown. Here, we show that activated PSCs expressed PAR-2 and that PAR-2 agonists induced proliferation and collagen synthesis in PSCs.

Materials and Methods

Activating and control reverse PAR-2 peptides were synthesized with amidated C termini (purity >95%); Thermo Hybaid, Ulm, Germany). The sequences of the active and control peptides of PAR-2 were SLIGRL-NH₂ and LGRLS-NH₂, respectively (Nystedt et al., 1994). Bovine trypsin was from Wako Pure Chemicals (Osaka, Japan). Collagenase P and recombinant human interleukin (IL)-18 were from Roche Diagnostics (Mannheim, Germany). [3H]-proline and [γ-32P]ATP were from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Rat recombinant platelet-derived growth factor-BB and human transforming growth factor (TGF)-β1 were from R&D Systems (Minneapolis, MN). Double-stranded oligonucleotide probes for nuclear factor κB (NF-κB) and activator protein-1 (AP-1) were from PromoGen (Madison, WI). Mouse monoclonal anti-PAR-2 antibody (SAM11; sc-13504) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antibodies against MAP kinases (phosphorylated and total) and inhibitor of NF-κB (IκB-α) and anti-β-tubulin protein ladder detection peptide were from Cell Signaling Technology Inc. (Beverly, MA). U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), SP600125 [anthracene-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] were from Calbiochem (San Diego, CA). Rabbit antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Trevigen (Bethesda, MD).

Reverse Transcription.

Total RNA was prepared from PSCs using the RNAeasy total RNA preparation kit (QIAGEN, Valencia, CA). Total RNA (200 ng) was reverse transcribed, and the resultant cDNA was subjected to PCR in a volume of 30 μl. Specific primer sets were as follows (listed 5′ to 3′; forward and reverse, respectively): PAR-2, GCGTGGCTGCTGGGAGGTATC and GGAA-CAGAAAGACTCCAATG; and GAPDH, ACATCATCCCTGCATC-3′. Double-stranded oligonucleotide primers were synthesized by SigmaGenosys, Ishikari, Japan) at room temperature for 2 h in a 5-fold (by weight) excess of the antigen to the antibody. The levels of α-SMA and GAPDH were determined in a similar manner. Densitometric analysis was carried out using the NIH Image 1.63 program (National Institute of Health, Bethesda, MD).

Reverse Transcription-PCR.

Reverse transcription-PCR was performed as previously described (Masamune et al., 1996). Double-stranded oligonucleotide probes for NF-κB (5′-AGTTGAGGACCTTTCCAGGaGC-3′) and AP-1 (5′-CGCTGATAGTCAGGCGGA-3′) were end labeled with [γ-32P]ATP. Nuclear extracts (approximately 5 μg) were incubated with the labeled oligonucleotide probe for 20 min at 37°C and electrophoresed through a 4% polyacrylamide gel. The gel was dried and autoradiographed at −80°C overnight. A 100-fold excess of unlabeled oligonucleotide was incubated with nuclear extracts for 10 min prior to the addition of the radiolabeled probe in the competition experiments.

Assessment of MAP Kinase Activation.

Activation of MAP kinases was examined by Western blot analysis using anti-phosphospecific MAP kinase antibodies as previously described (Masamune et al., 1992d). These antibodies recognize only phosphorylated forms of MAP kinases, thus allowing the assessment of activation of the kinases. The levels of total MAP kinases and IκB-α were also determined by Western blotting.

Cell Proliferation Assay.

Cell proliferation was assessed using two parameters: rate of DNA synthesis as measured by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) and cell counting.

Cells were passaged using a 0.5 mM EDTA in phosphate-buffered saline. All experiments were performed using culture-activated cells between passages two and five except for those using freshly isolated PSCs. Unless specifically described, we incubated PSCs in serum-free medium for 24 h before the addition of experimental reagents. For some experiments, trypsin was boiled in a water bath for 10 min.

Expression of PAR-2 in PSCs.

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DNA Synthesis. DNA synthesis was assessed using a commercial kit (cell proliferation enzyme-linked immunosorbent assay, BrdU; Roche Diagnostics) according to the manufacturer’s instruction. This is a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. After 24-h incubation, cells were labeled with BrdU for 3 h at 37°C. Cells were fixed and incubated with peroxidase-conjugated anti-BrdU antibody. Then, the peroxidase substrate 3,3',5,5'-tetramethylbenzidine was added, and BrdU incorporation was quantitated by differences in absorbance at wavelength 370 to 492 nm.

Cell Counting. After 72-h incubation, cells were detached by gentle trypsinization and counted using a hemocytometer.

Chemokine Measurement. After 24-h incubation, cell culture supernatants were harvested and stored at −80°C until the measurement. The levels of monocyte chemotactic protein-1 (MCP-1) and cytokine-induced neutrophil chemotactic protein-1 (CINC-1) in the culture supernatants were measured using commercial kits (Pierce Chemical, Rockford, IL; Panapharm Laboratories, Udo, Japan) according to the manufacturers’ instructions.

Collagen Assay. New collagen synthesis in PSCs was determined by measuring the incorporation of [3H]-proline into collagenase-sensitive proteins as previously described (Agelli and Wahl, 1988). PSCs were treated with or without PAR-2 agonists for 20 h in serum-free medium. Then, PSCs were labeled for 24 h with 2 μCi/ml [3H]-proline in Ham’s F-12 medium containing the PAR-2 agonist plus 100 μg/ml ascorbic acid and 100 μg/ml β-aminopropionitrile. At the end of the incubation, cells were harvested, pooled with the culture supernatant, and sonicated. Proteins were precipitated with 10% trichloroacetic acid, and the pellet was solubilized with 0.2 N NaOH. After neutralization, samples were divided into two equal aliquots. One aliquot was incubated with 25 μg/ml collagenase for 2 h at 37°C, and the other was treated with vehicle. Collagenase-resistant proteins were separated from the released collagen peptides by trichloroacetic acid precipitation. Radioactivity in the trichloroacetic acid-soluble fraction was measured in a liquid scintillation counter, and new collagen synthesis was determined by the difference between the radioactivities found in the collagenase- and vehicle-treated aliquots and normalized for total cellular DNA.

Effect of PAR-2 Agonists on the Transformation of Freshly Isolated PSCs in Culture. Freshly isolated PSCs were treated with or without trypsin (at 10 nM), activating peptide (at 25 μM), or TGF-β1 (at 10 ng/ml) in serum-free medium. After 7-day incubation, morphological changes characteristic of PSC transformation were assessed after staining with glial fibrillary acidic protein (GFAP) as previously described (Masumune et al., 2003d) using a streptavidin-biotin-peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo, Japan). Briefly, cells were fixed with ice-cold methanol, and then endogenous peroxidase activity was blocked by incubation in methanol with hydrogen peroxide for 5 min. After immersion in normal rabbit serum, the slides were incubated with mouse anti-GFAP antibody overnight at 4°C. The slides were incubated with biotinylated goat anti-mouse Ig antibody, followed by peroxidase-conjugated streptavidin. Finally, color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto, Japan). As a control, the primary antibody was replaced with phosphate-buffered saline. In addition, total cellular proteins (approximately 25 μg) were prepared after 7-day incubation, and the levels of α-SMA and GAPDH were determined by Western blotting.

Statistical Analysis. The results were expressed as mean ± S.D. Experiments were performed at least three times, and similar results were obtained. Representative luminograms and autoradiograms are shown. Differences between the groups were evaluated by ANOVA, followed by Fisher’s test for post hoc analysis. A p value less than 0.05 was considered statistically significant.

Results

PAR-2 Was Expressed in Culture-Activated, but Not Freshly Isolated, PSCs. Freshly isolated PSCs were induced to transform to a myofibroblast-like phenotype by culture on plastic in serum-containing medium. Progressive activation of PSCs following plating on plastic was demonstrated by their expression of α-SMA (Fig. 1A). Western blotting showed that PAR-2 was undetectable in PSCs on day 1 but was highly expressed in culture-activated PSCs on day 14 (Fig. 1, B and C). PAR-2 was detected as a single band of ~55 kDa, and this band could be eliminated from the blot by prior incubation of the antibody with the relevant PAR-2 peptide used to generate the antibody. In agreement with the result of Western blotting, mRNA of PAR-2 was undetectable in PSCs on day 1 but was highly expressed in PSCs on day 14 (Fig. 1D).

PAR-2 Agonists Induced Proliferation of PSCs. Trypsin increased DNA synthesis in a dose-dependent manner, with peaking around 10 nM (Fig. 2A). In this experiment, trypsin up to 25 nM did not affect the cell viability during the incubation as assessed by trypan blue exclusion test (data not shown). However, when PSCs were treated with trypsin above 25 nM, cytotoxic effects were observed. Other PAR-2 agonists, tryptase and PAR-2 activating peptide, also induced DNA synthesis, whereas control peptide was ineffective (Fig. 2B). Similar to the results obtained with DNA synthesis assay, PAR-2 agonists increased the cell numbers (Fig. 2C). Trypsin-induced mitogenic effect was abrogated if trypsin was heat-inactivated or in the presence of nafamostat.
mesilate (at 10 μM), a serine protease inhibitor (Aoyama et al., 1984) (data not shown).

PAR-2 Agonist Increased Collagen Synthesis. PAR-2 agonists significantly increased the collagen synthesis (Fig. 3A). Trypsin-induced effect on the collagen synthesis was abrogated if trypsin was heat-inactivated or in the presence of nafamostat mesilate. On the other hand, IL-1β induced MCP-1 and CINC-1 production, but PAR-2 agonists did not (Fig. 3B).

PAR-2 Agonists Activated AP-1 but Not NF-κB. Both trypsin and activating peptide, as well as IL-1β, increased the AP-1 binding activity (Fig. 4A). The specificity of AP-1-specific DNA binding activity was demonstrated by the addition of a 100-fold molar excess of unlabeled AP-1 oligonucleotide but not by unrelated NF-κB oligonucleotide in competition assays (data not shown). In contrast, NF-κB binding activity was induced by IL-1β, but not by trypsin or activating peptide (Fig. 4B). Phosphorylation and degradation of the inhibitory protein IκB-α and subsequent dissociation of this protein from NF-κB are thought to be necessary for the activation (Grilli et al., 1993). We also examined the effect of trypsin and activating peptide on the degradation of IκB-α by Western blotting. IL-1β, but not PAR-2 agonists, induced degradation of IκB-α, further supporting that PAR-2 agonists did not activate NF-κB (Fig. 4C).

PAR-2 Agonists Activated MAP Kinases. Trypsin and activating peptide activated extracellular signal-regulated kinase (ERK), JNK, and p38 MAP kinase in a time-dependent manner around 5 to 15 min (Fig. 5, A and B). Tryptase also activated these MAP kinases, whereas boiled trypsin, control peptide, and trypsin in the presence of nafamostat mesilate all failed to activate MAP kinases (Fig. 5C).

Differential Roles of MAP Kinases for Proliferation and Collagen Synthesis. To clarify the role of activated MAP kinases for PAR-2-mediated proliferation, we employed specific inhibitors of MAP kinases. We have previously shown that SP600125 specifically inhibited JNK (Masamune et al., 2004) and that SB203580 specifically inhibited p38 MAP kinase (Masamune et al., 2003c) in PSCs. Activation of ERK by trypsin and activating peptide was abolished in the presence of U0126 (data not shown), which specifically inhibits MAP kinase kinase and consequent ERK activation. U0126 inhibited PSC proliferation in response to PAR-2 agonists (Fig. 6A). SP600125 and SB203580 did not affect proliferation.

Fig. 2. PAR-2 agonists induced proliferation of PSCs. Serum-starved, culture-activated PSCs were treated with trypsin (at the indicated concentrations or at 10 nM, Try in panel C), PAR-2 activating peptide (at 25 μM, AP), reverse peptide (at 25 μM, Rev), trypase (at 1 μU/ml, Trypt), or platelet-derived growth factor (at 25 ng/ml, PD) in serum-free medium. A and B, after 24-h incubation, DNA synthesis was assessed by BrdU incorporation enzyme-linked immunosorbent assay. C, after 72-h incubation, cells were detached and counted. Data are shown as mean ± S.D. (percentage of the control, n = 6). **, p < 0.01 versus trypsin at 0 nM (A) or control (Con in B and C).

Fig. 3. PAR-2 agonists induced collagen synthesis. A, culture-activated PSCs were left untreated (control, column 1) or treated with trypsin (at 10 nM, column 2), trypsin in the presence of nafamostat mesilate (at 10 μM, column 3), boiled trypsin (at 10 nM, column 4), activating peptide (at 25 μM, column 5), reverse peptide (at 25 μM, column 6), or tryptase (at 1 mU/ml, column 7). After 48-h incubation, new collagen synthesis was determined by measuring the incorporation of [3H]-proline into collagenase-sensitive proteins. Data are shown as mean ± S.D. (percentage of the control, n = 6). **, p < 0.01 versus control. B, culture-activated PSCs were treated with IL-1β (at 2 ng/ml), trypsin (at 10 nM, Try), activating peptide (at 25 μM, AP), or tryptase (at 1 μU/ml, Trypt) in serum-free medium. After 24-h incubation, culture supernatants were harvested, and the levels of MCP-1 and CINC-1 were determined. Data shown as means ± S.D. (n = 6). **, p < 0.01 versus control (Con).
Discussion

There is accumulating evidence that activated PSCs play a key role in the pathogenesis of pancreatic fibrosis and inflammation (Apte et al., 1998; Bachem et al., 1998; Haber et al., 1999; Masamune et al., 2002a,c). Here, we showed for the first time that PAR-2 agonists induced proliferation and collagen synthesis of culture-activated PSCs, the two major hallmarks in the development of pancreatic fibrosis. All of the PAR-2 agonists examined showed similar effects. Control peptide, heat-inactivated trypsin, and trypsin in the presence of the serine protease inhibitor nafamostat mesilate were ineffective, suggesting that the effects of trypsin were mediated through its proteolytic action on PAR-2. MAP kinases play differential roles in the PAR-2-mediated proliferative and fibrotic responses; PAR-2 agonists induced PSC proliferation through the activation of ERK, whereas collagen synthesis was mediated by JNK and p38 MAP kinase. PAR-2 was expressed in culture-activated PSCs, whereas it was almost undetectable in freshly isolated PSCs. Therefore, prior activation of PSCs might be necessary for PAR-2 agonists to elicit effects on PSCs. Indeed, PAR-2 agonists failed to induce the transformation of quiescent PSCs to the activated, myofibroblast-like phenotype. The finding that activated PSCs expressed PAR-2 is in agreement with the recent report showing that strong PAR-2 expression was observed in the tissues of chronic pancreatitis accompanied by severe fibrosis (Ikeda et al., 2003). Previous studies have suggested that cytokines and growth factors such as TGF-β1, tumor necrosis factor-α, and oxidative stress induced the transformation of quiescent PSCs (Schneider et al., 2000). During the course of pancreatitis, these mediators are released or produced from inflammatory cells, platelets, and pancreatic acinar cells (Gukovskaya et al., 1997; Apte et al., 1999; Schneider et al., 2000), leading to the activation of PAR-2 expression in PSCs. Once activated, PSCs are sensitive to PAR-2 agonists and show proliferative and fibrogenic responses.

Previous studies have suggested that the effects of PAR-2 activation on cell growth varied, depending on the cell type. PAR-2 agonists inhibited colony formation of A549 lung adenocarcinoma cells (Bohm et al., 1996), whereas PAR-2 agonists increased proliferation of hepatic stellate cells (Gaca et al., 2002). The molecular mechanism responsible for PAR-2-mediated proliferation remains to be fully elucidated, but the role of ERK has been suggested in several cells (Belham et al., 2000; Haber et al., 2001). Along this line, we here showed that PAR-2 agonists induced the activation of ERK and U0126 abolished PAR-2-mediated proliferation of PSCs, suggesting a key role of this pathway. It should be noted that

SP600125 and SB203580 inhibited collagen synthesis in response to PAR-2 agonists (Fig. 6B). In contrast, U0126 did not affect the collagen production. Thus, PAR-2 agonists increased the collagen synthesis through the activation of JNK and p38 MAP kinase but not ERK.

PAR-2 Agonists Did Not Initiate the Activation of Freshly Isolated PSCs. After 7 days, PSCs treated with TGF-β1 showed transformation into cells with a myofibroblast-like phenotype (Fig. 7A). In contrast, PSCs treated with trypsin or activating peptide were small and circular, with lipid droplets present in many cells and with slender dendritic processes (Fig. 7, B and C), as in the case of PSCs treated with serum-free medium only (Fig. 7D). In agreement with the morphological changes, significant expression of α-SMA was observed on day 7 in TGF-β1-treated PSCs, whereas trypsin and activating peptide did not increase the expression (Fig. 7E).

**Fig. 4.** PAR-2 agonists activated AP-1 but not NF-κB. A and B, culture-activated PSCs were treated with IL-1β (2 ng/ml, lane 2), trypsin (at 10 nM, lane 3), activating peptide (at 25 μM, lane 4), or reverse peptide (at 25 μM, lane 5) for 1 h. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay using AP-1 (A) or NF-κB (B) oligonucleotide probes. Lane 1, control (serum-free medium only). C, culture-activated PSCs were left untreated (lane 1) or treated with IL-1β (2 ng/ml, lane 2), trypsin (at 10 nM, lane 3), activating peptide (at 25 μM, lane 4), or reverse peptide (at 25 μM, lane 5) in serum-free medium for 15 min. Total cell lysates were prepared, and the levels of IκB-α and GAPDH were determined by Western blotting.

**Fig. 5.** PAR-2 agonists activated MAP kinases. A and B, culture-activated PSCs were treated with trypsin (A, at 10 nM) or activating peptide (B, at 25 μM) in serum-free medium for the indicated time. C, PSCs were left untreated (lane 1) or treated with trypsin (at 10 nM, lane 2), trypsin in the presence of nafamostat mesilate (at 10 μM, lane 3), boiled trypsin (at 10 nM, lane 4), activating peptide (at 25 μM, lane 5), reverse peptide (at 25 μM, lane 6), or trypase (at 1 μU/ml, lane 7) for 5 min. Total cell lysates (approximately 100 μg) were prepared, and the levels of phosphorylated and total ERK1/2, JNK, and p38 MAP kinase were determined by Western blotting.
trypsin and tryptase might show their effects on cell proliferation and MAP kinase activation by not only PAR-2-dependent but also PAR-2-independent mechanisms (Belham et al., 1996; Akers et al., 2000; Brown et al., 2002). In vascular smooth muscle cells, trypsin stimulated the activation of MAP kinase cascade by PAR-2-dependent mechanism, whereas activation of MAP kinase by trypsin in vascular fibroblasts was independent of PAR-2 (Belham et al., 1996). Although tryptase stimulated proliferation of human adult dermal fibroblasts, the PAR-2-activating peptides had no effect, suggesting that tryptase was acting via PAR-2-independent mechanisms (Akers et al., 2000). Tryptase-induced mitogenic responses in human airway smooth muscle cells were shown to be largely via nonproteolytic mechanisms (Brown et al., 2002). On the other hand, Frungieri et al. (2002) showed that the proliferative effects of tryptase and PAR-2-activating peptide in human fibroblasts were inhibited by a cyclooxygenase-2 inhibitor and a peroxisome proliferator-activated receptor-γ antagonist, suggesting a role of cyclooxygenase-2 and peroxisome proliferator-activated receptor-γ in the proliferative response. However, the role of peroxisome proliferator-activated receptor-γ for proliferative response is unlikely in PSCs because our previous study showed that peroxisome proliferator-activated receptor-γ ligands did not increase, but decreased, the proliferation of PSCs (Masamune et al., 2002a).

It has been shown that PAR-2 is abundantly expressed in the pancreas, both in pancreatic acinar cells (Kawahata et

**Fig. 6.** Differential roles of MAP kinases for proliferation and collagen production. A, culture-activated, serum-starved PSCs were left untreated or treated with trypsin (at 10 nM) or AP (at 25 μM) in the absence or presence of MAP kinase inhibitors (a JNK inhibitor SP600125 at 10 μM, a p38 MAP kinase inhibitor SB203580 at 10 μM, or an inhibitor of MAP kinase kinase U0126 at 5 μM). After 24-h incubation, DNA synthesis was assessed by BrdU incorporation enzyme-linked immunosorbent assay. B, culture-activated PSCs were treated with trypsin (at 10 nM) or AP (at 25 μM) in the absence or presence of MAP kinase inhibitors. After 48-h incubation, new collagen synthesis was determined by measuring the incorporation of [3H]-proline into collagenase-sensitive proteins. Data are shown as mean ± S.D. (percentage of the control, n = 6). ***, p < 0.01 versus respective positive control (trypsin or activating peptide only).**

**Fig. 7.** PAR-2 agonists did not initiate the activation of freshly isolated PSCs. A to D, freshly isolated PSCs were incubated with TGF-β1 (at 10 ng/ml, A), trypsin (at 10 nM, B), activating peptide (at 25 μM, C), or serum-free medium only (D) for 7 days. Morphological changes characteristic of PSC activation were assessed after staining with GFAP. Original magnification: ×10 objective. E, total cell lysates (approximately 25 μg) were prepared from cells treated with serum-free medium (Con), TGF-β1, trypsin (Try), or AP for 7 days, and the levels of α-SMA and GAPDH were determined by Western blotting.
PAR-2 plays a physiological role in the secretory functions in the pancreas; PAR-2 activation resulted in pancreatic amylase secretion (Nystedt et al., 1994; Kawabata et al., 2002) and increased Ca\(^{2+}\)-activated Cl\(^-\) and K\(^+\) conductances in pancreatic duct epithelial cells (Nguyen et al., 1999). PSCs are located mainly in the interlobular areas and interacinar regions of the pancreas and comprise about 4% of all pancreatic cells (Apte et al., 1998; Bachem et al., 1998). Because quiescent PSCs do not express PAR-2, it seems unlikely that PAR-2 plays a physiological role in the cell functions of quiescent PSCs in normal pancreas. Of note, PAR-2 expression was strongly induced upon activation of PSCs. The underlying mechanism of PAR-2 up-regulation in PSCs upon activation remains unknown. It has been shown that the expression of PAR-2 in endothelial cells was up-regulated as a response to inflammatory mediators such as IL-1 and tumor necrosis factor-\(\alpha\) (Nystedt et al., 1996). Interestingly, D’Andrea et al. (2001) showed the transformation of PAR-2-negative to \(\alpha\)-positive fibroblasts in response to scrape wound-inducing in vitro, indicating that cell damage relays a signal for PAR-2 induction.

PAR-2 may play a proinflammatory role through the cytokine production. It has been shown that PAR-2 agonists induced the activation of NF-\(\kappa\)B and consequent cytokine production in human dermal microvascular endothelial cells (Shpacovitch et al., 2002). Activated PSCs acquire the proinflammatory phenotype; they may modulate the recruitment and activation of inflammatory cells. We have shown that proinflammatory cytokines such as IL-1\(\beta\) and tumor necrosis factor-\(\alpha\) induced the expression of MCP-1 and intercellular adhesion molecule-1 in PSCs (Masamune et al., 2002a,c). In this study, PAR-2 activation activated MAP kinases but failed to induce the activation of NF-\(\kappa\)B and the consequent expression of MCP-1. This is in agreement with our previous findings that activation of MAP kinases was required but not sufficient for optimal MCP-1 expression in PSCs (Masamune et al., 2002b, 2004). The failure of MCP-1 induction does not exclude the role of PAR-2 in the development of pancreatic inflammation and fibrosis because MCP-1 might be induced by proinflammatory cytokines released from inflammatory cells and pancreatic acinar cells during the course of pancreatitis (Gukovskaya et al., 1997; Apte et al., 1999; Schneider et al., 2000).

Very recently, Namkung et al. (2004) reported that PAR-2 plays a dual role in acute pancreatitis; PAR-2 protected acinar cells against pancreatitis-induced cell damage and death, whereas it mediated or aggravated the systemic complications of acute pancreatitis. They showed that PAR-2-activating peptide protected against a relatively mild form of intraparacrine pancreatitis induced by a single intraperitoneal injection of cerulein. In vitro, PAR-2-activating peptide decreased acinar cell death induced by bile acids at low concentrations, whereas cell death induced by bile acids at high concentrations was not altered. Thus, PAR-2 is likely to serve as an acute local defense mechanism, which appears not to be very strong. Once the PAR-2 defense mechanism is overwhelmed by excessive trypsin secretion and/or accumulation of proinflammatory mediators, pancreatitis ensues, and PAR-2 plays an opposite role; it mediates or aggravates the systemic complications of acute pancreatitis. Our results suggest that PAR-2 plays a detrimental role in the development of pancreatic fibrosis. Diverse effects of PAR-2 in inflammation have also been reported in colon; PAR-2 activation protected against 2,4,6-trinitrobenzene sulfonic acid-induced colitis (Fiorucci et al., 2001), whereas PAR-2 plays a detrimental role in radiation-induced fibrosis (Wang et al., 2003). Further studies using neutralizing antibodies, PAR-2 antagonists, or PAR-2 knockout mice (Ferrell et al., 2003) will be required to establish the role of PAR-2 in the development of pancreatic fibrosis in vivo.

There is accumulating evidence that proliferative action of mast cell tryptase plays a role in the pathogenesis of human fibrotic disorders (Akers et al., 2000; Frugieri et al., 2002). For example, mast cell tryptase stimulated the proliferation of human lung fibroblasts via PAR-2, thus playing a role in the fibro-proliferative response observed in bronchial asthma, chronic obstructive pulmonary disease, and pulmonary fibrosis (Akers et al., 2000). Tryptase may contribute to the development of the renal interstitial fibrosis through the proliferation and extracellular matrix protein production of renal interstitial fibroblasts (Kondo et al., 2001). Zimnoch et al. (2002) showed that the number of degranulated mast cells was increased in chronic pancreatitis with more severe fibrosis. Our results support a role of tryptase for the development of pancreatic fibrosis via PAR-2-mediated proliferative and fibrogenic actions on PSCs. Elucidation of the role of PAR-2 activation in the pancreas would provide better understanding and rational approaches for the control of pancreatic fibrosis and inflammation.

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References


