

Endothelin-1 stimulates contraction and migration of rat pancreatic stellate cells

Atsushi Masamune, Masahiro Satoh, Kazuhiro Kikuta, Noriaki Suzuki, Kennichi Satoh, Tooru Shimosegawa

Atsushi Masamune, Masahiro Satoh, Kazuhiro Kikuta, Noriaki Suzuki, Kennichi Satoh, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan

Supported by Grant-in-Aid for Encouragement of Young Scientists from Japan Society for the Promotion of Science, No. 16590572 (to AM.), by Pancreas Research Foundation of Japan, No. 01-01 (to AM.), by the Kanae Foundation for Life and Socio-Medical Science (to AM), and by the Uehara Memorial Foundation (to AM)

Correspondence to: Dr. Atsushi Masamune, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574,

Japan. amasamune@int3.med.tohoku.ac.jp

Telephone: +81-22-717-7171 Fax: +81-22-717-7177

Received: 2004-12-19 Accepted: 2005-02-18

Abstract

AIM: To examine the ability of ET-1 to affect the cell functions of PSCs and the underlying molecular mechanisms.

METHODS: PSCs were isolated from the pancreas of male Wistar rats after perfusion with collagenase, and cells between passages two and five were used. Expression of ET-1 and ET receptors was assessed by reverse transcription-PCR and immunostaining. Phosphorylation of myosin regulatory light chain (MLC), extracellular-signal regulated kinase (ERK), and Akt was examined by Western blotting. Contraction of PSCs was assessed on hydrated collagen lattices. Cell migration was examined using modified Boyden chambers. Cell proliferation was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine.

RESULTS: Culture-activated PSCs expressed ET_A and ET_B receptors, and ET-1. ET-1 induced phosphorylation of MLC and ERK, but not Akt. ET-1 induced contraction and migration, but did not alter proliferation of PSCs. ET-1-induced contraction was inhibited by an ET_A receptor antagonist BQ-123 and an ET_B receptor antagonist BQ-788, whereas migration was inhibited by BQ-788 but not by BQ-123. A Rho kinase inhibitor Y-27632 abolished both contraction and migration.

CONCLUSION: ET-1 induced contraction and migration of PSCs through ET receptors and activation of Rho-Rho kinase. ET_A and ET_B receptors play different roles in the regulation of these cellular functions in response to ET-1.

Key words: Pancreatitis; Pancreatic fibrosis; Pancreatic stellate cells; Endothelin-1; Rho kinase

Masamune A, Satoh M, Kikuta K, Suzuki N, Satoh K, Shimosegawa T. Endothelin-1 stimulates contraction and migration of rat pancreatic stellate cells. *World J Gastroenterol* 2005; 11(39): 6144-6151

<http://www.wjgnet.com/1007-9327/11/6144.asp>

INTRODUCTION

In 1998, star-shaped cells in the pancreas, namely pancreatic stellate cells (PSCs), were identified and characterized^[1,2]. 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 myofibroblast-like cells, which actively proliferate, express the cytoskeletal protein α -smooth muscle actin, and produce extracellular matrix components. Many of the morphological and metabolic changes associated with the activation of PSCs in animal models of fibrosis also occur when these cells are grown in serum-containing medium in culture on plastic. There is accumulating evidence that activated PSCs play a pivotal role in the development of pancreatic fibrosis^[1-4]. In addition, PSCs may participate in the pathogenesis of acute pancreatitis^[3,5]. The activation of signal transduction pathways such as p38 mitogen-activated protein (MAP) kinase^[6], Rho-Rho kinase^[7], and c-Jun N-terminal kinase^[8] is likely to play a role in PSC activation. But intracellular signal transduction pathways in PSCs remain largely unknown.

Endothelin-1 (ET-1), which acts through G-protein coupled ET_A and ET_B receptors, was initially described as a potent endothelial cell-derived vasoconstrictor isolated from the culture medium of porcine aortic endothelial cells^[9]. Subsequent studies have shown that ET-1 is produced by a variety of cells and exhibits several biological activities such as vasoconstriction, regulation of peptide secretion, migration, and positive or negative effects on cell growth^[10,11]. ET-1 has been implicated in the pathogenesis of cardiovascular, pulmonary, renal, and developmental disorders^[11]. In addition, ET-1 plays a key role in the development of portal hypertension and hepatic fibrosis through its effects on hepatic stellate cells^[12,13]. ET-1 has been shown to stimulate contraction via ET_A receptor^[14], and inhibit proliferation via ET_B receptor^[15] in hepatic stellate cells. But, the roles of ET-1 and ET receptors in the regulation of cell functions of PSCs remain largely unknown. In this study, we examined

the ability of ET-1 to affect the cell functions of PSCs and the underlying molecular mechanisms. We here report that ET-1 stimulated contraction and migration, but not proliferation, of PSCs through ET receptors and Rho-Rho kinase pathway.

MATERIALS AND METHODS

Materials

ET-1 was purchased from Peptide Institute (Osaka, Japan). Collagenase P was obtained from Roche Diagnostics (Mannheim, Germany). Rat recombinant platelet-derived growth factor (PDGF)-BB was purchased from R&D Systems (Minneapolis, MN). Rhodamine-labeled phalloidin was purchased from Molecular Probes (Eugene, OR). BQ-123, BQ-788, and U0126 were purchased from Calbiochem (La Jolla, CA). Y-27632 was a generous gift from Mitsubishi Pharma Co. (Osaka, Japan). Rabbit antibodies against extracellular-signal regulated kinase (ERK; phosphorylated and total), Akt (phosphorylated at Ser⁴⁷³ and total), and myosin regulatory light chain II (MLC; phosphorylated at Ser¹⁹ and total) were purchased from Cell Technologies, Inc. (Beverly, MA). Rabbit antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Trevigen (Gaithersburg, MD). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specifically described.

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, Japan) weighing 200–250 g using the Nycodenz solution (Nycomed Pharma, Oslo, Norway) after perfusion with 0.3 g/L collagenase P as previously described^[16]. The cells were resuspended in Ham's F-12 medium containing 100 mL/L heat-inactivated fetal bovine serum (MP Biomedicals, Irvine, CA), penicillin sodium, and streptomycin sulfate. Cell purity was always more than 90% as assessed by a typical star-like configuration and by detecting vitamin A autofluorescence. All experiments were performed using cells between passages two and five. We incubated PSCs in serum-free medium for 24 h before the addition of experimental reagents. For some experiments, BQ-123, BQ-788, U0126, or Y-27632 were added 30 min prior to the ET-1 treatment.

Reverse transcription-PCR

Total RNA was isolated using RNeasy total RNA preparation kit (Qiagen, Chatsworth, CA). Total RNA was reverse-transcribed, and the resultant cDNA was subjected to PCR as previously reported^[17]. Specific primer sets were as follows (listed 5'-3'; forward and reverse, respectively). ET-1 precursor (preproET-1): TTGCTCCTGCTCCTC-CTTGAG and GGTCTTGATGCTGTTGCTGATG; ET_A: CGTCTTCTGCTTGGTTGTCA and TGGTTC-TGCTCCTGGTTCTT; ET_B: CAAGACAGTATTCTGC CTGG and CAAGCAGGATTGCTTCTCCT. They were amplified using 30 cycles at 94 °C (for 1 min), at 50 °C (for 1 min), and at 72 °C (for 1 min). The products of PCR

were separated by 15 g/L agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. The expected sizes of the PCR products were 382 bp for preproET-1, 321 bp for ET_A receptor, and 297 bp for ET_B receptor.

Immunostaining

PSCs were grown directly on slides, serum-starved for 24 h, and immunostaining for ET-1 was performed using a streptavidin-biotin peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo, Japan) as previously described^[18]. Briefly, cells were fixed with 1 000 mL/L methanol at -20 °C, and then endogenous peroxidase activity was blocked by incubation with hydrogen peroxide in methanol. After immersion in normal rabbit serum, the slides were incubated with mouse anti-ET-1 antibody (at 1:200 dilution) at 4 °C overnight. The slides were incubated with biotinylated anti-mouse immunoglobulin antibody, followed by peroxidase-conjugated streptavidin. Finally, color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto, Japan). The expression of ET_A and ET_B was examined in a similar manner.

Western blotting

The level of activated, phosphorylated ERK was determined by Western blotting as previously described^[19]. Cells were lysed in sodium dodecyl sulfate buffer. Cellular proteins (approximately 100 µg) were fractionated on a 100 g/L sodium dodecyl sulfate-polyacrylamide gel. They were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membrane was incubated overnight at 4 °C with rabbit anti-phosphospecific ERK antibody. After incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody for 1 h, proteins were visualized using an ECL kit (Amersham Biosciences, UK). The levels of total ERK, Akt (phosphorylated and total), and MLC (phosphorylated and total) were determined in a similar manner.

Fluorescence microscopy

Stress fibers were stained with rhodamine-labeled phalloidin as previously described^[20]. After fixation with 40 g/L paraformaldehyde for 5 min, PSCs were permeabilized with 1.5 mL/L Triton X-100 in PBS for 5 min. Cells were then incubated for 2 h at room temperature in a dark place with rhodamine-labeled phalloidin in blocking buffer (10 g/L bovine serum albumin in PBS). Cells were then washed thrice with PBS for 10 min each, and coverslipped with Fluoromount (Vector Laboratories, Burlingame, CA). Cells were observed with a Leica fluorescence microscope. Images were captured with a Leica QFISH system (Wetzlar, Germany).

Collagen gel contraction assay

Contraction of PSCs on collagen lattices was examined in six-well flat-bottom tissue culture plates (Becton Dickinson, Bedford, MA) as previously described^[21]. Culture vessels were preincubated with PBS containing 10 g/L bovine serum albumin for 2 h at 37 °C, washed, and air-dried. The gel mixture consisted of eight parts Vitrogen-100 (Collagen Corp., Palo Alto, CA), one part 10×minimal essential medium,

and one part 0.2 mol/L HEPES, pH 9.0, which resulted in a final collagen concentration of 2.4 g/L. It was prepared at 4 °C, added to the culture vessel, and incubated at 37 °C for 2 h to allow gelation. PSCs were trypsinized, resuspended in serum-free medium (at 1×10^9 cells/L), plated on top of the gels (1 mL/well), and allowed to attach for 2 h. The collagen lattices were detached from the plate by gentle circumferential dislodgement using a fine needle, and ET-1 (at 100 nmol/L) was added to elicit contraction. After 24-h incubation, we measured the diameter of the collagen lattices, and calculated the “% of the original diameter (= 35 mm)”. In addition, cell-free collagen lattices were incubated with 100 nmol/L ET-1 or serum-free medium alone.

Cell migration assay

Cell migration was assessed as previously described^[16]. Serum-starved PSCs were trypsinized, and resuspended at the concentration of 3×10^8 cells/L in serum-free medium containing 10 g/L bovine serum albumin. For the assay, we used modified Boyden chambers with 8- μ m pore filters (Iwaki Glass Co. Ltd., Funabashi, Japan) coated with rat-tail type I collagen. ET-1 was added to the lower chamber, and 250 μ L of cell suspension was added to the upper chamber. The chambers were then incubated at 37 °C for 24 h. 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, Japan), viewed, and counted at 200 \times magnification.

Cell proliferation assay

Serum-starved PSCs (approximately 80% density) were treated with ET-1 in the presence or absence of 100 mL/L fetal bovine serum or PDGF-BB (at 25 μ g/L). Cell proliferation was assessed using a commercial kit (Cell proliferation ELISA, BrdU; Roche Diagnostics) according to the manufacturer's instruction. This is a colorimetric immunoassay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis. After 24-h incubation with experimental reagents, 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 $A_{370-492}$.

Statistical analysis

The results were expressed as mean \pm SD. Luminograms and autoradiograms are representative of at least three experiments. 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

Activated PSCs expressed ET-1 and ET receptors

We first examined whether culture-activated PSCs expressed ET-1 and ET receptors. ET-1 is a product of the gene coding for a large precursor protein (i.e. preproET-1)^[9]. Culture-activated PSCs expressed ET_A and ET_B receptors, and ET-1, as assessed by reverse transcription-PCR and immunostaining (Figure 1).

ET-1 induced phosphorylation of ERK and MLC, but not Akt

In an attempt to elucidate the effect of ET-1 on cell functions of PSCs, we examined whether ET-1 activated intracellular signaling pathways. We examined the activation of ERK, Akt, and MLC, all of which are implicated in ET-1-induced cellular contraction and/or migration^[22-24]. PSCs were treated with ET-1 at 100 nmol/L, and the activation of the signaling pathways by Western blotting using anti-phosphospecific antibodies. We chose this ET-1 concentration because previous studies have shown that ET-1 acts on ET receptors in a dose-dependent manner and ET-1 at this concentration effectively elicited effects in a variety of cell types^[14,15,25,26]. PDGF-BB induced phosphorylation of ERK, Akt, and MLC whereas ET-1 induced phosphorylation of ERK and MLC but not Akt (Figure 2).

Y-27632 decreased stress fiber formation and MLC phosphorylation

It has been shown that RhoA, acting through its downstream effector Rho kinase, stimulates MLC phosphorylation, stress fiber formation, and contractile force generation^[27,28]. The fact that ET-1 induced phosphorylation of MLC prompted us to examine the effect of Y-27632, a specific Rho kinase inhibitor^[29], on the phosphorylation of MLC and stress fiber formation. Y-27632 decreased phosphorylation of MLC in a dose-dependent manner (Figure 3A). When cells were treated with Y-27632 at 25 μ mol/L for 24 h, disassembly of stress fibers was observed (Figures 3B and 3C). In these experiments, Y-27632 up to 25 μ mol/L did not affect the cell viability during the incubation as assessed by trypan blue exclusion test (data not shown). In addition, the effects of Y-27632 were reversible within 48 h after the removal of Y-27632 (Figures 3A and 3D). However, when PSCs were treated with Y-27632 at 50 μ mol/L, cytotoxic effects were observed during the incubation (data not shown).

ET-1 induced contraction of PSCs

We examined whether ET-1 induced contraction of PSCs using collagen lattices. With no stellate cells, the size of the collagen lattices did not change in response to ET-1 (data not shown). When PSCs were plated on the lattices, and incubated with serum-free medium alone for 24 h, there was a slight reduction in the diameter of the lattices to $89 \pm 3\%$ of their original size (Figure 4A). By incubation with 100 nmol/L ET-1, the diameter of the lattices decreased to $54 \pm 8\%$ (Figure 4B). ET-1-induced contraction of PSCs was inhibited by an ET_A receptor antagonist BQ-123^[30], an ET_B receptor antagonist BQ-788^[31]. Y-27632 abolished ET-1-induced contraction, whereas a MAP kinase inhibitor U0126^[32] was ineffective.

ET-1 induced migration, but did not alter proliferation of PSCs

ET-1 induced migration of PSCs, which was inhibited by BQ-788, Y-27632, and U0126, but not BQ-123 (Figure 5).

Previous studies have shown that ET-1 showed positive or negative effects on cell growth^[15,25,26]. In agreement with the previous study^[16], PDGF-BB and 100 mL/L fetal bovine serum increased proliferation of PSCs (Figure 6). ET-1 did not affect basal proliferation or proliferation in response to PDGF or serum.

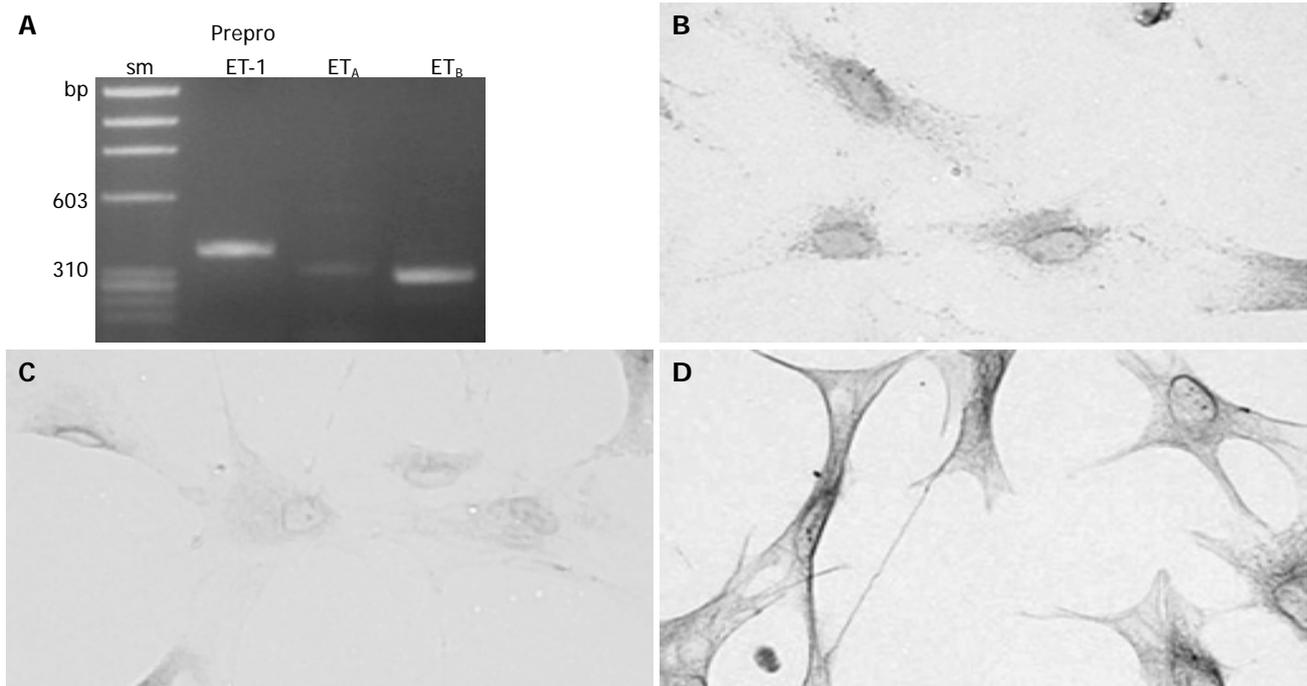


Figure 1 Culture-activated PSCs expressed ET and ET receptors. **A:** Total RNA was prepared from culture-activated, serum-starved PSCs. The expression of preproET-1, ET_A receptor and ET_B receptor was examined by reverse transcription-PCR. The expected sizes of the PCR products were 382 bp for preproET-1, 321 bp for ET_A receptor, and 297 bp for ET_B receptor. sm: size

marker (ϕ X174 *Hae*III digest). bp: bp; **B-D:** Serum-starved PSCs were grown directly on slides. Immunostaining for ET-1 (panel B), ET_A receptor (panel C), and ET_B receptor (panel D) was performed using a streptavidin-biotin peroxidase complex detection kit. Original magnification: $\times 20$ objective.

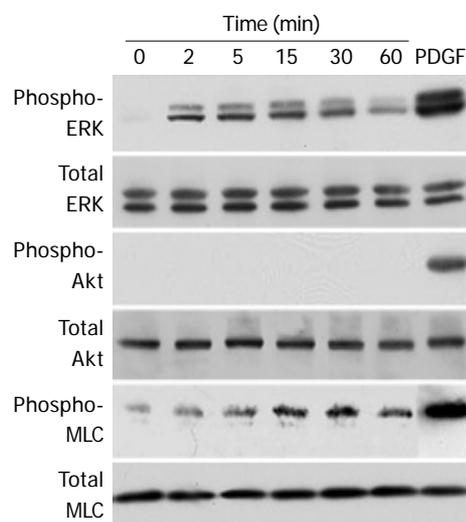


Figure 2 ET-1 induced phosphorylation of ERK and MLC, but not Akt. PSCs were treated with ET-1 (at 100 nmol/L) for the indicated time. Total cell lysates (approximately 100 μ g) were prepared, and the levels of phosphorylated ERK, Akt, and MLC were determined by Western blotting. As positive controls, total cell lysates were prepared from PSCs treated with PDGF-BB (at 25 μ g/L) for 5 min, and Western blotting was performed in a similar manner. Levels of total ERK, Akt, and MLC were also determined.

DISCUSSION

There is accumulating evidence that activated PSCs play a pivotal role in the pathogenesis of pancreatic fibrosis and inflammation^[1-5]. We here showed that culture-activated PSCs expressed ET_A and ET_B receptors, and ET-1. ET-1

stimulated contraction and migration of PSCs, suggesting autocrine and paracrine mechanisms of ET-1-regulated cellular functions of PSCs. ET-1-induced contraction was inhibited by ET_A and ET_B receptor antagonists whereas ET-1-induced migration was inhibited by ET_B receptor antagonist but not by ET_A receptor antagonist. Thus, ET_A and ET_B receptors play different roles in the regulation of these cellular functions in response to ET-1.

Cellular migration and contraction are complex processes involving dynamic changes in the actin-myosin cytoskeleton, including generation of contractile forces^[33]. It has been shown that contractile force is powered by myosin, which is activated by phosphorylation of MLC^[33]. Phosphorylation of MLC is regulated by the balance of two enzymatic activities, i.e., MLC kinase(s) and myosin phosphatase. For example, MLC is phosphorylated by MLC kinase in a Ca²⁺/calmodulin-dependent manner in smooth muscle cells^[34]. RhoA, acting through its downstream effector Rho kinase, stimulates MLC phosphorylation and consequently contractile force generation through the inhibition of myosin phosphatase^[27,28]. Activated Rho kinase promotes the contraction of isolated stress fibers and generates a long-lasting tensile activity by the maintained inhibition of myosin phosphatase^[35]. On the other hand, contractile force generation by certain non-muscle cell types including fibroblasts may occur independently of myosin phosphorylation^[36,37]. To clarify the role of myosin in ET-1-induced contraction and migration of PSCs, we examined the effect of ET-1 on the phosphorylation of MLC. We here showed that ET-1 induced the phosphorylation of MLC, supporting a role of myosin in contractile force generation in PSCs. In addition, an inhibitor of Rho kinase

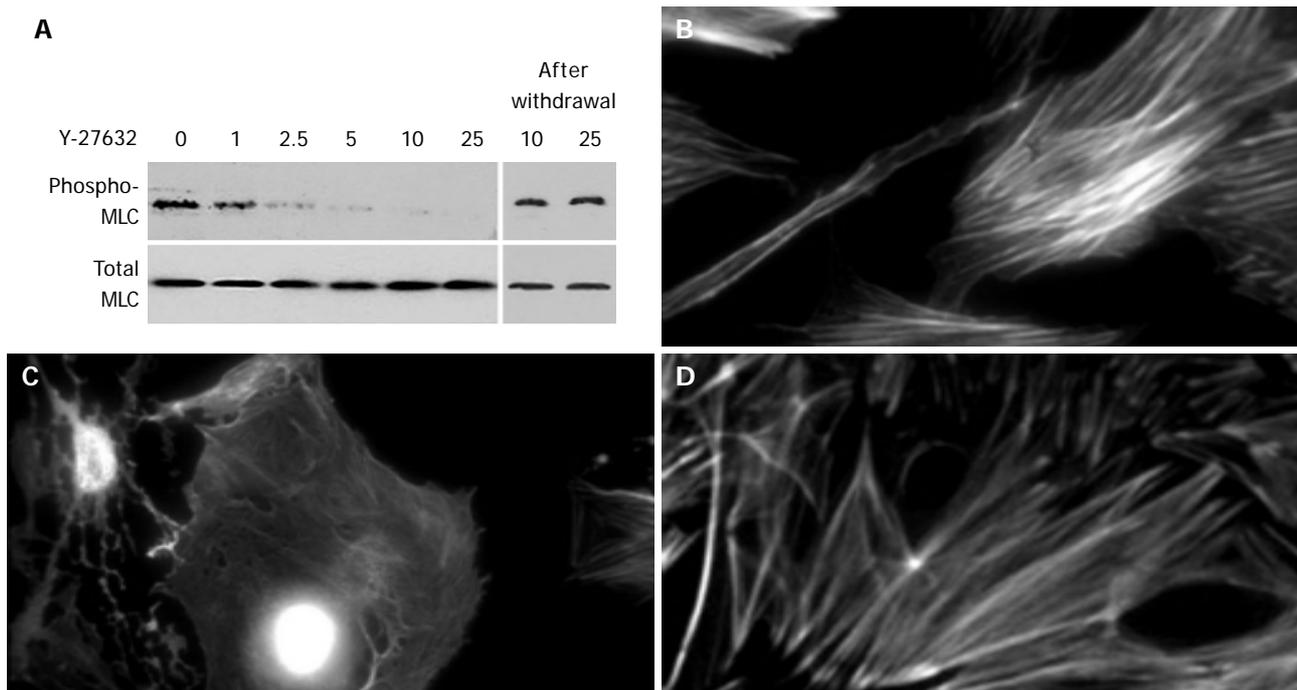


Figure 3 Y-27632 decreased stress fiber formation. **A:** PSCs were treated with ET-1 (at 100 nmol/L) in the presence of Y-27632 at the indicated concentrations (at $\mu\text{mol/L}$) for 30 min. Total cell lysates (approximately 100 μg) were prepared, and the levels of phosphorylated and total MLC were determined by Western blotting. In parallel experiments, Y-27632 was withdrawn after 30-min incubation, and replaced with 10% FBS. After 48-h incubation with 10% FBS, total cell lysates were prepared, and the levels of phosphorylated and total MLC

were determined by Western blotting; **B-D:** PSCs were incubated with ET-1 (at 100 nmol/L) in the absence (**B**) or presence (**C**) of Y-27632 (at 25 $\mu\text{mol/L}$) for 24 h, and stained with rhodamine-labeled phalloidin. In parallel experiments, Y-27632 was withdrawn and replaced with 10% FBS. After 48-h incubation with 10% FBS, PSCs were stained with rhodamine-labeled phalloidin (**D**). Original magnification: $\times 60$ objective.

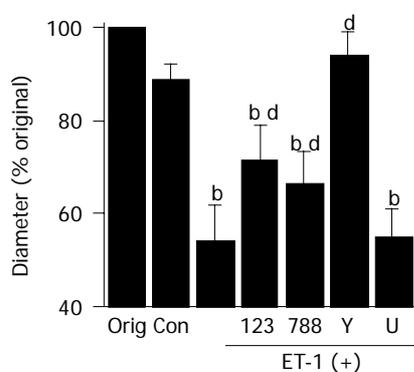


Figure 4 ET-1 induced contraction of PSCs. PSCs were plated on the surface of collagen lattices prepared in each well of a six-well plate and incubated for 2 h to allow adherence. The collagen lattices were then detached from the plate by gentle circumferential dislodgment. Serum-starved PSCs were left untreated ("Con") or were treated with ET-1 (at 100 nmol/L) in the absence or presence of BQ-123 ("123" at 10 $\mu\text{mol/L}$), BQ-788 ("788" at 10 $\mu\text{mol/L}$), Y-27632 ("Y" at 25 $\mu\text{mol/L}$), or U0126 ("U" at 5 $\mu\text{mol/L}$). Lattices were then detached from the plate, and incubated in serum-free medium (control, "Con") with or without 100 nmol/L ET-1. After 24-h incubation, we measured the diameter of the collagen lattices, and calculated the "% of the original diameter ("Orig" = 35 mm)". Data are shown as mean \pm SD (% of the original diameter, $n = 8$). ^b $P < 0.01$ vs control (serum-free medium only), ^d $P < 0.01$ vs ET-1 treatment only.

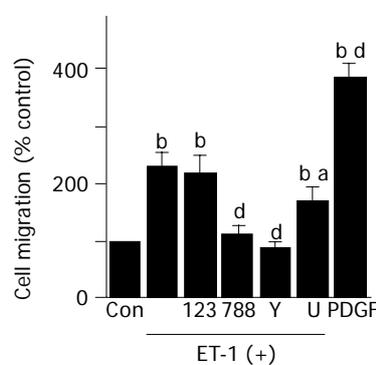


Figure 5 ET-1 induced migration of PSCs. Cell migration was assessed using modified Boyden chambers with 8- μm pore filters. Serum-starved PSCs were left untreated ("Con") or were treated with ET-1 (at 100 nmol/L) in the lower chamber in the absence or presence of BQ-123 ("123" at 10 $\mu\text{mol/L}$), BQ-788 ("788" at 10 $\mu\text{mol/L}$), Y-27632 ("Y" at 25 $\mu\text{mol/L}$), or U0126 ("U" at 5 $\mu\text{mol/L}$). After 24-h incubation, the cells migrated to the underside of the filter were stained, and counted under a light microscopy. As positive controls, cells were treated with PDGF-BB (at 25 $\mu\text{g/L}$) in the lower chamber, and cell migration was assessed in a similar manner. Data are shown as mean \pm SD (% of the control, $n = 6$). ^b $P < 0.01$ vs control (serum-free medium only), ^a $P < 0.05$, ^d $P < 0.01$ vs ET-1 treatment only.

Y-27632 abolished the phosphorylation of MLC, stress fiber formation, contraction, and migration. These results agree with the idea that force generation by the myofibroblasts depends on the isometric contraction of stress fibers containing α -smooth muscle actin, and is mediated through Rho-Rho kinase^[38]. Indeed, myosin mediates contractile force generation

by hepatic stellate cells in response to ET-1^[24]. We have previously shown that PDGF-BB induced migration of PSCs through the activation of phosphatidylinositol 3-kinase/Akt pathway^[16]. Unlike PDGF-BB, ET-1 did not activate Akt but did induce migration of PSCs, excluding a role of phosphatidylinositol 3-kinase/Akt pathway in ET-

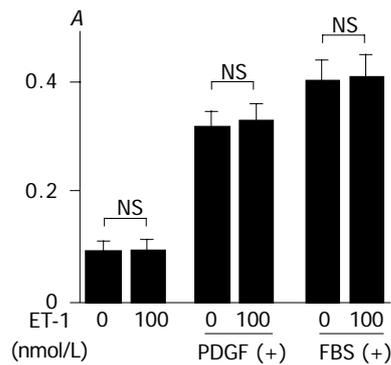


Figure 6 ET-1 did not alter basal or inducible proliferation of PSCs. Serum-starved PSCs were treated with ET-1 at 0 or 100 nmol/L in the absence or presence of PDGF-BB (at 25 μ g/L) or 100 mL/L fetal bovine serum (*FBS*). After 24-h incubation, DNA synthesis was assessed by BrdU incorporation enzyme-linked immunosorbent assay. Data are shown as mean \pm SD ($n = 6$). NS: not significant, A: optical density.

1-mediated migration.

In this study, ET-1 induced contraction of PSCs through both ET_A and ET_B receptors as assessed by the contraction of collagen lattices. This is in agreement with the previous study showing that both ET_A and ET_B receptors play an important role in contraction of colonic myofibroblast cells^[26], but disagrees with hepatic stellate cells where ET-1 induced contraction is through ET_A receptor^[14]. Investigation of cellular contraction using collagen lattices (gels) was introduced by Bell *et al.*^[39], and has been validated as a model of study of cellular contraction in a variety of cell types. Fibroblasts seeded on top of type I collagen gels bind and organize underlying collagen fibrils, both in close proximity and at a distance from cells^[40]. After such reorganization, lattices are converted into a dense mass with the morphological appearance of dermal scar. Kawada *et al.*^[41], reported a morphological study of ET-1-induced contraction of hepatic stellate cells on hydrated collagen gels. They demonstrated that contracted form of the stellate cells was morphologically characterized by the retraction of dendritic processes and the bending of the extended cell bodies that enfold the underlying collagen matrix. Because the cell body and processes are both closely attached to the collagen fibers, their contraction generates effective forces for the contraction of collagen gels.

ET-1 did not alter the proliferation of PSCs. The effect of ET-1 on growth and the underlying mechanisms depend on cell type. For example, ET-1 promotes the proliferation of human pulmonary artery smooth muscle cells through ET_A and ET_B receptors^[25]. ET-1 did not affect the proliferation of human colonic myofibroblast cells^[26]. In human hepatic stellate cells, ET-1 has been shown to inhibit the serum-induced proliferation through ET_B receptor, whereas ET-1 did not induce proliferation by itself^[15]. Of note, Pinzani *et al.*^[14], reported that ET-1 induced proliferation of early-passaged human hepatic stellate cells (passages 1 and 2), but the effect was less evident at a later stage of culture. This phenomenon is attributable at least in part to the progressive shift from a relative predominance of ET_A receptor to ET_B receptor during culture^[14]. Because the effects of ET-1 on cell growth might be mediated by distinct ET

receptors, the ratio between ET_A and ET_B receptors in the cells might constitute an additional factor that could affect the sensitivity of cells to the growth regulatory effects of ET-1. We and others have shown that ERK is a key mediator of PDGF-BB-induced mitogenic signals in PSCs^[16,42]. In spite of the ability to activate ERK, ET-1 failed to alter the proliferation, suggesting that activation of ERK is required but not sufficient for the proliferation of PSCs. This is also in the case of stimulation by ethanol, acetaldehyde, and 4-hydroxy-2,3-nonenal in PSCs^[43], and in agreement with the concept that activation of ERK, although necessary, may not be sufficient to justify the mitogenic properties of an agonist^[44].

Klonowski-Stumpe *et al.*^[45], reported that activated PSCs expressed ET_A receptor and ET-1, and that ET-1 increased cytosolic Ca²⁺ concentration^[45]. Exogenous ET-1 did not induce contraction, and, even in the absence of exogenous addition of ET-1, PSCs reduced the area of hydrated collagen lattices to 75% of the original value within 6 h, which was significantly attenuated by BQ-123, but not by another ET_B receptor antagonist IRL-1 038. The reason for discrepancy of the results between our study and theirs remain unknown, but the different experimental systems (e.g., different cell passages, different ET-1 concentrations, different ET_B receptor antagonist, and different serum-starvation condition) employed may be one explanation. As the ET_A receptor antagonist inhibited basal contraction of PSCs in their study, the endogenous ET-1 from PSCs or ET-1 that remained in the culture supernatant appeared to be responsible for the basal contraction of PSCs. In addition to ET_A receptor, we here have shown for the first time that activated PSCs express ET_B receptor, and that exogenously added ET-1 induces migration of PSCs.

It has been accepted that hepatic stellate cell contraction in response to ET-1 plays an important role in the regulation of sinusoidal blood flow, and in the development of portal hypertension and hepatic fibrosis^[12,13]. ET-1 is markedly overexpressed in different cellular elements in cirrhotic liver tissue, particularly in sinusoidal endothelial cells and activated hepatic stellate cells located in the sinusoids of the regenerating nodules at the edge of fibrous septa^[16]. On the other hand, the pathophysiological implication of ET-1-induced contraction of PSCs remains obscure. Although PSCs are located predominantly in the periacinar regions in normal pancreas^[1,2], perivascular and periductal localization has been also reported^[46]. Ikejiri^[46] showed that vitamin A-storing cells (now recognized as PSCs) were perivascularly localized in the pancreas of rats fed with vitamin A. Perivascular PSCs might participate in the regulation of blood flow and the development of microvascular ischemia during pancreatitis through ET-1 production^[47]. Izumi *et al.*^[48], reported that PSCs were found in the pancreatic duct wall of normal pancreas, and that the amount of such cells was excessively increased in patients with focal pancreatitis. They assumed that circular contraction of the ductal wall by proliferating PSCs might induce the stenosis of the main pancreatic duct and block the efflux of pancreatic juice from branch pancreatic ducts at the stenotic portion, leading to localized acinar atrophy and fibrosis^[48]. In addition, PSCs might participate in control of the intraductal pressure, epithelial

cell reconstitution, and duct remodeling during the healing of ductal injury. Along this line, it is now well accepted that the contractile activity of myofibroblasts is responsible for connective tissue remodeling during wound healing^[38]. Obviously, further studies are necessary to clarify the pathophysiological relevances of ET-1-mediated cell functions of PSCs.

ACKNOWLEDGMENTS

The authors thank Dr. Kenzo Kaneko for fluorescence microscopy, and Mitsubishi Pharma Co. for Y-27632.

REFERENCES

- 1 Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation and culture. *Gut* 1998; **43**: 128-133
- 2 Bachem MG, Schneider E, Gross H, Weidenbach H, Schmidt RM, Menke A, Siech M, Beger H, Grunert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology* 1998; **115**: 421-432
- 3 Haber PS, Keogh GW, Apte MV, Moran CS, Stewart NL, Crawford DH, Pirola RC, McCaughan GW, Ramm GA, Wilson JS. Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis. *Am J Pathol* 1999; **155**: 1087-1095
- 4 Masamune A, Kikuta K, Satoh M, Sakai Y, Satoh A, Shimosegawa T. Ligands of peroxisome proliferator-activated receptor-gamma block activation of pancreatic stellate cells. *J Biol Chem* 2002; **277**: 141-147
- 5 Masamune A, Sakai Y, Kikuta K, Satoh M, Satoh A, Shimosegawa T. Activated rat pancreatic stellate cells express intercellular adhesion molecule-1 (ICAM-1) *in vitro*. *Pancreas* 2002; **25**: 78-85
- 6 Masamune A, Satoh M, Kikuta K, Sakai Y, Satoh A, Shimosegawa T. Inhibition of p38 mitogen-activated protein kinase blocks activation of rat pancreatic stellate cells. *J Pharmacol Exp Ther* 2003; **304**: 8-14
- 7 Masamune A, Kikuta K, Satoh M, Satoh K, Shimosegawa T. Rho kinase inhibitors block activation of pancreatic stellate cells. *Br J Pharmacol* 2003; **140**: 1292-1302
- 8 Masamune A, Kikuta K, Masamune A, Kikuta K, Suzuki N, Satoh M, Satoh K, Shimosegawa T. A c-Jun NH2-terminal kinase inhibitor SP600125 (anthra[1,9-cd]pyrazole-6 (2H)-one) blocks activation of pancreatic stellate cells. *J Pharmacol Exp Ther* 2004; **310**: 520-527
- 9 Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411-415
- 10 Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 1992; **13**: 103-108
- 11 Levin ER. Endothelins. *N Engl J Med* 1995; **333**: 356-363
- 12 Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
- 13 Rockey DC, Chung JJ. Endothelin antagonism in experimental hepatic fibrosis. Implications for endothelin in the pathogenesis of wound healing. *J Clin Invest* 1996; **98**: 1381-1388
- 14 Pinzani M, Milani S, De Franco R, Grappone C, Caligiuri A, Gentilini A, Tosti-Guerra C, Maggi M, Failli P, Ruocco C, Gentilini P. Endothelin 1 is overexpressed in human cirrhotic liver and exerts multiple effects on activated hepatic stellate cells. *Gastroenterology* 1996; **110**: 534-538
- 15 Mallat A, Fouassier L, Preaux AM, Gal CS, Raufaste D, Rosenbaum J, Dhumeaux D, Jouneaux C, Mavier P, Lotersztajn S. Growth inhibitory properties of endothelin-1 in human hepatic myofibroblastic Ito cells. *J Clin Invest* 1995; **96**: 42-49
- 16 Masamune A, Kikuta K, Satoh M, Kume K, Shimosegawa T. Differential roles of signaling pathways for proliferation and migration of rat pancreatic stellate cells. *Tohoku J Exp Med* 2003; **199**: 69-84
- 17 Wendel-Wellner M, Noll T, Konig P, Schmeck J, Koch T, Kummer W. Cellular localization of the endothelin receptor subtypes ET_A and ET_B in the rat heart and their differential expression in coronary arteries, veins, and capillaries. *Histochem Cell Biol* 2002; **118**: 361-369
- 18 Masamune A, Satoh M, Kikuta K, Suzuki N, Shimosegawa T. Establishment and characterization of a rat pancreatic stellate cell line by spontaneous immortalization. *World J Gastroenterol* 2003; **9**: 2751-2758
- 19 Masamune A, Satoh K, Sakai Y, Yoshida M, Satoh A, Shimosegawa T. Ligands of peroxisome proliferator-activated receptor-gamma induce apoptosis in AR42J cells. *Pancreas* 2002; **24**: 130-138
- 20 Kaneko K, Satoh K, Masamune A, Satoh A, Shimosegawa T. Myosin light chain kinase inhibitors can block invasion and adhesion of human pancreatic cancer cell lines. *Pancreas* 2002; **24**: 34-41
- 21 Rocky DC, Housset CN, Friedman SL. Activation-dependent contractility of rat hepatic lipocytes in culture and *in vivo*. *J Clin Invest* 1993; **92**: 1795-1804
- 22 Ishihata A, Tasaki K, Katano Y. Involvement of p44/42 mitogen-activated protein kinases in regulating angiotensin II- and endothelin-1-induced contraction of rat thoracic aorta. *Eur J Pharmacol* 2002; **445**: 247-256
- 23 Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell* 2004; **15**: 2707-2719
- 24 Saab S, Tam SP, Tran BN, Melton AC, Tangkijvanich P, Wong H, Yee HF Jr. Myosin mediates contractile force generation by hepatic stellate cells in response to endothelin-1. *J Biomed Sci* 2002; **9**: 607-612
- 25 Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, Wharton J. ETA and ETB receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2002; **165**: 398-405
- 26 Kernochan LE, Tran BN, Tangkijvanich P, Melton AC, Tam SP, Yee HF Jr. Endothelin-1 stimulates human colonic myofibroblast contraction and migration. *Gut* 2002; **50**: 65-70
- 27 Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996; **273**: 245-248
- 28 Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 2000; **522**: 177-185
- 29 Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997; **389**: 990-994
- 30 Ihara M, Noguchi K, Saeki T, Fukuroda T, Tsuchida S, Kimura S, Fukami T, Ishikawa K, Nishikibe M, Yano M. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. *Life Sci* 1992; **50**: 247-255
- 31 Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T, Nishikibe M, Yano M. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci USA* 1994; **91**: 4892-4896
- 32 Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998; **273**: 18623-18632

- 33 **Lauffenburger DA**, Horwitz AF. Cell migration: A physically integrated molecular process. *Cell* 1996; **84**: 359-369
- 34 **Somlyo AP**, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994; **372**: 231-236
- 35 **Katoh K**, Kano Y, Amano M, Onishi H, Kaibuchi K, Fujiwara K. Rho-kinase-mediated contraction of isolated stress fibers. *J Cell Biol* 2001; **153**: 569-584
- 36 **Obara K**, Nikcevic G, Pestic L, Nowak G, Lorimer DD, Guerriero V Jr, Elson EL, Paul RJ, de Lanerolle P. Fibroblast contractility without an increase in basal myosin light chain phosphorylation in wild type cells and cells expressing the catalytic domain of myosin light chain kinase. *J Biol Chem* 1995; **270**: 18734-18737
- 37 **Skuta G**, Ho CH, Grinnell F. Increased myosin light chain phosphorylation is not required for growth factor stimulation of collagen matrix contraction. *J Biol Chem* 1999; **274**: 30163-30168
- 38 **Hinz B**, Gabbiani G. Mechanisms of force generation and transmission by myofibroblasts. *Curr Opin Biotechnol* 2003; **14**: 538-546
- 39 **Bell E**, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc Natl Acad Sci USA* 1979; **76**: 1274-1278
- 40 **Guidry C**, Grinnell F. Contraction of hydrated collagen gels by fibroblasts: evidence for two mechanisms by which collagen fibrils are stabilized. *Coll Relat Res* 1987; **6**: 515-529
- 41 **Kawada N**, Harada K, Ikeda K, Kaneda K. Morphological study of endothelin-1-induced contraction of cultured hepatic stellate cells on hydrated collagen gels. *Cell Tissue Res* 1996; **286**: 477-486
- 42 **Jaster R**, Sparmann G, Emmrich J, Liebe S. Extracellular signal regulated kinases are key mediators of mitogenic signals in rat pancreatic stellate cells. *Gut* 2002; **51**: 579-584
- 43 **Masamune A**, Kikuta K, Satoh M, Satoh A, Shimosegawa T. Alcohol activates activator protein-1 and mitogen-activated protein kinases in rat pancreatic stellate cells. *J Pharmacol Exp Ther* 2002; **302**: 36-42
- 44 **Blenis J**. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc Natl Acad Sci USA* 1993; **90**: 5889-5892
- 45 **Klonowski-Stumpe H**, Reinehr R, Fischer R, Warskulat U, Luthen R, Haussinger D. Production and effects of endothelin-1 in rat pancreatic stellate cells. *Pancreas* 2003; **27**: 67-74
- 46 **Ikejiri N**. The vitamin A-storing cells in the human and rat pancreas. *Kurume Med J* 1990; **37**: 67-81
- 47 **Kakugawa Y**, Paraskevas S, Metrakos P, Giaid A, Qi SJ, Duguid WP, Rosenberg L. Alterations in pancreatic microcirculation and expression of endothelin-1 in a model of chronic pancreatitis. *Pancreas* 1996; **13**: 89-95
- 48 **Izumi M**, Suda K, Torii A, Inadama E. Pancreatic ductal myofibroblasts. Proliferative patterns in various pathologic situations. *Virchows Arch* 2001; **438**: 442-450