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Curcumin Blocks Activation of Pancreatic Stellate Cells

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Abstract Activated pancreatic stellate cells (PSCs) play a pivotal role in the pathogenesis of pancreatic fibrosis and inflammation. Inhibition of activation and cell functions of PSCs is a potential target for the treatment of pancreatic fibrosis and inflammation. The polyphenol compound curcumin is the yellow pigment in curry, and has anti-inflammatory and anti-fibrotic properties. We here evaluated the effects of curcumin on the activation and cell functions of PSCs. PSCs were isolated from rat pancreas tissue and used in their culture-activated, myofibroblast-like phenotype unless otherwise stated. The effects of curcumin on proliferation, α-smooth muscle actin gene expression, monocyte chemoattractant protein (MCP)-1 production, and collagen expression were examined. The effect of curcumin on the activation of freshly isolated cells in culture was also assessed. Curcumin inhibited platelet-derived growth factor (PDGF)-induced proliferation, α-smooth muscle actin gene expression, interleukin-1β- and tumor necrosis factor (TNF)-α-induced MCP-1 production, type I collagen production, and expression of type I and type III collagen genes. Curcumin inhibited PDGF-BB-induced cyclin D1 expression and activation of extracellular signal-regulated kinase (ERK). Curcumin inhibited interleukin-1β- and TNF-α-induced activation of activator protein-1 (AP-1) and mitogen-activated protein (MAP) kinases (ERK, c-Jun N-terminal kinase (JNK), and p38 MAP kinase), but not of nuclear factor-κB (NF-κB). In addition, curcumin inhibited transformation of freshly isolated cells to myofibroblast-like phenotype. In conclusion, curcumin inhibited key cell functions and activation of PSCs. J. Cell. Biochem. 97: 1080–1093, 2006.

Key words: pancreatitis; pancreatic fibrosis; pancreatic stellate cells; curcumin; polyphenol

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, PSCs 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 (α-SMA), and produce collagen and other 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 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]. In addition, PSCs may participate in the pathogenesis of acute pancreatitis [Haber et al., 1999; Masamune et al., 2002c]. The activation of signaling pathways such as p38 mitogen-activated protein (MAP) kinase [Masamune et al., 2003c], Rho-Rho kinase [Masamune et al., 2003b], and c-Jun N-terminal kinase (JNK) [Masamune et al., 2004] is likely to play a role in PSC activation. Obviously, control of activation and cell functions of PSCs is a potential target for the development of new treatment for pancreatic fibrosis and inflammation.

Curcumin (diferuloyl-methane), the yellow color of curry, is a natural product of plants obtained from *Curcuma longa* Linn (turmeric)

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[Govindarajan, 1980]. Curcumin has a variety of biological activities including anti-inflammatory [Sugimoto et al., 2002; Gukovsky et al., 2003], antioxidant [Rajakumar and Rao, 1995], antifibrotic [Punithavathi et al., 2000; Kang et al., 2002], and anticancer properties [Ruby et al., 1995; Jiang et al., 1996; Pereira et al., 1996]. Curcumin protected against trinitrobenzene sulfonic acid-induced colitis in mice [Sugimoto et al., 2002], acute pancreatitis in rats [Gukovsky et al., 2003], bleomycin-induced pulmonary fibrosis in mice [Punithavathi et al., 2000], and carbon tetrachloride-induced liver fibrosis [Kang et al., 2002]. The anti-cancer properties of curcumin include induction of cell cycle arrest and apoptosis [Jiang et al., 1996], and inhibition of tumor formation and growth in vivo [Pereira et al., 1996]. The molecular mechanisms responsible for curcumin's action remain largely unknown, but include effects on signal transduction pathways. Previous studies have shown that curcumin inhibited the activation of nuclear factor-κB (NF-κB) [Singh and Aggarwal, 1995], activator protein-1 (AP-1) [Pendurthi et al., 1997], JNK [Chen and Tan, 1998], and extracellular signal-regulated kinase (ERK) [Chen and Tan, 1998]. Because the activation of these signal transduction pathways plays an important role in PSCs, it would be interesting to see whether curcumin affects the activation and cell functions of PSCs. But no previous studies have addressed this issue. We here report that curcumin inhibited key cell functions of PSCs and spontaneous activation of freshly isolated PSCs in culture.

MATERIALS AND METHODS

Materials

Curcumin was dissolved in dimethysulfoxide and stocked at 25 mM. 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo (Kumamoto, Japan). Rat recombinant platelet-derived growth factor (PDGF)-BB was purchased from R&D Systems (Minneapolis, MN). Collagenase P, recombinant interleukin (IL)-1β and tumor necrosis factor (TNF)-α were obtained from Roche Diagnostics (Mannheim, Germany). Double-stranded consensus oligonucleotides probes for NF-κB and AP-1 were purchased from Promega (Madison WI). Rabbit antibodies against ERK (phosphorylated and total), JNK (phosphorylated), p38 MAP kinase (phosphorylated), and inhibitor of NF-κB (IκB)-α were purchased from Cell Technologies, Inc. (Beverly, MA). Rabbit antibody against cyclin D1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-type I collagen antibody was purchased from Southern Biotechnology (Birmingham, AL). Rabbit antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Trevigen (Gaithersburg, MD). Bay11-7082, SP600125, U0126, and SB203580 were purchased from Calbiochem (La Jolla, CA). 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) weighting 200–250 g using the Nycodenz solution (Nycomed Pharma, Oslo, Norway) after perfusion with 0.03% collagenase P as previously described [Masamune et al., 2003a]. The cells were resuspended in Ham's F-12 containing 10% heat-inactivated fetal bovine serum (FBS; 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 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, curcumin or inhibitors of signal transduction pathways was added at 1 h before the stimulation with PDGF-BB, IL-1β, TNF-α, or 5% FBS.

Cell Viability Assay

Cell viability was assessed by the MTT assay and by the release of lactose dehydrogenase (LDH) into the culture supernatant using a colorimetric kit (Takara Bio, Otsu, Japan) according to the manufacturer's instruction. PSCs were treated with curcumin at the indicated concentrations in serum-free medium for 48 h. For the MTT assay, culture supernatants were aspirated, MTT solution was added to the cells at a final concentration of

500 μg/ml, and the incubation continued at 37°C for 4 h. The medium was then aspirated and the formazan product was solubilized with dimethylsulfoxide. Cell viability was determined by OD 570–OD 690. For the LDH assay, LDH activity in the culture supernatant was determined by OD 492–OD 690. The background values of the corresponding concentration of curcumin without cells were deduced from the sample values.

Cell Proliferation Assay

Serum-starved PSCs (~80% density) were left untreated or treated with PDGF-BB (at 25 ng/ml) in the presence of curcumin at the indicated concentrations. 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 for 90 min at room temperature. Then the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine was added, and BrdU incorporation was determined by OD 370–OD 492.

Cell Cycle Analysis

The cell cycle of PSCs was analyzed by flow cytometry as previously described [Masamune et al., 2002a]. Briefly, serum-starved PSCs (~60%–70% density) were left untreated or treated with PDGF-BB (at 25 ng/ml) in the absence or presence of curcumin (at 25 μM). After 24 h, cells were harvested, and suspended in phosphate-buffered saline solution containing 40 μg/ml propidium iodide, 0.02% Triton X-100, and 50 μg/ml ribonuclease A. Samples were incubated in the dark at room temperature for 30 min and stored at 4°C until the analysis. Cell fluorescence was measured by FACSCaliber flow cytometer (Becton Dickinson Co. Ltd., Tokyo, Japan), and analyzed using ModFit LT software (Verity Software House, Topsham, ME) to determine the distribution of cells in the various phases of the cell cycle. In addition, the percentage of apoptotic cells after serum starvation for 48 h was determined by evaluating hypodiploid nuclei.

Real-Time PCR

The levels of α_1(I)procollagen, α_1(III)procollagen, and α-SMA mRNAs were examined by real-time PCR as previously described [Masamune et al., 2005c]. PSCs were incubated in the absence or presence of curcumin at the indicated concentrations in serum-free medium for 24 h. Total RNA (~100 ng) was prepared using RNeasy total RNA preparation kit (Qiagen, Chestsworth, CA) and reverse-transcribed in a volume of 20 μl using the Reverse Transcription System (Promega). Two microliters of the resultant cDNA was subjected to real-time PCR with the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics) using the LightCycler instrument (Roche Diagnostics). Specific primer sets were as follows (listed 5′-3′; forward and reverse, respectively). α_1(I)-procollagen: TCACCTACAGCACGCTTG and GGTCTGTTTCCAGGGTTG; α_1(III)procollagen: ATATCAAACACGCAAGGC and GATTAAAGCAAGAGGAACAC; α-SMA: TGTGCTGGACTCTGGAGATG and GATCACCTGCCCATCAGG; GAPDH: ACATCATCCCTGCATCCACT and GGGAGTTGCTGTTGAAGTCA. Reactions were performed in a volume of 20 μl containing 0.5 μM primers and 2.5 mM $MgCl_2$. The PCR protocol consisted of an initial denaturation step at 95°C for 10 min and 50 cycles of denaturation (95°C for 15 s), annealing (60°C for 10 s), and extension (72°C for 10 s). For each step, the temperature transition rate was 20°C/s. Melting curve analyses were performed to confirm the PCR product identity and to differentiate specific amplification from non-specific products by denaturation (95°C for 10 s), annealing (65°C for 10 s), and a slow heating to 95°C (temperature transition rate, 0.1°C/s) combined with a continuous fluorescence measurement at 0.2°C increments. After completion of PCR, the copy number of the target molecules was calculated by plotting fluorescence versus cycle number. As a standard curve, we used the linear regression line based on the data of standard crossing points (threshold cycle) versus the logarithm of standard sample concentrations. The expression levels of target genes were evaluated by the ratio of the target mRNA to that of GAPDH.

Quantification of Type I Collagen in the Culture Supernatant

The type I collagen concentration in the culture supernatant was determined after 48 h

by an enzyme-linked immunosorbent assay (ELISA) as previously reported [Moshage et al., 1990] with slight modifications. Briefly, ELISA immunoassay plates (Becton Dickinson, Franklin Lakes, NJ) were coated overnight at 4°C with diluted samples. After coating, the plates were incubated with 5% drymilk to block uncoated sites. Goat anti-type I collagen antibody was added and incubated for 1 h at room temperature. After washes, anti-goat IgG antibody conjugated with horseradish peroxidase (Southern Biotechnology) was added and incubated for 1 h. Finally, soluble substrate 1,2-phenylenediamine and 0.03% hydrogen peroxide were added, and the type I collagen concentration was determined by OD 492−OD 690. The type I collagen levels in each sample were normalized for total cellular DNA content.

Monocyte Chemoattractant Protein (MCP)-1 Assay

After 24-h incubation, cell culture supernatants were harvested and stored at −80°C until the measurement. MCP-1 level in the culture supernatants was measured by ELISA (Pierce Chemical, Rockford, IL) according to the manufacturer's instruction.

Electrophoretic Mobility Shift Assay

Following 1-h incubation with IL-1β or TNF-α, $\sim 5 \times 10^6$ cells were harvested and nuclear extracts were prepared, and electrophoretic mobility shift assay was performed as previously described [Masamune et al., 1996]. Double-stranded oligonucleotides probes for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were endlabeled with [γ-^{32}P]ATP. Nuclear extracts (~5 μg) were incubated with the labeled oligonucleotide probe for 20 min 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 min prior to the addition of the radiolabeled probe in the competition assays.

Luciferase Assay

Luciferase assay was performed as previously described [Masamune et al., 2002b] using the luciferase expression vector containing two consensus NF-κB binding sites (GGGACTTTCC), which was kindly provided by Dr. Naofumi Mukaida (Kanazawa University, Japan). $\sim 1 \times 10^6$ PSCs were transfected with 2 μg of the luciferase expression vector, along with 40 ng of pRL-TK vector (Promega) as an internal control, using the FuGENE6 reagent (Roche Diagnostics). After 24 h, the transfected cells were treated within IL-1β (at 2 ng/ml) in the presence of curcumin (at 0, 10, or 25 μM) or Bay11-7082 (at 10 μM) for additional 24 h. At the end of the incubation, cell lysates were prepared using Pica Gene kit (Toyo Ink Co., Tokyo, Japan), and the light intensities were measured using a model Lumat LB9507 Luminescence Reader (EG&G Berthold, Bad Wildbad, Germany).

Western Blotting

Activation of MAP kinases was examined by Western blot analysis using anti-phosphospecific MAP kinase antibodies as previously described [Masamune et al., 2002d]. These antibodies recognize only phosphorylated form of MAP kinases, thus allowing the assessment of activation of these kinases. Briefly, cells were lysed in sodium dodecyl sulfate buffer, and total cell lysates (~100 μg) were fractionated on a 10% 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 antibodies against phosphorylated MAP kinases. After incubation with peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h, proteins were visualized using an ECL kit (Amersham Biosciences UK, Ltd.). Levels of total ERK, cyclin D1, and IκB-α were determined in a similar manner.

Effect of Curcumin on Spontaneous Activation of PSCs in Culture

Freshly isolated PSCs were left untreated or treated with 5% FBS in the absence or presence of curcumin at 10 μM. After 7-day incubation, morphological changes characteristic of PSC activation were assessed after staining with glial fibrillary acidic protein (GFAP) as previously described [Masamune et al., 2005b] 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 immunoglobulin antibody for 45 min at room temperature, 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 (~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 ± SD. 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

Curcumin Was Not Cytotoxic up to 25 μM

We first examined whether curcumin was cytotoxic to PSCs. PSCs were treated with increasing concentrations of curcumin for 48 h, and the cell viability was assessed by the MTT assay and by the release of LDH into the culture supernatant. Curcumin up to 25 μM did not affect the cell viability, but above 25 μM, curcumin was cytotoxic (Fig. 1). The results were also confirmed by trypan blue dye exclusion test (data not shown). We also examined whether serum-starvation itself induced apoptosis by evaluating hypodiploid nuclei using flow cytometry after staining with propidium iodide. Less than 0.5% of cells were hypodiploid after serum-starvation for 48 h (data not shown), suggesting that serum-starvation itself did not induce apoptosis in our experimental system. Based on these results, we used curcumin up to 25 μM in the subsequent experiments.

Curcumin Inhibited PDGF-Induced Proliferation

In agreement with the previous studies showing that PDGF-BB is a potent mitogen of PSCs in vitro [Apte et al., 1999; Masamune et al., 2003a], PDGF-BB increased proliferation of PSCs (Fig. 2A). PDGF-induced proliferation

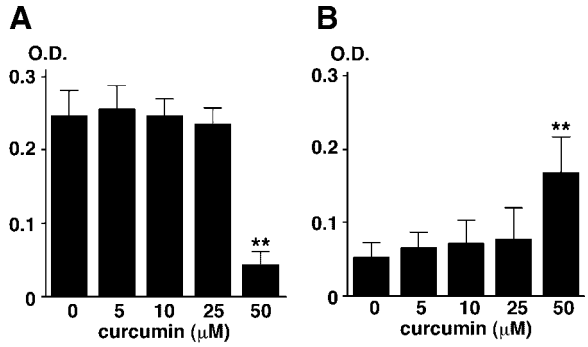

Fig. 1. Curcumin was not cytotoxic up to 25 μM. PSCs were treated with curcumin at the indicated concentrations in serum-free medium for 48 h. Cytotoxicity of curcumin was determined by the MTT assay (**A**) and by the release of LDH into the culture supernatant (**B**). OD 570–OD 690 ("OD," optical density) for the MTT assay and OD 492–OD 690 for the LDH assay of the samples are shown. Data are shown as mean ± SD (n = 6). $^{**}P < 0.01$ versus curcumin at 0 μM.

was inhibited by curcumin in a dose-dependent manner. The inhibitory effect was significant at as low as 5 μM, and the proliferative effect of PDGF was abolished by curcumin at 25 μM, whereas the vehicle (dimethylsulfoxide) did not.

We analyzed the cell cycle in PSCs. Exposure to PDGF was associated with a marked decrease in the percentage of cells in the G_0/G_1 phase together with an increase in the number of cells in the S phase (Fig. 2B). The addition of curcumin before PDGF reduced the number of cells in the S phase, and the percentage of cells in the G_0/G_1 phase was similar to the percentage observed in untreated cells. Thus, curcumin inhibited PDGF-induced progression of the cell cycle beyond the G_1 phase. In addition, we examined the effects of curcumin treatment on cyclin D1 expression. The level of cyclin D1 in serum-starved cells was low, and PDGF-BB induced the expression (Fig. 2C). Curcumin reduced the PDGF-induced cyclin D1 expression.

It has been shown that activation of ERK plays a key role in PDGF-induced PSC proliferation [Jaster et al., 2002; Masamune et al., 2003a]. Curcumin inhibited PDGF-induced phosphorylation of ERK (Fig. 2D).

Curcumin Decreased the Expression of α-SMA and Collagen

It has been shown that culture-activated PSCs express α-SMA and produce extracellular matrix such as type I and type III collagens [Apte et al., 1998; Bachem et al., 1998]. α-SMA expression has been accepted as a marker of

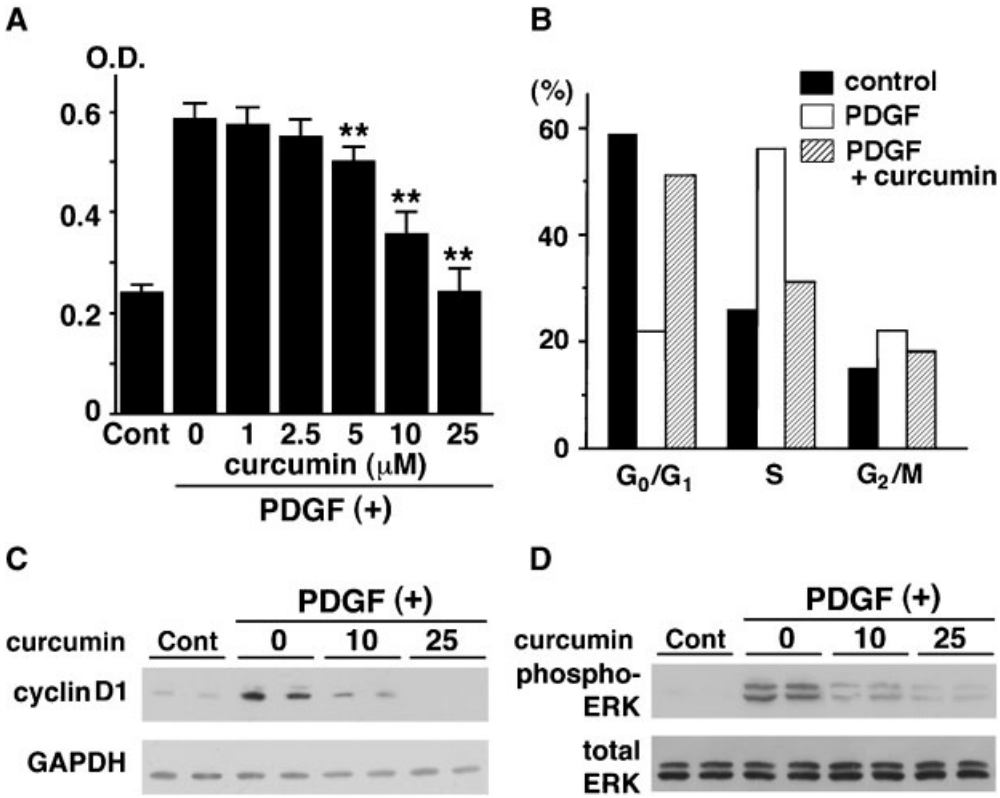

Fig. 2. Curcumin inhibited PDGF-induced proliferation of PSCs. **A**: Serum-starved PSCs were left untreated (''Cont'') or treated with PDGF-BB (at 25 ng/ml) in the presence or absence of curcumin at the indicated concentrations. After 24-h incubation, DNA synthesis was assessed by BrdU incorporation ELISA, and the OD 370–OD 492 (''OD'', optical density) of the sample is shown. Data are shown as mean ± SD (n = 6). $^{**}P < 0.01$ versus PDGF-BB only. **B**: PSCs were left untreated or treated with PDGF-BB (at 25 ng/ml) in the absence or presence of curcumin (at 25 μM). After 24-h incubation, cells were harvested, and cell cycle analysis was performed by flow cytometry after staining with propidium iodide. Data show the percentage of cells in each phase of the cell cycle in a representative experiment. **C**, **D**: PSCs were left untreated (''Cont,'' control) or treated with PDGF-BB (at 25 ng/ml) in the absence or presence of curcumin at the indicated concentrations. After 24-h (C) or 5-min (D) incubation with PDGF-BB, total cell lysates (~100 μg) were prepared, and the levels of cyclin D1, GAPDH, phosphorylated ERK, and total ERK were determined by Western blotting.

PSC activation [Apte et al., 1998] and in situ hybridization techniques showed that α-SMA-positive cells were the principal source of collagen in fibrotic pancreas [Haber et al., 1999]. Cuircumin decreased the levels of α-SMA, α_1(I)procollagen, and α_1(III)procollagen mRNAs in a dose-dependent manner (Fig. 3A,B). In addition, curcumin decreased type I collagen production in a dose-dependent manner (Fig. 3C).

Curcumin Inhibited Activation of MAP Kinases and AP-1, But Not NF-κB

Previous studies showing that curcumin inhibited the activation of NF-κB, AP-1, and MAP kinases [Singh and Aggarwal, 1995; Pendurthi et al., 1997; Chen and Tan, 1998] prompted us to examine the effect of curcumin on the activation of these signaling pathways. We examined the effects of curcumin on the activation of AP-1 and NF-κB by electrophoretic mobility shift assay. IL-1β induced activation of AP-1 and NF-κB (Fig. 4A,B). Curcumin inhibited IL-1β-induced activation of AP-1, but not of NF-κB. Curcumin did not affect IL-1-induced degradation of IκB-α (Fig. 4C). To further confirm that curcumin did not inhibit the activation of NF-κB, we examined the effect of curcumin on the NF-κB-dependent transcriptional activity. Curcumin did not decrease IL-1-induced NF-κB-dependent transcriptional activity whereas Bay11-7082, an inhibitor of NF-κB [Pierce et al., 1997], decreased the activity (Fig. 4D).

IL-1β induced activation of ERK, JNK, and p38 MAP kinases, and curcumin inhibited the activation of all these MAP kinases in a dose-dependent manner (Fig. 4E). Curcumin also

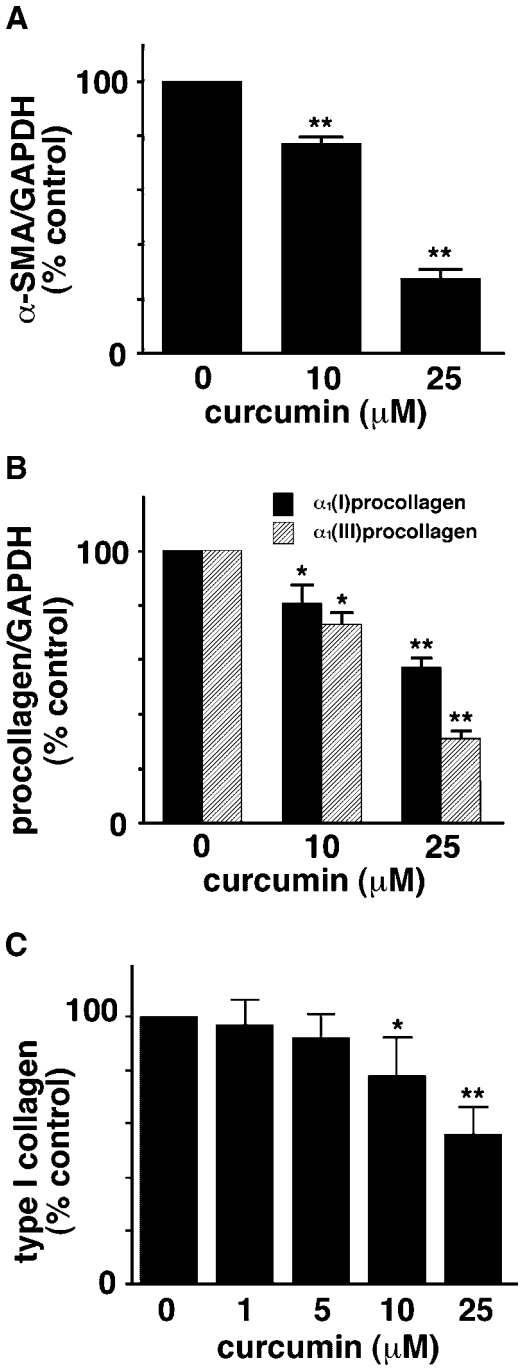

Fig. 3. . Curcumin decreased expression of α-SMA and collagen. PSCs were incubated with curcumin at the indicated concentrations in serum-free medium. **A**, **B**: After 24-h incubation, total RNA was prepared, reverse-transcribed, and the resultant cDNA was subjected to real-time PCR with the LightCycler Instrument. The levels of α-SMA, α_1(I)procollagen, and α_1(III)procollagen mRNAs were evaluated by the ratio of the target mRNA to that of GAPDH. **C**: After 48-h incubation, the level of type I collagen in the culture supernatant was determined by ELISA. Data are shown as mean ± SD (% of the control, n = 6). $^{**}P < 0.01$ versus curcumin at 0 μM (control).

inhibited TNF-α-induced activation of AP-1 and MAP kinases, but not of NF-κB (data not shown).

Curcumin Inhibited Ethanol-Induced Activation of MAP Kinases

We have previously shown that ethanol activated ERK, JNK, and p38 MAP kinase through the generation of oxidative stress [Masamune et al., 2002b]. Because curcumin is known to be a potent antioxidant [Rajakumar and Rao, 1995], we examined whether curcumin affected ethanol-induced activation of MAP kinases. Curcumin inhibited ethanol-induced activation of MAP kinases (Fig. 5), suggesting that curcumin has an anti-oxidant action in PSCs.

Curcumin Inhibited MCP-1 Production

Activated PSCs may acquire the ability to modulate the recruitment and activation of inflammatory cells at least in part through the expression of MCP-1 [Masamune et al., 2002a]. As previously reported [Masamune et al., 2002a], IL-1β and TNF-α induced MCP-1 production in PSCs, and curcumin decreased the inducible MCP-1 production in a dose-dependent manner (Fig. 6A).

We have previously shown that activation of NF-κB plays a key role in MCP-1 expression and that activation of AP-1, ERK, JNK, and p38 MAP kinase is all required for optimal MCP-1 expression in PSCs [Masamune et al., 2003c, 2004]. To clarify the role of MAP kinases and NF-κB in curcumin's action on MCP-1 production, we employed specific inhibitors of MAP kinases and NF-κB. Inhibitors of MAP kinases did not further enhance the inhibitory effect of curcumin on MCP-1 production whereas Bay 11-7082 did (Fig. 6B). Thus, curcumin inhibited MCP-1 production through its inhibition of MAP kinases but not of NF-κB.

Curcumin Blocked the Transformation of Freshly Isolated PSCs

We finally examined whether curcumin blocked the transformation of PSCs to myofibroblast-like phenotype in culture. Freshly isolated PSCs were incubated with curcumin in 5% serum-containing medium for 7 days. Morphological changes characteristic of PSC activation were assessed after staining with GFAP. PSCs cultured in serum-containing medium without curcumin showed transforma-

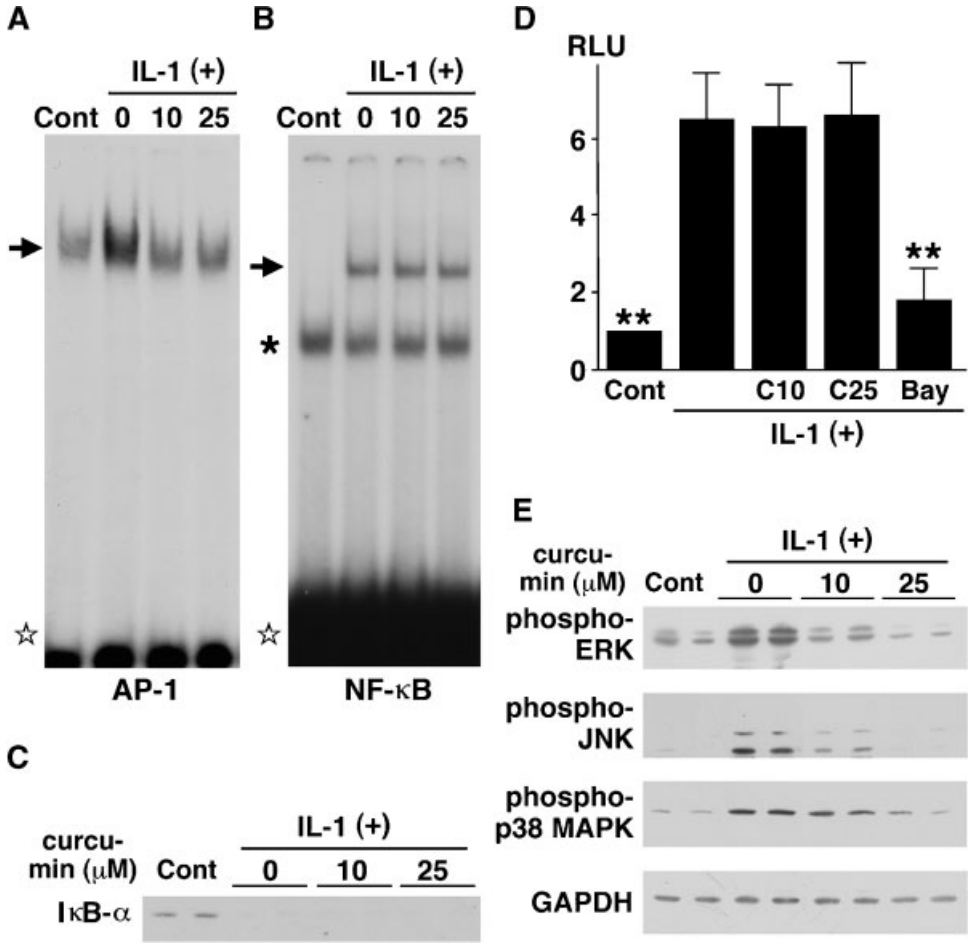

Fig. 4. Curcumin inhibited the activation of AP-1 and MAP kinases, but not of NF-κB. **A**, **B**: Serum-starved PSCs were left untreated ''Cont,'' or treated with IL-1 (at 2 ng/ml) in the presence of curcumin at the indicated concentrations (μM)) for 1 h. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay using AP-1 (**panel A**) or NF-κB (**panel B**) oligonucleotide probes. Arrows denote specific complexes competitive with cold double-stranded oligonucleotide probes. *non-specific band. ☆: free probe. **C**: PSCs were left untreated ''Cont,'' or treated with IL-1β (at 2 ng/ml) in the presence of curcumin at the indicated concentrations (μM) for 15 min. Total cell lysates were prepared, and the level of IκB-α was determined by Western blotting. **D**: PSCs were transfected with the 2 X NF-*K*B luciferase expression vector along with pRL-TK vector as an internal control. After 24 h, the transfected cells were left untreated ''Cont,'' or treated with IL-1β (at 2 ng/ml) in the presence of curcumin (''C'' at 0, 10, or 25 μM) or Bay11-7082 (''Bay'' at 10 μM). After another 24-h incubation, intracellular luciferase activity was determined. The data represent mean values ± SD (n = 6), calculated as fold induction compared with the activity observed in control (serun-free medium only, ''Cont''). $^{**}P < 0.01$ versus IL-1β. RLU: relative light units. **E**: PSCs were left untreated ''Cont,'' or treated with IL-1β (at 2 ng/ml) in the presence of curcumin at the indicated concentrations (μM) for 10 min. Total cell lysates were prepared, and the levels of phosphorylated MAP kinases and GAPDH were determined by Western blotting.

tion into cells with a myofibroblast-like phenotype whereas PSCs cultured in serum-free medium remained quiesecent (Fig. 7A,B). PSCs cultured in the presence of curcumin (at 10 μM) were still small and circular, with slender dendritic processes (Fig. 7C). To rule out the possibility that the effects of curcumin might have been due to cytotoxicity, curcumin was withdrawn from PSCs that had been treated with it for 7 days. Within 72 h after withdrawal of curcumin, PSCs had acquired the activated phenotype (Fig. 7D). In addition, curcumin decreased serum-induced expression of α-SMA (Fig. 7E), further supporting that curcumin blocked the transformation of freshly isolated cells to activated, myofibroblast-like phenotype.

DISCUSSION

There is accumulating evidence that activated PSCs play a pivotal role in the pathogenesis of pancreatic fibrosis and inflammation [Apte et al., 1998; Bachem et al., 1998; Haber

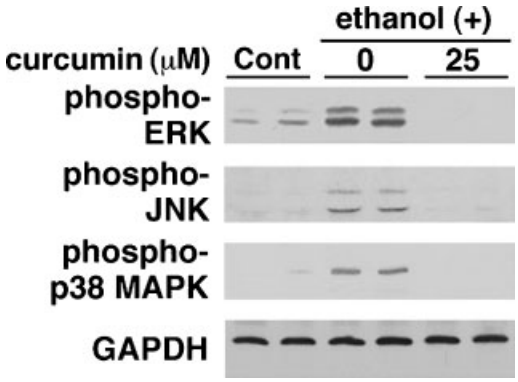

Fig. 5. Curcumin inhibited ethanol-induced activation of MAP kinases. PSCs were left untreated "Cont," or treated with ethanol (at 50 mM) in the absence or presence of curcumin (at 25 μM) for 10 min. Total cell lysates were prepared, and the levels of phosphorylated and total MAP kinases were determined by Western blotting.

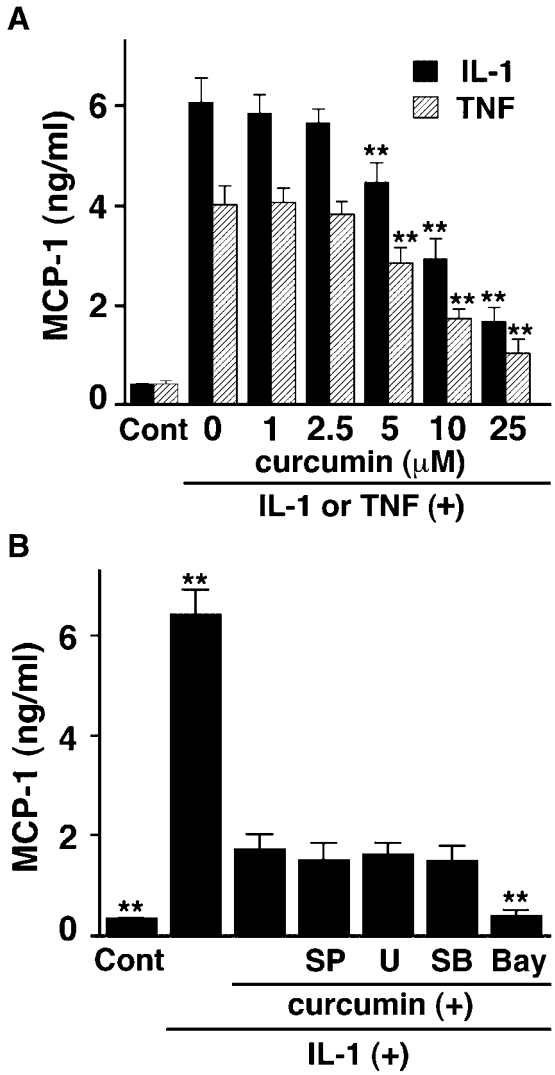

Fig. 6. Curcumin decreased MCP-1 expression. **A**: Serum-starved PSCs were left untreated ("Cont," control), or treated with IL-1β (at 2 ng/ml) or TNF-α (at 10 ng/ml) in the absence or presence of curcumin at the indicated concentrations. **B**: PSCs were left untreated ("Cont," control), or treated with IL-1β (at 2 ng/ml) in the absence or presence of curcumin (at 25 μM) and inhibitors of signal transduction pathways ("SP," SP600125 at 10 μM; "U," U0126 at 5 μM; "SB," SB203580 at 10 μM; "Bay," Bay11-7082 at 10 μM). After 24 h, MCP-1 levels in the culture supernatant were determined by ELISA. Data shown are expressed as means ± SD (n = 6). $^{**}P < 0.01$ versus respective positive control (IL-1β or TNF-α treatment only) in **panel A**, or versus IL-1β and curcumin treatment in **panel B**.

et al., 1999; Masamune et al., 2002a]. Control of the activation of PSCs and their cell functions are potential targets for the development of new treatments for pancreatic fibrosis and inflammation. The present study demonstrated that curcumin, the yellow pigment in curry, inhibited several key parameters of PSC activation including PDGF-induced proliferation, α-SMA gene expression, MCP-1 production, and collagen expression. In addition, curcumin inhibited spontaneous transformation of freshly isolated cells to an activated, myofibroblast-like phenotype. These inhibitory effects were not due to the non-specific cytotoxicity, because the concentrations of curcumin used in this study did not cause cell death. In this study, curcumin was cytotoxic at concentrations above 25 μM. This is relatively low compared to hepatic stellate cells where curcumin was not cytotoxic up to 100 μM [Xu et al., 2003]. This may reflect differences in experimental conditions, including differences in cell types. Indeed, Xu et al. [2003] treated hepatic stellate cells with curcumin in the presence of 10% FBS whereas we treated PSCs with curcumin in serum-free medium.

Stellate cell proliferation and the expansion of their pool are fundamental features of pancreatic fibrosis [Haber et al., 1999]. PDGF-BB potently induces proliferation of PSCs mainly through the activation of ERK pathway [Jaster et al., 2002; Masamune et al., 2003a]. Indeed, inhibition of proliferation by curcumin was correlated with the inhibition of ERK activation. We here showed that PDGF-BB induced the expression of cyclin D1. The effect of PDGF-BB on this cell cycle-related molecule was blocked by curcumin treatment, resulting in the inhibition of cell cycle progression beyond the G_1 phase. Inhibition of proliferation by curcumin correlated with the down-regulation of the cyclin D1 expression in several types of

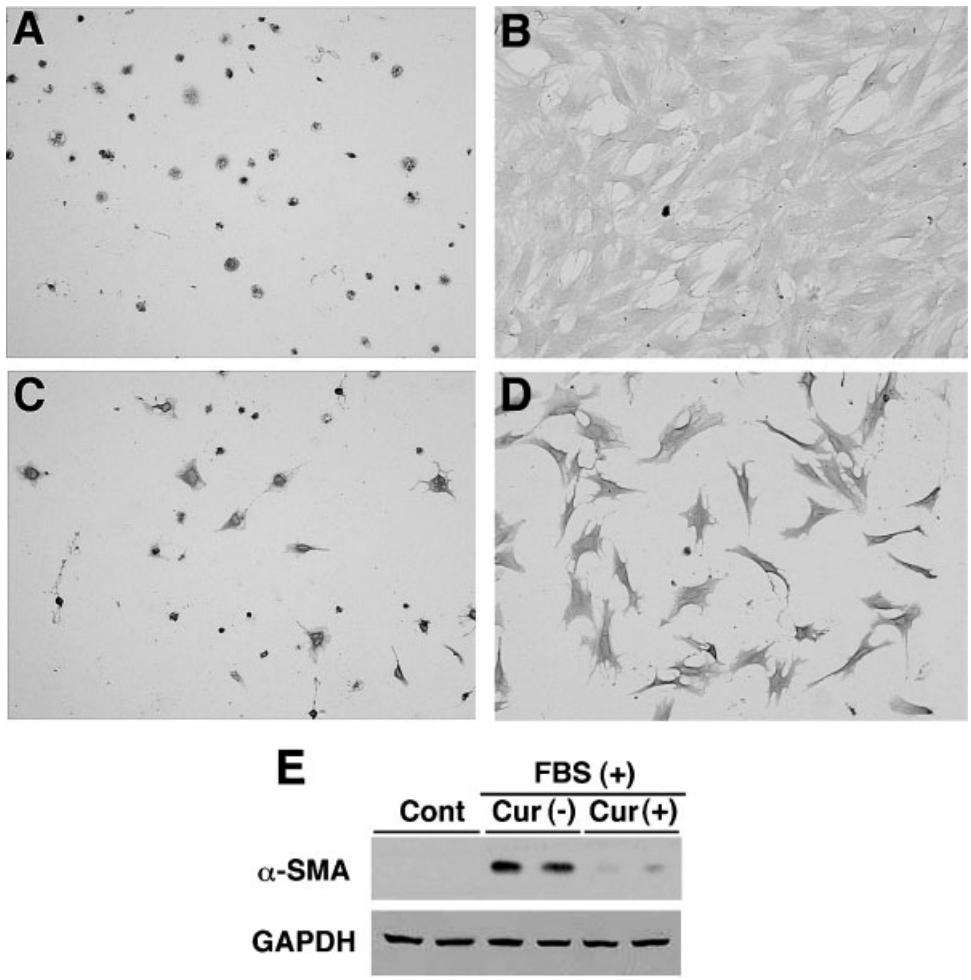

Fig. 7. Curcumin inhibited the transformation of freshly isolated PSCs. **A–D**: Freshly isolated PSCs were incubated with serum-free medium only (**panel A**), or 5% FBS in the absence (**panel B**) or presence (**panel C**) of curcumin (at 10 μM) for 7 days. Morphological changes characteristic of PSC activation were assessed after staining with GFAP. D: Curcumin (at 10 μM) was withdrawn from PSCs that had been treated with it in the presence of 5% FBS for 7 days, and the cells were treated with 5% FBS only. Three days after the withdrawal of curcumin, PSCs showed the typical phenotype of activated PSCs. Original magnification 10× objective. (E) Freshly isolated PSCs were incubated with serum-free medium only (''Cont,'' control), or 5% FBS in the presence (''+'') or absence (''−'') of curcumin (''Cur'' at 10 μM) for 7 days. Total cellular proteins (~25 μg) were prepared after 7-day-incubation, and the levels of α-SMA and GAPDH were determined by Western blotting.

cells [Mukhopadhyay et al., 2002; Xu et al., 2003]. The D-group cyclin proteins play critical roles in the progression of cells through the G_1 phase of the cell cycle [Baldin et al., 1993; Jiang et al., 1993]. It has been reported that overexpression of cyclin D1 in cultured cells shortened G_1 phase and caused more rapid entry into S phase [Jiang et al., 1993]. Conversely, microinjection of antisense cyclin D1 oligonucleotide or cyclin D1 antibody arrested the cells at G_1 phase [Baldin et al., 1993]. Although regulation of cell cycle progression in PSCs remains largely unknown, our results suggested for the first time that induction of cyclin D1 might be a prerequisite for PSC proliferation. We have recently observed that another antioxidant (-)-epigallocatechin-3-gallate, a major and active component in green tea extracts, also inhibited proliferation and altered the expression of cyclin D1 in PSCs [Masamune et al., 2005a]. Further experiments will elucidate the mechanisms by which antioxidants regulate the expression of cell cycle-related proteins.

Activated PSCs are the principal source of collagen, mainly type I, during pancreatic fibrosis. Inhibition of collagen expression by curcumin agrees with the previous studies

showing oxidative stresses such as acetaldehyde and 4-hydroxynonenal induced type I collagen gene expression in PSCs [Masamune et al., 2002b; Kikuta et al., 2004]. The precise mechanism of type I collagen gene expression is unclear in PSCs. Activation of p38 MAP kinase is critical for acetaldehyde- and 4-hydroxynoneal-induced type I collagen gene expression [Masamune et al., 2002b; Kikuta et al., 2004]. It has been reported that treatment of rat hepatic stellate cells with a 5-lipoxygenase-specific inhibitor reduced α_1(I) procollagen mRNA transcript abundance, suggesting that leukotriene production might be involved in maintaining the high level of collagen production in activated stellate cells [Chen et al., 1996]. Suppression of the gene transcription was localized to a nuclear factor-1 binding domain in the proximal promoter and an AP-2 binding domain adjacent to it. An increase in AP-2 binding adjacent to the nuclear factor-1 site was probably the transmodulator responsible for the suppression of the nuclear factor-1-dependent gene expression [Chen et al., 1996]. It remains to be clarified whether curcumin inhibited collagen gene expression by a similar mechanism in PSCs. Very recently, the inhibitory effects of curcumin on multiple sites of the transforming growth factor (TGF)-β signaling cascade have been reported in rat mesangial cells [Gaedeke et al., 2004]. Curcumin blocked TGF-β′s profibrotic actions through down-regulation of TGF-β receptor type II expression and inhibition of c-jun activity [Gaedeke et al., 2004]. Because TGF-β plays a key role in the development of pancreatic fibrosis [Nagashio et al., 2004], it is interesting to see whether curcumin affects the TGF-β signaling cascade in PSCs.

Upon activation, PSCs acquire proinflammatory phenotype; they modulate the recruitment and activation of inflammatory cells through the expression of MCP-1 and intercellular adhesion molecule-1. The previous study showed that MCP-1 expression by activated PSCs was increased in fibrous tissue sections from patients with chronic pancreatitis [Saurer et al., 2000]. MCP-1 may also act as a fibrosis-promoting chemokine; MCP-1 stimulated collagen gene expression via endogenous upregulation of transforming growth factor β in rat lung fibroblasts [Gharaee-Kermani et al., 1996]. Therefore, control of MCP-1 expression is an important therapeutic target for pancreatic fibrosis as well as inflammation. In this study, curcumin inhibited IL-1β- and TNF-α-induced MCP-1 expression. We have previously shown that activation of NF-κB plays a key role in MCP-1 expression and that activation of ERK, JNK, and p38 MAP kinase is all required for optimal MCP-1 expression in PSCs [Masamune et al., 2002c, 2004]. Because curcumin inhibited IL-1β- and TNF-α-induced activation of MAP kinases, but not NF-κB, it is logical to assume that curcumin inhibited MCP-1 production through the inhibition of MAP kinases activation. Because curcumin inhibited ethanol-induced activation of MAP kinases, inhibition of MAP kinases by curcumin might be due to its anti-oxidant property. In contrast, despite of its antioxidant property, curcumin did not inhibit the activation of NF-κB in PSCs. This is in striking contrast to other types of cells where curcumin inhibited the activation of NF-κB [Singh and Aggarwal, 1995; Mukhopadhyay et al., 2002; Xu et al., 2003]. How curcumin downregulates NF-κB in other types of cells is not clear, but the suppression of IκB kinase activity, the kinase required for the phosphorylation and degradation of IκB-α, has been demonstrated [Jobin et al., 1999]. We here showed that curcumin did not inhibit degradation of IκB-α or NF-κB-dependent transcriptional activity, further supporting that curcumin did not inhibit the activation of NF-κB in PSCs. It has been recognized that redox-dependent activation of NF-κB is cell and stimulus specific as opposed to the concept that oxidative stress is a common mediator of diverse NF-κB activators [Li and Karin, 1999; Hoare et al., 1999]. In human aortic smooth muscle cells, hydrogen peroxide failed to activate NF-κB or induce degradation of IκB-α [Hoare et al., 1999]. IL-1β did not increase intracellular oxidative stress and IL-1β-induced NF-κB activation was not inhibited by an antioxidant N-acetylcysteine, excluding a role of oxidative stress in IL-1β-induced activation of NF-κB at least in human aortic smooth muscle cells [Hoare et al., 1999]. Li and Karin [1999] reported that when a redox-regulated effect on NF-κB is observed, it appears to occur downstream from the IκB kinase at the level of ubiquitination and/or degradation of IκB.

α-SMA expression has been accepted as a marker of PSC activation [Apte et al., 1998]. Curcumin here decreased α-SMA gene expression in culture-activated PSCs. From the therapeutic point of view, this is an important

finding because deactivation of established myofibroblasts is desirable for the resolution of pancreatic fibrosis. Regulation of α-SMA expression in PSCs remains largely unknown, but activation of p38 MAP kinase [Masamune et al., 2003c] and Rho-Rho-kinase [Masamune et al., 2003b] might be involved. Of note, it has been shown that c-Myb modulates α-SMA gene transcription in association with enhanced oxidative stress by interacting with the proximal E box of the α-SMA gene in activated hepatic stellate cells [Lee et al., 1995]. Transfection of c-*myb* antisense RNA inhibited both expression of the endogenous α-SMA gene and myofibroblastic transformation of quiescent hepatic stellate cells, whereas transfection of c-*myb* stimulated α-SMA expression in quiescent hepatic stellate cells [Buck et al., 2000]. Thus, c-myb modulates not only the α-SMA gene expression but also the activation of quiescent hepatic stellate cells. The intracellular events that signal the transformation of PSCs from a quiescent phenotype to a myofibroblast-like cells are largely unknown, but peroxisome proliferator-activated receptor-γ (PPAR-γ) has been implicated as a repressor for maintaining PSCs in the quiescent state [Masamune et al., 2002a]. It has been shown that activation of PPAR-γ by curcumin inhibited proliferation and extracellular matrix synthesis in hepatic stellate cells [Xu et al., 2003; Zheng and Chen, 2004]. Blocking of PPAR-γ activation abrogated the effects of curcumin on the cell functions of activated hepatic stellate cells, suggesting an essential role of PPAR-γ activation on the inhibitory effects of curcumin. Along this line, we have previously shown that PPAR-γ ligands inhibited proliferation, α-SMA expression, collagen gene expression, and MCP-1 production in a similar manner to curcumin [Masamune et al., 2002a]. Further studies, which are beyond the scope of this study, are required to clarify the roles of c-myb and PPAR-γ in the inhibitory effects of curcumin on the activation and cell functions of PSCs.

In summary, we here showed that curcumin inhibited key cell functions and activation of PSCs. It has been increasingly recognized that PSCs are potential targets of anti-fibrogenic and anti-inflammatory strategies. The characteristics of curcumin, including antioxidant potential, inhibition of cell functions and activation of PSCs, and protection against acute pancreatitis, as well as the long history of dietary consumption of curry without adverse health effects, make it a potential candidate for the treatment of pancreatic fibrosis. Although curcumin itself is a highly lipophilic and has a poor oral bioavailability, the development of water-soluble curcumin analogs [Tonnesen et al., 2002] might aid in the further in vivo studies.

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