Establishment and Characterization of a Simian Virus 40-Immortalized Rat Pancreatic Stellate Cell Line

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SATOH, M., MASAMUNE, A., SAKAI, Y., KIKUTA, K., HAMADA, H. and SHIMOSEGAWA, T. *Establishment and Characterization of a Simian Virus 40-Immortalized Rat Pancreatic Stellate Cell Line.* Tohoku J. Exp. Med., 2002, 198 (1), 55-69 — Activated pancreatic stellate cells (PSCs) have recently been implicated in the pathogenesis of pancreatic fibrosis and inflammation. Primary PSCs can be subcultured only several times because of their limited growth potential. A continuous cell line would be valuable in studying molecular mechanisms of these pancreatic disorders. The aim of this study was to establish an immortalized cell line of rat PSCs. PSCs were isolated from the pancreas of male Wistar rats, and the simian virus 40 T antigen was introduced to PSCs by retrovirus-mediated gene transfer. This procedure yielded an actively growing cell line, designated as SAM-K. This cell line has been passaged repeatedly for almost 2 years, and is thus likely immortalized. SAM-K cells retained morphological characteristics of primary PSCs, and expressed α -smooth muscle actin, glial fibrillary acidic protein, type I collagen, fibronectin, and prolyl hydroxylases. The level of p53 expression was very high in SAM-K cells. Proliferation of SAM-K cells was stimulated by serum and platelet-derived growth factor-BB. Interleukin-1 β (IL-1 β) activated nuclear factor- κ B, activator protein-1, and three classes of mitogen-activated protein (MAP) kinases: extracellular signal-regulated kinase1/2, c-Jun N-terminal kinase, and p38 MAP kinase. IL-1 β induced expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1, both of which were abolished in the presence of pyrrolidine dithiocarbamate, a

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Received September 13, 2002; revision accepted for publication September 27, 2002.

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Abbreviations: AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; ICAM-1, intercellular adhesion molecule-1; I κ B- α , inhibitor of nuclear factor κ B; IL, interleukin; JNK, c-Jun N-terminal kinase; MAP kinases, mitogen-activated protein kinases; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor- κ B; PSCs, pancreatic stellate cells; PDGF-BB, platelet-derived growth factor-BB isoform; PDTTC, pyrrolidine dithiocarbamate; α -SMA, α -smooth muscle actin; SV40, simian virus 40; TNF- α , tumor necrosis factor- α

specific inhibitor of nuclear factor- κ B activation. IL-1 β -induced monocyte chemoattractant protein-1 was partially inhibited by specific inhibitors of MAP kinase kinase (U0126) and of p38 MAP kinase (SB203580) whereas intercellular adhesion molecule-1 expression was not altered by the inhibitors. Thus, SAM-K would be useful for in vitro studies of cell biology and signal transduction of PSCs. ————— pancreatic stellate cells; chronic pancreatitis; pancreatic fibrosis; SV40 large T antigen; signal transduction
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Chronic pancreatitis as well as pancreatic cancer are accompanied by a progressive fibrosis that is characterized by loss of functional tissue and its replacement by extracellular matrix rich connective tissue (Suda et al. 1990; Etemad and Whitcomb 2001; Lankisch 2001). In contrast to liver fibrosis, the molecular mechanisms of pancreatic fibrogenesis remain to be elucidated. In 1998, star-shaped cells in the pancreas, namely pancreatic stellate cells (PSCs), was identified and characterized (Apte et al. 1998; Bachem et al. 1998). They are morphologically very similar to the hepatic stellate cells that play a central role in fibrosis of the liver (Friedman 2000). 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 α -smooth muscle actin (α -SMA), and produce type I collagen and other extracellular matrix components. There is accumulating evidence that PSCs, like hepatic stellate cells, are responsible for the development of pancreatic fibrosis (Apte et al. 1998; Bachem et al. 1998; Haber et al. 1999). Furthermore, PSCs might participate in the pathogenesis of acute pancreatitis through the expression of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule (ICAM)-1 (Andoh et al. 2000; Masamune et al. 2002c). The molecular mechanisms responsible for PSC activation

remain to be elucidated, but the activation of signaling pathways is likely to play a central role as in the case of hepatic stellate cells (Mann and Smart 2002). However, the precise intracellular signaling pathways in PSCs are largely unknown.

Although primary stellate cell cultures are a useful tool for studying molecular mechanisms of pancreatic fibrosis and inflammation, their isolation is time-consuming, yields are relatively low, and there is considerable preparation-to-preparation variability. Therefore, several weeks in culture are required to obtain sufficient cell numbers to perform experiments. In addition, primary cells can be subcultured only several times because of their limited growth potential in vitro. A variety of pancreatic ductal, islet, and acinar cell lines have been established, but a continuous stellate cell line has not been established (Ulrich et al. 2002). We here report an establishment of an immortalized cell line of rat PSC with introduction of the simian virus 40 (SV40) large T antigen.

MATERIALS AND METHODS

Materials

Poly(dI.dC)-poly(dI.dC), and [γ - ^{32} P]ATP were purchased from Amersham Biosciences UK, Ltd. (Buckinghamshire, England). Collagenase P and recombinant human interleukin (IL)-1 β were from Roche Applied Science (Mannheim, Germany). Rat recombinant platelet-derived growth factor-BB (PDGF-BB) was from R & D Systems (Minneapolis, MN, USA). Rabbit antibodies against phosphospecific

mitogen-activated protein kinases (MAP kinases), total MAP kinases, and inhibitor of nuclear factor κ B (NF- κ B)- α (I κ B- α) were purchased from Cell Technologies, Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies against SV40 large T antigen and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibodies against rat type I collagen and prolyl hydroxylases (α , β) were from LSL Cosmo Bio (Tokyo). Mouse monoclonal anti-rat ICAM-1 antibody was from Genzyme (Cambridge, MA, USA). Rabbit anti-rat fibronectin antibody was from Chemicon International (Temecula, CA, USA). SB203580 and U0126 were from Calbiochem (La Jolla, CA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless specifically described.

Cell culture and establishment of the immortalized cell line

All animal procedures were performed in accordance with the National Institutes of Health Animal Care and Use Guidelines. The procedure for the isolation of PSCs was a modification of that used for obtaining pancreatic ducts (Githens et al. 1980). The pancreas tissues of male Wistar rats (Japan SLC Inc., Hamamatsu) weighting 200–250 g were minced with sharp scissors, and incubated at 37°C for 30 minutes with gentle shaking in Hanks' balanced salt solution containing 0.05% collagenase P, 0.02% pronase, 0.1% deoxyribonuclease, 0.02% bovine serum albumin (fraction V), 0.01% soybean trypsin inhibitor, and 0.02% α -chymotrypsin. The digested tissues were pipetted through narrow orifices, and centrifuged at 800 rpm for 5 minutes. The supernatants were discarded and the pellets were washed twice with Ham's F-12 medium. Finally, the resultant pellets were resuspended, and cultured in Ham's F-12 medium containing 10% heat-inactivated fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), penicillin sodium and streptomycin sulfate. The culture

medium was changed the day after seeding and each second day thereafter. This procedure resulted in the isolation of star-shaped cells with lipid droplets in the cytoplasm, which is the typical characteristics of primary PSCs (Apte et al. 1998; Bachem et al. 1998). With the first two medium changes (24 and 48 hours after seeding), most of the contaminating cells were removed, and almost (>95%) pure PSC cultures were obtained. The retrovirus expression vector pMFGtsT (Dranoff et al. 1993) contains the partial coding region (~260 base pairs) of the SV40 T antigen gene. The retroviral packaging cells, ψ -CRIP-MFGtsT (Wakimoto et al. 1996), were grown to subconfluence, and PSCs at passage 2 were treated for 12 hours with the diluted culture supernatant of ψ -CRIP-MFGtsT in the presence of PDGF-BB at 25 ng/ml and polybrene (hexadimethrine bromide) at 12 μ g/ml. Then medium was replaced with fresh medium containing 10% heat-inactivated FBS, and incubation was continued. Isolation of individual clones was carried out by limiting dilution. After reaching confluency, cells were subcultured at the ratio of 1:4 by trypsinization using a 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid solution.

Western blotting

The levels of activated, phosphorylated MAP kinases were determined by Western blotting as previously described (Masamune et al. 2002d). Briefly, cells were lysed in sodium dodecyl sulfate buffer (62.5 mM Tris-HCl at pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) for 15 minutes on ice. The samples were then sonicated for 2 seconds, heated at 100°C for 5 minutes, and centrifuged at 12 000 $\times g$ for 5 minutes to remove insoluble cell debris. Cellular proteins (approximately 100 μ g) were fractionated on a 10% sodium dodecyl sulfate-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 MAP kinases antibodies (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.). Levels of total MAP kinases, α -SMA, SV40 large T antigen, p53, and I κ B- α were determined in a similar manner.

Immunostaining

SAM-K cells were grown directly on glass coverslips, serum-starved for 24 hours, and immunostaining for α -SMA was performed using a streptavidin-biotin-peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo) as previously described (Masamune et al. 2000). Briefly, cells were fixed with 100% methanol at -20°C, and then endogenous peroxidase activity was blocked by incubation in methanol with hydrogen peroxide. After immersion in normal rabbit serum, the slides were incubated with mouse anti- α -SMA antibody (at 1:100 dilution) overnight. The slides were incubated with biotinylated anti-mouse immunoglobulin antibody, followed by with peroxidase-conjugated streptavidin. Finally, color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto). Expression of desmin, glial acidic fibrillary protein, vimentin, fibronectin, type I collagen, and prolyl hydroxylases (α , β) was examined in a similar manner.

Cell proliferation assay

Serum-starved cells (approximately 20–30% density) were treated with FBS or PDGF-BB at the indicated concentrations. Cell proliferation was assessed using a commercial kit (CellTiter non-radioactive cell proliferation assay; Promega, Madison, WI, USA) according to the manufacturer's instruction. Briefly, after 72-hour-incubation with FBS or PDGF-BB, the dye solution was added to cells and

incubation continued at 37°C for 4 hours. Then, the formazan product was solubilized with the solubilization/stop solution. Cell viability was determined by differences in absorbance at wavelength 570 vs. 690 nm.

Electrophoretic mobility shift assay

Following 1-hour-incubation, approximately 5×10^6 cells were harvested and nuclear extracts were prepared, and electrophoretic mobility shift assay was performed as previously described (Masamune et al. 1996). Double-stranded oligonucleotide probes for NF- κ B (5'-AGTTGAGGGGACTTCCAGGC-3'), and activator protein-1 (AP-1) (5'-CGCTTGATGAGTCAGCCGGA-3') were endlabeled with [γ - ^{32} P]ATP. Nuclear extracts (approximately 5 μ g) were incubated with the labeled oligonucleotide probe for 20 minutes at 22°C, and electrophoresed through a 4% polyacrylamide gel. Gels were dried and autoradiographed at -80°C overnight. A 100-fold excess of unlabeled oligonucleotide was incubated with nuclear extracts for 10 minutes prior to the addition of the radiolabeled probe in the competition assays.

Enzyme-linked immunosorbent assay

After a 24 hour-incubation, cell culture supernatants were harvested and stored at -80°C until the measurement. MCP-1 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (Endogen, Woburn, MA, USA) according to the manufacturer's instructions. Cell-surface expression of ICAM-1 was determined by enzyme-linked immunosorbent assay as previously reported (Masamune et al. 1999).

Statistical analysis

Differences between experimental groups were evaluated by the two-tailed unpaired Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Establishment of Immortalized Rat PSC Line

It has been shown that PSCs are activated after approximately 48 hours of culture on plastic (Apte et al. 1998). PSCs at passage 2 were treated with the culture supernatant of the retroviral packaging cells, ψ -CRIP-MFGtsT, in the presence of PDGF-BB and polybrene. PDGF-BB was added because retrovirus-mediated gene transfer requires at least one cell division for gene integration (Miller et al. 1990) and PDGF-BB has been shown to be the most potent mitogen for PSCs in vitro (Apte et al. 1999; Luttenberger et al. 2000). This procedure yielded actively growing cells. Then they were subjected to limiting dilution, and one clone was obtained and designated as SAM-K. This cell line has been passaged repeatedly over 100 population doublings without showing any evidence of senescence for almost 2 years, and thus immortalized. SAM-K cells were myofibroblast-like shaped, and morphologically very similar to the primary PSCs (Figs. 1A and 1B). We examined the expression of SV40 large T antigen and α -SMA in SAM-K cells by Western blotting. SAM-K cells expressed both SV40 large T antigen and α -SMA (Fig. 1C). Parental primary PSCs expressed α -SMA, but not SV40 large T antigen, whereas ψ -CRIP-MFGtsT expressed SV40 large T antigen, but not α -SMA. In agreement with the result of Western blotting, the characteristic fiber-like pattern of positive α -SMA staining was observed throughout the cytoplasm in SAM-K cells (Fig. 1D).

Phenotyping of SAM-K cells

In addition to α -SMA, SAM-K cells stained positively for cytoskeletal protein glial acidic fibrillary protein and vimentin (Figs. 2A and 2B). Accumulation of extracellular matrix is a prominent feature of chronic pancreatitis, and activated PSCs play a critical role in the pathogenesis of pancreatic fibrosis through the pro-

duction of extracellular matrix proteins such as type I collagen and other extracellular matrix components (Apte et al. 1998; Bachem et al. 1998). SAM-K cells expressed extracellular matrix proteins type I collagen (Fig. 2C) and fibronectin (Fig. 2D) as assessed by immunostaining. In addition, SAM-K cells expressed prolyl hydroxylases (α , β) (Figs. 2E and 2F), that are key enzymes in the hydroxylation of the proline residues in procollagen and are useful markers of collagen synthesis (Masamune et al. 2002a). These results suggest that collagen synthesis was actively occurring in SAM-K cells, and that SAM-K cells shared many phenotypical and functional characteristics with primary, activated PSCs.

Expression of p53

The mechanisms by which SV40 large T antigen leads to immortalization are not fully resolved. But the most important contribution of the SV40 large T antigen during the process of immortalization may be its ability to bind to p53 molecules, thus considerably stabilizing the protein by increasing its effective concentration (Banerjee et al. 1992). We examined the expression of p53 in primary PSCs and SAM-K cells by Western blotting. The level of p53 expression was undetectable in primary PSCs and apparently very high in SAM-K cells (Fig. 3).

Proliferation of SAM-K was serum-dependent and stimulated by PDGF-BB

Fully confluent parental cell cultures showed a density of approximately 10 000 cells/cm², whereas confluent SAM-K cells were more numerous: approximately 30 000 cells/cm². Previous studies have shown that proliferation of primary PSCs was stimulated by growth factors such as serum and PDGF-BB (Apte et al. 1999; Luttenberger et al. 2000; Masamune et al. 2002a). Especially, PDGF-BB has been shown to be the most potent mitogen of PSCs, and is likely to be an important mediator of the

Fig. 1.

Fig. 1. SAM-K expressed SV40 large T antigen and α -SMA.

(A, B) Morphological characteristics of primary PSCs (panel A) and SAM-K cells (panel B) in culture demonstrated by phase contrast microscopy. Original magnification: $\times 40$. (C) Total cell lysates (approximately 100 μ g) were prepared from primary PSCs, SAM-K, and ψ -CRIP-MFGtsT cells. The levels of SV40 large T antigen and α -SMA were determined by Western blotting. (D) Cells were grown directly on glass coverslips. Immunostaining for α -SMA in serum-starved SAM-K cells was performed using a streptavidin-biotin-peroxidase complex detection kit. Original magnification: $\times 200$.

Fig. 2. Immunostaining of cytoskeletal proteins and extracellular matrix proteins in SAM-K cells. Cells were grown directly on glass coverslips, and serum-starved for 24 hours. Immunostaining for glial fibrillary acidic protein (A), vimentin (B), type I collagen (C), fibronectin (D), and prolyl hydroxylases (α) (E) and (β) (F) was performed using a streptavidin-biotin-peroxidase complex detection kit. Original magnification: $\times 200$.

Fig. 2.

Fig. 3. The level of p53 expression was very high in SAM-K cells.

Total cell lysates (approximately 100 μ g) were prepared from primary PSCs and SAM-K cells. The level of p53 was determined by Western blotting.

Fig. 4. Serum and PDGF-BB stimulated proliferation of SAM-K cells.

SAM-K cells were cultured in serum-free medium for 24 hours. Then FBS (at the indicated concentrations) or PDGF-BB (at 10 ng/ml) was added. After 72 hours, cell proliferation was assessed. The absorbance at 570 nm ("O.D.") of the sample is shown. Data are shown as mean $\pm$ s.d. ($n=6$).

* $p<0.05$, ** $p<0.01$ vs. serum-free medium only. O.D.: optical density.

increased proliferation of the cells both in vivo and in vitro (Apte et al. 1999; Luttenberger et al. 2000). We examined whether proliferation of SAM-K cells were also stimulated by serum and PDGF-BB. SAM-K cells required serum for their growth, and eventually underwent apoptosis in the absence of serum (data not shown). Treatment of the cells with FBS stimulated proliferation in a dose-dependent manner; serum-induced proliferation was significant at as low as 1% ($p<0.05$) (Fig. 4). PDGF-BB stimulated proliferation of PSCs; PDGF-BB (at 10 ng/ml) induced more than five-fold increase of cell proliferation in serum-free medium after 72 hours (Fig. 4).

IL-1 β activated NF- κ B, AP-1 and MAP kinases

We have previously shown that proinflammatory cytokines IL-1 β and tumor necrosis factor (TNF)- α activated transcription factors NF- κ B and AP-1 in primary PSCs (Masamune et al. 2002a, 2002c). These transcription factors are important regulators of gene expression in response to many stimuli, including proinflammatory cytokines and growth factors (Grilli et al. 1993; Karin et al. 1997). We examined whether IL-1 β activated these transcription factors in SAM-K cells. Nuclear extracts were prepared from SAM-K cells treated with IL-1 β for 1 hour, and specific binding activities of NF- κ B and AP-1 were assessed by electrophoretic mobility shift assay. IL-1 β increased NF- κ B and AP-1 binding activities (Fig. 5A). The specificity of these binding activities was confirmed by competition assays using 100-fold excess of unlabeled oligonucleotides (data not shown). Phosphorylation and degradation of I κ B- α is necessary for the activation of NF- κ B (Grilli et al. 1993). We examined the effects of IL-1 β on the cellular I κ B- α levels by Western blotting. IL-1 β induced transient degradation of I κ B- α , further supporting that IL-1 β activated NF- κ B (Fig. 5B).

Fig. 5. IL-1 β activated NF- κ B and AP-1.

(A) SAM-K cells were treated with IL-1 β (at 2 ng/ml) for 1 hour. Nuclear extracts were prepared, and specific binding activities of NF- κ B and AP-1 were assessed by electrophoretic mobility shift assay. (B) SAM-K cells were treated with IL-1 β for indicated times. Total cell lysates (approximately 100 μ g) were prepared, and the level of I κ B- α were determined by Western blotting.

MAP kinases are key elements in the regulation of cellular responses to external inflammatory and proliferative signals (Karin et al. 1997; Robinson and Cobb 1997). Several studies have shown that MAP kinases are upstream mediators of NF- κ B and AP-1 activation and the gene expression of proinflammatory cytokines and adhesion molecules (Karin et al. 1997; Robinson and Cobb 1997). For example, induction of the expression of AP-1 components c-Fos and c-Jun by a variety of stimuli such as growth factors and

Fig. 6. IL-1 β activated MAP kinases.

(A) SAM-K cells were treated with IL-1 β (at 2 ng/ml) for the indicated time. Total cell lysates (approximately 100 μ g) were prepared, and the levels of activated, phosphorylated MAP kinases were determined by Western blotting using anti-phosphospecific MAP kinase antibodies. The levels of total MAP kinases were also determined. (B) Total cell lysates (approximately 100 μ g) were prepared from unstimulated, serum-starved, primary PSCs or SAM-K cells, and the basal level of phosphorylated ERK1/2 were determined by Western blotting.

cytokines is mediated by the activation of three distinct MAP kinases: extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal

kinase (JNK), p38 MAP kinase (Karin et al. 1997; Robinson and Cobb 1997). We have previously shown that IL-1 β and TNF- α activated these three classes of MAP kinases in primary PSCs (Masamune et al. 2002c). Because the activation of these kinases occurs through phosphorylation, we examined the activation of these MAP kinases in SAM-K cells by Western blotting using anti-phosphospecific MAP kinase antibodies. These antibodies recognize only phosphorylated form of MAP kinases, thus allowing the assessment of activation of these kinases. IL-1 β activated these three classes of MAP kinases in a time-dependent manner with peaking around 5 to 15 minutes (Fig. 6A). The levels of total MAP kinases were unaffected by the treatment, indicating that the lanes had been equally loaded. It should be noted that basal levels of phosphorylated ERK1/2 in SAM-K cells were apparently higher than those in parental primary PSCs (Fig. 6B).

Roles of NF- κ B and MAP kinases for MCP-1 expression

Activated PSCs acquire the proinflammatory phenotype; they may modulate the recruitment and activation of inflammatory cells through the expression of IL-8, MCP-1 (Andoh et al. 2000; Masamune et al. 2002a), and ICAM-1 (Masamune et al. 2002c). We examined whether SAM-K cells express MCP-1 and ICAM-1 in response to IL-1 β . SAM-K cells constitutively expressed significant amount of ICAM-1 whereas MCP-1 production was very low. IL-1 β significantly induced the expression of MCP-1 and ICAM-1 (Fig. 7). To elucidate the roles of NF- κ B and MAP kinases for MCP-1 and ICAM-1 expression, we used specific inhibitors to block these pathways. First, we treated SAM-K cells with IL-1 β in the presence of pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF- κ B activation (Schreck et al. 1992). As shown in Fig. 7, PDTC decreased the IL-1 β -induced expression of MCP-1 and ICAM-1 to near the

Fig. 7. IL-1 β induced the expression of MCP-1 and ICAM-1.

SAM-K cells were left untreated, or treated with IL-1 β (IL; at 2 ng/ml) in the absence or presence of PDTC ("PD" at 10 μ M), U0126 ("U" at 10 μ M) or SB203580 ("SB" 25 μ M). After 24 hours, MCP-1 levels in the culture supernatant (A) and cell-surface ICAM-1 expression (B) were determined by enzyme-linked immunosorbent assay. MCP-1 levels are presented as nanograms per milliliter, and ICAM-1 levels are presented as optical density (O.D.). Con: control (medium only). Data shown are expressed as means $\pm$ s.d. ($n=6$; **, $p<0.01$ vs. IL-1 β).

basal levels. We next examined the effects of U0126 (Favata et al. 1998), a specific inhibitor of MAP kinase kinase activation, which prevents the activation of ERK1/2. U0126 at 10 μ M partially inhibited inducible MCP-1 production, whereas ICAM-1 expression was not altered. Similarly, a selective p38 MAP kinase inhibitor, SB203580 (Cuenda et al. 1995), at 25 μ M partially inhibited inducible MCP-1 production, whereas ICAM-1 expression was not altered. Similar results were obtained with TNF- α -induced MCP-1 and ICAM-1 expression (data not shown). Above these concentrations, inhibitors of MAP kinases showed some cytotoxicity. These results suggested that activation of NF- κ B plays a central role in inducible MCP-1 and ICAM-1 expression in SAM-K cells. Activation of ERK and p38 MAP kinase pathways are required for optimal MCP-1 expression, but not ICAM-1 expression.

DISCUSSION

In response to pancreatic injury or inflammation, PSCs undergo a transformation from quiescent cells to activated, proliferating myofibroblast-like cells, which produce cytokines and extracellular matrix proteins. Recent studies have established that activated PSCs play critical roles in the pathogenesis of pancreatic fibrosis and inflammation (Apte et al. 1998; Bachem et al. 1998; Haber et al. 1999; Masamune et al. 2002c). Although primary stellate cell cultures are a useful tool for studying molecular mechanisms of pancreatic fibrosis and inflammation *in vitro*, their isolation is time-consuming, yields are relatively low, and there is considerable preparation-to-preparation variability. In addition, primary cells can be subcultured only several times because of their limited growth potential. In this study, we have described the first successful immortalization of PSCs by retrovirus-mediated transfection of the cells with the SV40 T antigen gene. The immortalized SAM-K cells have been passaged repeatedly for almost 2 years, and thus

likely immortalized. SAM-K cells retained the morphologic characteristics of primary PSCs as well as the expression of the cytoskeletal proteins, α -SMA and glial acidic fibrillary protein. SAM-K cells maintained the functional characteristics of primary PSCs; SAM-K cells were strongly positive for type I collagen, prolyl hydroxylases, and fibronectin as assessed by immunostaining. SAM-K cells responded to IL-1 β and TNF- α , resulting in the activation of NF- κ B, AP-1, and MAP kinases. In addition, SAM-K cells exhibited proinflammatory responses: SAM-K cells expressed MCP-1 and ICAM-1 in response to IL-1 β and TNF- α . Thus, SAM-K cells appeared to retain several aspects of the primary PSC phenotype.

In culture, PSCs are morphologically very similar to the hepatic stellate cells. To date, several hepatic stellate cell lines have been established. LI90 human hepatic stellate cell line from a mesenchymal tumor of the liver (Murakami et al. 1995). Kim et al. (1998) established a hepatic stellate cell line, named HSC-T6, by introducing the SV40 large T antigen. The resulting clone HSC-T6 retains all features of activated hepatic stellate cells, including expression of desmin, α -SMA, and glial acidic fibrillary protein, and it can esterify retinol into retinyl esters. HSC-T6 cells were successfully used to stably overexpress exogenous genes including cytochrome P450E1 (Nieto et al. 1999), and discoidin domain tyrosine kinase receptor 2 (Olaso et al. 2001). Schnabl et al. (2002) reported immortal activated human hepatic stellate cells generated by ectopic telomerase expression. They showed that activated human hepatic stellate cells had little, if any, detectable telomerase activity. Telomerase-induced hepatic stellate cells did not undergo oncogenic transformation and showed morphologic characteristics of activated stellate cells. In addition, mRNA expression in telomerase-positive cells and -negative cells were very similar. It should be noted that some of the hepatic stellate cell lines were

established by spontaneous immortalization (Greenwel et al. 1991; Sauviant et al. 2002). Greenwel et al. reported the characterization of two hepatic stellate cell lines, one from normal and the other from cirrhotic liver (Greenwel et al. 1991). Although both cell lines resembled primary cultures of hepatic stellate cells, significant differences in cell proliferation and IL-6 production between the two cell lines were found. It is unknown whether PSCs undergo spontaneous transformation in culture.

The mechanism by which SV40 large T antigen leads to immortalization has not yet been solved completely. Immortalization of mammalian cells by SV40 large T antigen is thought to result, at least in part, from interactions of T antigen with cellular tumor suppressor proteins that block the cell cycle in G1 (Truckenmiller et al. 1998). The proteins include retinoblastoma protein, p300, and p53. In this study, the expression of SV40 T antigen in SAM-K cells was associated with elevated cellular p53 protein expression; the level of p53 expression was undetectable in primary PSCs and apparently very high in SAM-K cells. The most important contribution of the SV40 large T antigen during the process of immortalization may be its ability to bind to p53 molecules, thus considerably stabilizing the protein by increasing its effective concentration (Banerjee et al. 1992). However, it was also demonstrated that a truncated SV40 large T antigen lacking the p53 binding domain overcomes p53-induced growth arrest and immortalizes primary mesencephalic cells (Truckenmiller et al. 1998). It was also shown that large T antigen did lead to a decrease in chromosome stability, and this alteration of the genome may finally cause immortalization by activation of growth stimulatory genes and inactivation of genes that control the cell cycles or cellular senescence (Ray et al. 1990). The resulting genetic manipulations might limit the use of SV40-transfected cells for molecular biologic studies, especially for defining genetic changes that occur during

cell differentiation and transformation.

Activated PSCs acquire the proinflammatory phenotype; they may modulate the recruitment and activation of inflammatory cells. We and others have reported that activated PSCs express MCP-1 (Andoh et al. 2000; Masamune et al. 2002a) and ICAM-1 (Masamune et al. 2002c) in response to IL-1 β and TNF- α in vitro. In this study, IL-1 β induced expression of MCP-1 and ICAM-1 in SAM-K cells, and the inducible expression was abolished by PDTC, a specific inhibitor of NF- κ B activation. This is in agreement with the previous studies in PSCs indicating a central role of NF- κ B in MCP-1 and ICAM-1 expression (Andoh et al. 2000; Masamune et al. 2002a). On the other hand, roles of MAP kinases for proinflammatory responses in PSCs are not fully understood. We here showed that IL-1 β and TNF- α activated three classes of MAP kinases in SAM-K cells. Inhibition of ERK pathway by U0126 and of p38 MAP kinase by SB203580 partially inhibited IL-1 β -induced MCP-1 production. In contrast, IL-1 β -induced ICAM-1 expression was not affected by these inhibitors, suggesting a differential role of MAP kinases for proinflammatory responses in SAM-K cells. This is in agreement with the previous study showing that IL-1 β -induced MCP-1 expression was partially inhibited in primary PSCs (Masamune et al. 2003). There is accumulating evidence that roles of MAP kinases for MCP-1 expression depend on the types of cells and stimuli. For example, in human umbilical vein endothelial cells, activation of p38 MAP kinase pathway is important for TNF- α -induced MCP-1 expression, whereas JNK and ERK pathways are not of major relevance (Goebeler et al. 1999). In colonic subepithelial myofibroblasts, IL-17-induced MCP-1 expression was partially blocked by SB203580 and U0126 (Hata et al. 2002). In a similar manner to PSCs (Masamune et al. 2002b), ethanol and acetaldehyde at clinically relevant concentrations activated ERK and p38

MAP kinase, but failed to induce MCP-1 and ICAM-1 expression in SAM-K (Masamune et al. unpublished data). Thus, activation of ERK and p38 MAP kinase is required, but not sufficient for MCP-1 and ICAM-1 expression in SAM-K cells. It should be noted that basal levels of activated ERK1/2 in SAM-K cells were significantly higher than those in parental PSCs. It has been reported that activation of ERK1/2 pathway and stimulation of cell growth are dependent on the interaction between SV40 small *t* antigen and protein phosphatase 2A (Sontag et al. 1993).

In summary, SAM-K cells are a newly established PSC lines morphologically, phenotypically and functionally similar to primary PSCs. SAM-K cells can be easily used for both transient and stable transfections (Masamune et al. unpublished observation), facilitating the overexpressing genes. Its phenotypic characteristics as well as its responsiveness to IL-1 β make it a useful tool for understanding of the pathogenesis of pancreatic inflammation and fibrosis and thereby future therapeutic development.

Acknowledgments

This work was supported in part by Grant-in-Aid for Encouragement of Young Scientists from Japan Society for the Promotion of Science (to A. M.), and by Pancreas Research Foundation of Japan (to A.M.). Presented in part at the annual meeting of the American Gastroenterological Association in San Francisco, CA, USA in May 2002.

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