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Ellagic acid blocks activation of pancreatic stellate cells

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

Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

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Abstract

Activated pancreatic stellate cells (PSCs) play a pivotal role in the pathogenesis of pancreatic fibrosis and inflammation. Ellagic acid is a plant-derived polyphenol found in fruits and nuts, and has anti-oxidant and anti-inflammatory properties. But, little is known about the effects of ellagic acid on PSCs as well as on the activation of signal transduction pathways. We here evaluated the effects of ellagic acid 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. Ellagic acid inhibited platelet-derived growth factor (PDGF)-BB-induced proliferation and migration, interleukin (IL)-1β- and tumor necrosis factor (TNF)-α-induced monocyte chemoattractant protein-1 production, and expression of α-smooth muscle actin and collagen genes. Ellagic acid inhibited PDGF-BB-induced tyrosine phosphorylation of PDGF β-receptor and the downstream activation of extracellular signal-regulated kinase and Akt. Ellagic acid inhibited IL-1β- and TNF-α-induced activation of activator protein-1 and mitogen-activated protein kinases (extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase), but not of nuclear factor-κB. In addition, ellagic acid inhibited transformation of freshly isolated cells to an activated, myofibroblast-like phenotype. In conclusion, ellagic acid inhibited key cell functions and activation of PSCs.

Keywords: Pancreatitis; Pancreatic fibrosis; Pancreatic stellate cells; MAP kinase; Polyphenol; Platelet-derived growth factor

1. 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 α-smooth muscle actin (α-SMA), and produce type I 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 [1–4]. In addition, PSCs may participate in the pathogenesis of acute pancreatitis [3,5]. The activation of signaling pathways such as p38 MAP kinase [6], Rho-Rho kinase [7] and JNK [8] might play a role in the activation process. Obviously, control of the activation of PSCs and their cell functions are potential targets for the development of new treatments for pancreatic fibrosis and inflammation.

Ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3,–cde][1] benzopyran-5,10-dione) (Fig. 1) is a plant-derived polyphenol found in a wide variety of fruits and nuts such as raspberries, strawberries, walnuts, grapes and black currants [9]. Ellagic acid has a variety of biological activities including anti-oxidant [9], anti-inflammatory [10], anti-fibrosis [11] and anti-cancer [12,13] properties. Ellagic acid protected against ischemia/reperfusion-induced gastric injury [10] and carbon tetrachloride-induced liver fibrosis [11]. The anti-cancer properties of ellagic acid include induction of cell cycle arrest and apoptosis [12], and inhibition of tumor formation and growth in vivo [13]. The molecular mechanisms responsible for these effects remain largely unknown. But its

Abbreviations: AP-1, activator protein-1; BrdU, bromo-2′-deoxyuridine; EGCG, epigallocatechin-3-gallate; GFAP, glial fibrillary acidic protein; IκB, inhibitor of NF-κB; MCP-1, monocyte chemoattractant protein-1; PPAR, peroxisome proliferator-activated receptor; PSCs, pancreatic stellate cells; α-SMA, α-smooth muscle actin

* Corresponding author. Tel.: +81 22 717 7171; fax: +81 22 717 7177.
E-mail address: amasamune@int3.med.tohoku.ac.jp (A. Masamune).

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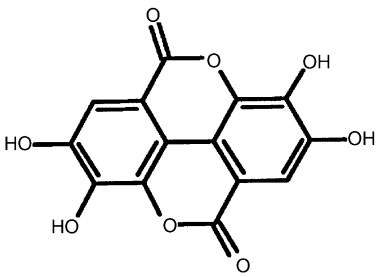

Fig. 1. Chemical structure of ellagic acid.

potent scavenging action on both superoxide anion and hydroxy anion might be involved [9,10]. Because oxidative stress plays a role in the development of pancreatic fibrosis and inflammation [14], it would be interesting to see whether ellagic acid affects the activation and cell functions of PSCs. But, little is known about the effects of ellagic acid on PSCs as well as on the activation of signal transduction pathways. We here report that ellagic acid inhibited key cell functions of PSCs and spontaneous activation of freshly isolated PSCs in culture.

2. Materials and methods

2.1. Materials

Ellagic acid (m.w. = 338.2) was dissolved in DMSO, and stocked at 10 mg/ml. Poly(dI-dC)-poly(dI-dC) and [γ-^{32}P]ATP were obtained from Amersham Biosciences, UK, Ltd. (Buckinghamshire, Little Chalfont, UK). Rat recombinant PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Recombinant human IL-1β and TNF-α were obtained from Roche Diagnostics (Mannheim, Germany). Double-stranded oligonucleotides probes for activator protein-1 (AP-1) or NF-κB were purchased from Promega (Madison, WI). Rabbit antibodies against ERK (phosphorylated and total), Akt (phosphorylated at Ser^{473} and total), JNK (phosphorylated and total), p38 MAP kinase (phosphorylated and total) and inhibitor of NF-κB (IκB)-α were purchased from Cell Technologies, Inc. (Beverly, MA). Rabbit antibodies against PDGF β-receptor (phosphorylated at Tyr^{857} and total) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibody against GAPDH was purchased from Trevigen (Gaithersburg, MD). Mouse anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless specifically described.

2.2. 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 as previously described using the Nycodenz solution (Nycomed Pharma, Oslo, Norway) after perfusion with 0.03% collagenase P [15]. The cells were resuspended in Ham's F-12 containing 10% heat-inactivated 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. Cells were treated with ellagic acid at the indicated concentrations for 1 h prior to the addition of PDGF-BB, IL-1β, TNF-α or 5% FBS.

2.3. Cell proliferation assay

Serum-starved PSCs (~80% density) were left untreated or treated with PDGF-BB (at 25 ng/ml) in the presence of ellagic acid at the indicated concentrations in a 96-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). 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 quantitated by differences in absorbance at wavelength 370 minus 492 nm.

2.4. Cell migration assay

Cell migration assay was performed as previously reported [15]. Serum-starved PSCs were trypsinized, and resuspended at a concentration of 3×10^5 cells/ml in serum-free medium containing 1% BSA and ellagic acid at 0, 10 or 25 μg/ml. For the assay, we used modified Boyden chambers with 8-μm-pore filters (Becton Dickinson) coated with rat-tail type I collagen. Cell suspension was added to the culture inserts (75,000 cells/insert), and the inserts were placed in 24-well culture plates containing the same medium and PDGF-BB (at 25 ng/ml). After 24-h incubation at 37 °C, 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), and counted at 200× magnification.

2.5. Western blotting

Activation of MAP kinases was examined by Western blot analysis using anti-phosphospecific MAP kinase antibodies as previously described [16]. These antibodies recognize only phosphorylated form of MAP kinases, thus allowing the assessment of activation of these kinases. Briefly, cells were lysed in SDS buffer (62.5 mM Tris–HCl at pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue) for 15 min on ice. Total cell lysates (~100 μg) were fractionated on a 10% SDS-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 immunoglobulin antibody for 1 h, proteins were visualized using an ECL kit (Amersham Biosciences, UK, Ltd.). The levels of total MAP kinases, Akt (phosphorylated and total), PDGF β-receptor (phosphorylated and total), phosphotyrosine, GAPDH and IκB-α were determined in a similar manner.

2.6. 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.

2.7. Electrophoretic mobility shift assay (EMSA)

Following 1-h incubation with IL-1β or TNF-α, ~5×10^6 cells were harvested and nuclear extracts were prepared, and electrophoretic mobility shift assay was performed as previously described [17]. Double-stranded consensus oligonucleotides probes for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) or 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 oligonucleotides was incubated with nuclear extracts for 10 min prior to the addition of the radiolabeled probe in the competition assays.

2.8. 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 [18]. PSCs were treated with ellagic acid at the indicated concentrations in serum-free medium for 24 h. Total RNA (~100 ng), prepared using RNeasy total RNA preparation kit (Qiagen, Chestsworth, CA), was 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.

2.9. Effect of ellagic acid on spontaneous activation of PSCs in culture

Freshly isolated PSCs were incubated with or without ellagic acid (at 10 μg/ml) in serum-free medium for 1 h, and then FBS was added at the final concentration of 5%. After 7-day incubation, morphological changes characteristic of PSC activation were assessed after staining with glial fibrillary acidic protein (GFAP) as previously described [19] 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, 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.

2.10. Statistical analysis

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

3. Results

3.1. Ellagic acid inhibited PDGF-induced proliferation and migration

As previously reported [15], PDGF-BB significantly increased proliferation of PSCs (Fig. 2A). PDGF-induced proliferation was inhibited by ellagic acid in a dose-dependent manner. The inhibitory effect was significant at as low as 1 μg/ml. Ellagic acid at 25 μg/ml (in 0.25% DMSO) virtually abolished the cell proliferation induced by PDGF-BB, whereas the vehicle (DMSO) did not. In these experiments, ellagic acid up to these concentrations did not affect cell viability during the incubation as assessed by a trypan blue exclusion test (data not shown). However, when PSCs were treated with the vehicle alone at concentrations more than 0.5%, cytotoxic effects were observed during the incubation. Therefore, we used ellagic acid up to 25 μg/ml in subsequent experiments.

Accumulation of PSCs in fibrotic pancreas also results from PSC migration, and PDGF-BB has been shown to be a potent inducer of PSC migration [15]. Ellagic acid inhibited PDGF-BB-induced PSC migration in a dose-dependent manner (Fig. 2B).

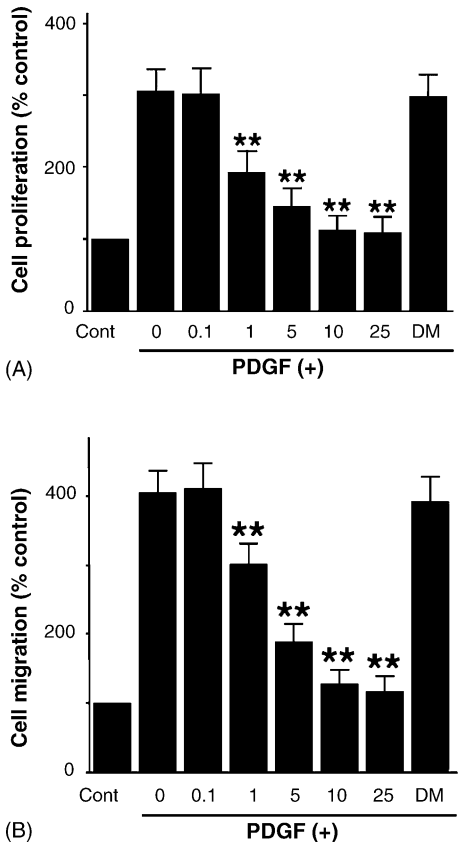

Fig. 2. Ellagic acid inhibited PDGF-induced proliferation and migration of PSCs. (A) Serum-starved PSCs were left untreated ("Cont") or treated with PDGF-BB (at 25 ng/ml) in the presence of ellagic acid at the indicated concentrations (μg/ml) or 0.25 % DMSO ("DM") in serum-free medium. After 24-h incubation, DNA synthesis was assessed by BrdU incorporation ELISA. Data are shown as mean ± S.D. (percent of the control, $n = 6$). $^{**}p < 0.01$ vs. PDGF-BB only. (B) Cell migration was assessed using modified Boyden chambers with 8-μm pore filters. Cell suspension was added to the culture inserts (75,000 cells/insert), and the inserts were placed in 24-well culture plates containing serum-free medium only ("Cont") or PDGF-BB (at 25 ng/ml) in the presence of ellagic acid at the indicated concentrations (μg/ml) or 0.25% DMSO ("DM"). After 24-h incubation with PDGF-BB, the cells migrated to the underside of the filter were stained, and counted. Data are shown as mean ± S.D. (percent of the control, $n = 6$). $^{**}p < 0.01$ vs. PDGF-BB only.

3.2. Ellagic acid inhibited PDGF-induced phosphorylation of PDGF β-receptor, ERK and Akt

Binding of PDGF to the receptors leads to dimerization of receptor subunits, phosphorylates itself on tyrosines (known as "autophosphorylation"), changes its cytoplasmic conformation, activates endogenous tyrosine phosphorylating activity and initiates intracellular signaling [20]. Ellagic acid inhibited tyrosine phosphorylation of PDGF β-receptor whereas the protein expression of PDGF β-receptor was not altered (Fig. 3). In PSCs, activation of c-Raf/MAPkinase kinase/ERK and phosphatidylinositol 3-kinase/Akt pathways plays key roles in PDGF-induced proliferation and migration, respectively [15]. We therefore examined whether ellagic acid affected the activation of ERK and Akt by Western blotting using anti-phosphospecific antibodies. Ellagic acid inhibited phosphorylation of ERK and Akt in response to PDGF-BB (Fig. 3). In addition, ellagic acid inhibited PDGF-induced tyrosine phosphorylation of several proteins. Thus, ellagic acid inhibited tyrosine phosphorylation of PDGF β-receptor and the activation of the downstream signaling pathways.

3.3. Ellagic acid decreased the expression of α-SMA and collagen genes

It has been shown that culture-activated PSCs express α-SMA and produce extracellular matrix such as type I and type III collagens [1,2]. α-SMA expression has been accepted as a marker of PSC activation [2], and in situ hybridization techniques showed that α-SMA-positive cells were the principal source of collagen in fibrotic pancreas [3]. Ellagic acid decreased the levels of α-SMA, α_1(I)procollagen and α_1(III)procollagen mRNAs in a dose-dependent manner as assessed by real-time PCR (Fig. 4).

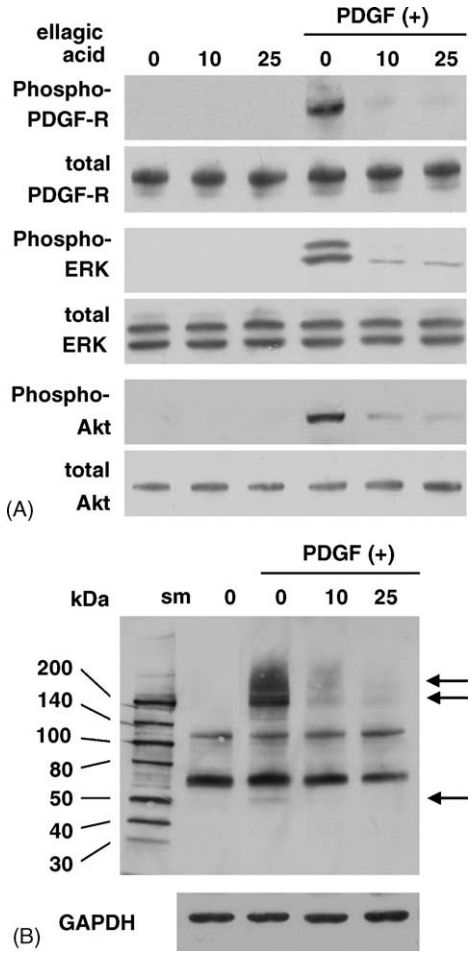

Fig. 3. Ellagic acid inhibited PDGF-induced phosphorylation of PDGF β-receptor, ERK and Akt. Serum-starved PSCs were treated with ellagic acid at the indicated concentrations (μg/ml) in the absence or presence of PDGF-BB (at 25 ng/ml) in serum-free medium. After 5-min incubation, total cell lysates (~100 μg) were prepared, and the levels of PDGF β-receptor (phosphorylated and total), ERK (phosphorylated and total), Akt (phosphorylated and total), phosphotyrosine (upper panel in panel B) and GAPDH were determined by Western blotting. Arrows denote PDGF-induced phosphotyrosine bands, which were inhibited by ellagic acid treatment.

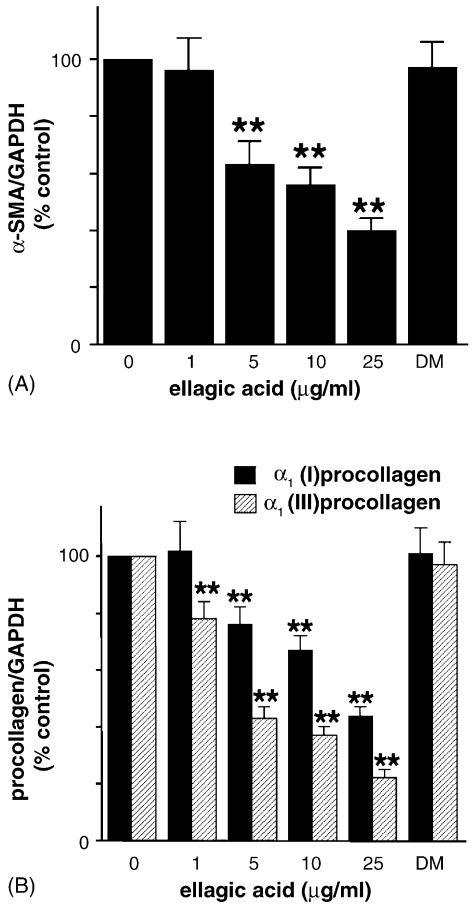

Fig. 4. Ellagic acid decreased expression of α-SMA and collagens. Serum-starved PSCs were treated with ellagic acid at the indicated concentrations or 0.25% DMSO (“DM”) in serum-free medium. After 24-h incubation, total RNA was prepared, reverse-transcribed and the resultant cDNA was subjected to real-time 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, and are shown as mean ± S.D. (percent of the control, $n = 6$). $^{**}p < 0.01$ vs. ellagic acid at 0 μg/ml.

3.4. Ellagic acid inhibited MCP-1 expression

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 [4]. As previously reported [4], IL-1β and TNF-α-induced MCP-1 production, and ellagic acid decreased the inducible MCP-1 production in a dose-dependent manner (Fig. 5). 0.25% DMSO, which is the vehicle of 25 μg/ml ellagic acid, did not affect IL-1β- and TNF-α-induced MCP-1 production (data not shown).

3.5. Ellagic acid inhibited activation of MAP kinases but not NF-κB

The expression of MCP-1 in PSCs is mediated at the transcription level through the coordinated activation of NF-κB and AP-1 [4]. We examined the effects of ellagic acid on the activation of these transcription factors by EMSA. IL-1β-induced activation of AP-1 and NF-κB (Fig. 6A and B). Ellagic acid inhibited IL-1β-induced activation of AP-1, but not NF-κB. Ellagic acid did not affect IL-1-induced degradation of IκB-α (Fig. 6C), further supporting that ellagic acid did not inhibit NF-κB activation.

Induction of the expression of AP-1 components c-Fos and c-Jun by a variety of stimuli such as growth factors and cytokines is mediated by the activation of three distinct MAP kinases: ERK, JNK and p38 MAP kinase [21]. IL-1β-induced activation of ERK, JNK and p38 MAP kinases, and ellagic acid inhibited the activation of all these MAP kinases in a dose-dependent manner (Fig. 7). Ellagic acid also inhibited TNF-α-induced activation of AP-1 and MAP kinases, but not of NF-κB (data not shown).

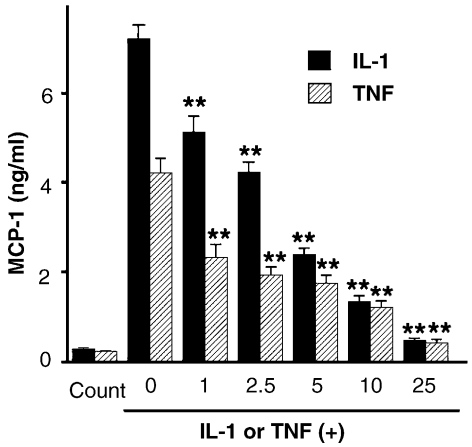

Fig. 5. Ellagic acid decreased MCP-1 expression. Serum-starved PSCs were treated with ellagic acid at the indicated concentrations (μg/ml) in the absence or presence of IL-1β (at 2 ng/ml) or TNF-α (at 10 ng/ml) in serum-free medium. After 24 h, culture supernatants were harvested, and the MCP-1 levels were determined by ELISA. Data shown are expressed as mean ± S.D. ($n = 6$). $^{**}p < 0.01$ vs. respective positive control (IL-1β or TNF-α treatment only).

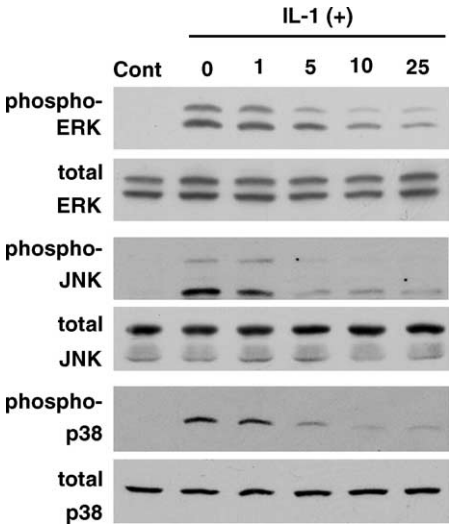

Fig. 7. Ellagic acid inhibited the activation of MAP kinases. Serum-starved PSCs were left untreated (“Cont”), or treated with IL-1β (at 2 ng/ml) in the presence of ellagic acid at the indicated concentrations (μg/ml) in serum-free medium for 15 min. Total cell lysates (~100 μg) were prepared, and the levels of phosphorylated and total MAP kinases were determined by Western blotting.

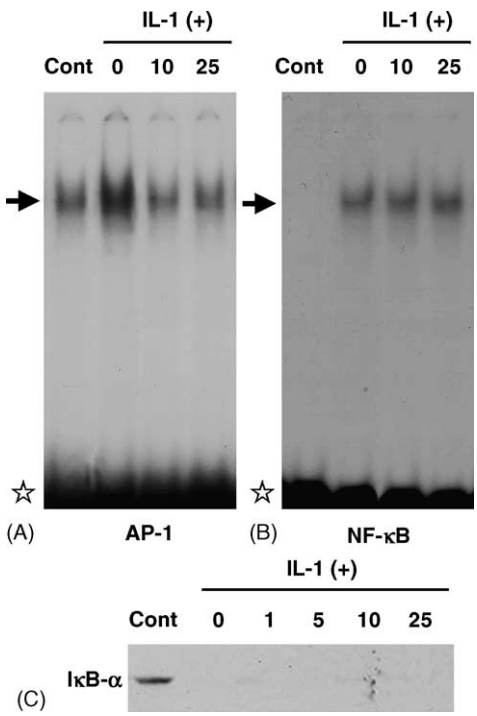

Fig. 6. Ellagic acid inhibited the activation of AP-1, but not NF-κB. (A and B) Serum-straved PSCs were left untreated (“Cont”), or treated with IL-1β (at 2 ng/ml) in the presence of ellagic acid at the indicated concentrations (μg/ml) for 1 h. Nuclear extracts were prepared and subjected to EMSA using AP-1 (panel A) or NF-κB (panel B) oligonucleotides probes. Arrows denote specific complexes competitive with cold double-stranded oligonucleotides probes; (☆) free probe. (C) PSCs were left untreated (“Cont”), or treated with IL-1β (at 2 ng/ml) in the presence of ellagic acid at the indicated concentrations (μg/ml) in serum-free medium for 15 min. Total cell lysates were prepared, and the level of IκB-α was determined by Western blotting.

3.6. Ellagic acid inhibited the transformation of freshly isolated PSCs

We finally examined whether ellagic acid blocked the transformation of PSCs from quiescent to myofibroblast-like phenotype in culture. Freshly isolated PSCs were incubated with ellagic acid in 5% serum-containing medium for 7 days. After 7 days, PSCs cultured in serum-containing medium without ellagic acid showed transformation into cells with a myofibroblast-like phenotype whereas PSCs cultured in serum-free medium remained quiesecent (Fig. 8A and B). PSCs cultured in the presence of ellagic acid (at 10 μg/ml) were small and circular, with lipid droplets present in many cells and with slender dendritic processes (Fig. 8C). To rule out the possibility that the effects of ellagic acid might have been due to cytotoxicity, ellagic acid was withdrawn from PSCs that had been treated with it for 7 days. Within 48 h after withdrawal of ellagic acid, PSCs had acquired the activated phenotype (Fig. 8D).

4. Discussion

Control of the activation of PSCs and their cell functions is a potential target for the development of new treatments for pancreatic fibrosis and inflammation. The present study demonstrated that a plant polyphenol ellagic acid inhibited several key functions of PSCs including PDGF-induced proliferation, migration, α-SMA gene expression, MCP-1 production and collagen gene expression. In addition, ellagic acid inhibited transformation of freshly isolated

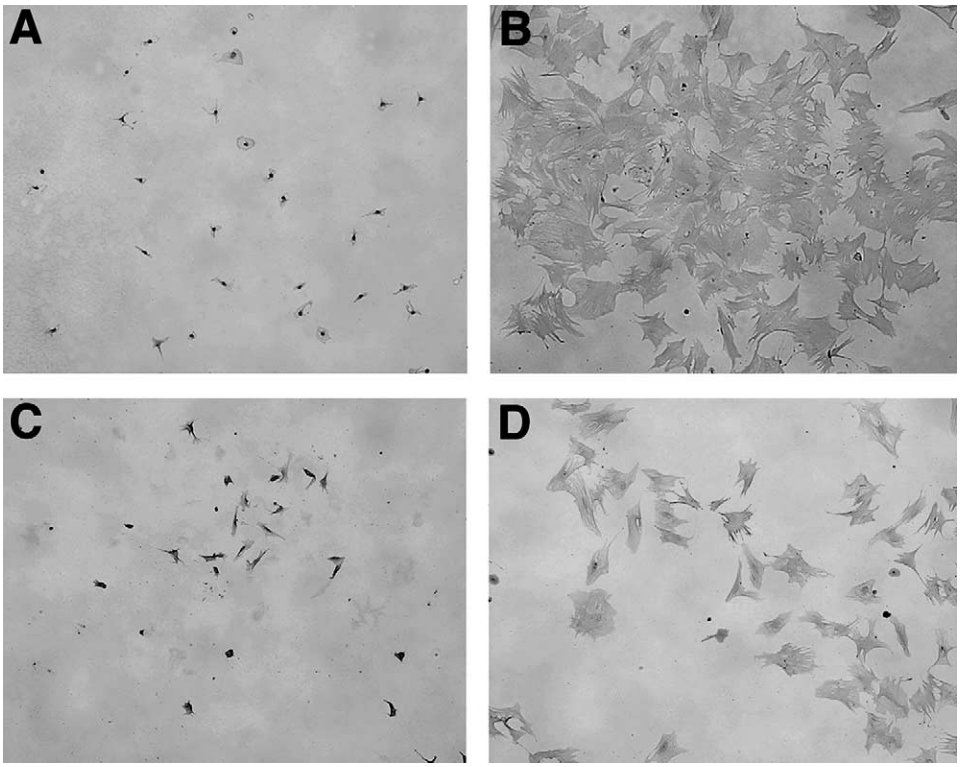

Fig. 8. Ellagic acid inhibited the transformation of freshly isolated PSCs. (A–D) Freshly isolated PSCs were incubated with serum-free medium only (panel A), or 5% FBS in the absence (panel B) or presence (panel C) of ellagic acid (at 10 μg/ml) for 7 days. Morphological changes characteristic of PSC activation were assessed after staining with GFAP. (D) Ellagic acid was withdrawn from PSCs that had been treated with it in the presence of 5% FBS for 7 days. Two days after the withdrawal of ellagic acid, PSCs showed the typical phenotype of activated PSCs. Original magnification: 10× objective.

cells to activated, myofibroblast-like phenotype. These inhibitory effects were not due to the potential cytotoxic effect of ellagic acid, because the concentrations of ellagic acid used in this study did not cause cell death and the effects of ellagic acid were reversible within 48 h after the removal of ellagic acid.

Stellate cell proliferation and the expansion of their pool are a fundamental feature of pancreatic fibrosis [3]. Accumulation of PSCs may result from not only proliferation but also migration. Ellagic acid here inhibited PDGF-BB-induced proliferation and migration of PSCs. These inhibitory effects appeared through the inhibition of tyrosine phosphorylation of PDGF β-receptor and the downstream activation of ERK and Akt. Tyrosine phosphorylation of PDGF β-receptor serves as a critical link between extracellualr PDGF stimulation and intracellular signaling [20]. Although this is the first study showing that ellagic acid inhibited tyrosine phosphorylation of PDGF β-receptor in PSCs, previous studies have shown that other types of polyphenolic compounds such as (-)-epigallocatechin-3-gallate (EGCG) inhibited tyrosine phosphorylation of PDGF β-receptor [22]. EGCG inhibited receptor-type protein tyrosine kinases (epidermal growth factor receptor, PDGF receptor and fibroblast growth factor receptor) whereas EGCG scarcely inhibited serine- and threonine-specific protein kinases such as protein kinases A and C [23]. On the other hand, decreased tyrosine phosphorylation of PDGF receptor by ellagic acid might be attributed to decreased binding of PDGF to the receptor. Weber et al. [24] showed that EGCG was incorporated into different cellular components including cell surface membranes, which leads to a trapping of PDGF to non-receptor binding sites and reduced PDGF-BB binding to the respective receptors. Ellagic acid, as in the case of EGCG, can form complexes with biologic macromolecule such as proteins and nucleic acids [25]. Thus, ellagic acid incorporated into plasma membrane or soluble ellagic acid might directly interact with PDGF-BB and PDGF β-receptor, thereby preventing specific receptor binding.

Activated PSCs are the principal source of collagen, mainly type I, during pancreatic fibrosis. Inhibition of collagen gene expression by ellagic acid agrees with the previous studies showing oxidative stresses such as acetaldehyde and 4-hydroxynonenal induced type I collagen gene expression in PSCs [26,27]. 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 [26,27]. In hepatic stellate cells, there are several reports dealing with this topic. For example, activation of JNK and AP-1 was required for the ultraviolet-induced increase in α_1(I) collagen gene expression [28]. The ultraviolet-responsive elements were located in the distal GC box, and the GC box was bound by a DNA-binding protein

termed basic transcription-binding protein. 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 [29]. 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 [29]. It remains to be clarified whether ellagic acid inhibited collagen gene expression by a similar mechanism 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 [4,5]. Saurer et al. [30] showed that MCP-1 expression by activated PSCs was increased in fibrous tissue sections from patients with chronic pancreatitis. 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 [31]. Therefore, control of MCP-1 expression is an important therapeutic target for pancreatic fibrosis as well as inflammation. In this study, ellagic acid 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 [5,7]. Because ellagic acid inhibited IL-1β- and TNF-α-induced activation of MAP kinases, but not NF-κB, it is logical to assume that ellagic acid inhibited MCP-1 production through the inhibition of MAP kinases activation. The underlying mechanisms responsible for inhibitory actions on MAP kinases activation by ellagic acid remain to be clarified. It is unlikely that ellagic acid prevented specific binding of IL-1β and TNF-α to the respective receptors, because activation of NF-κB was not altered. Recent cell-free study showed that EGCG could directly inhibit ERK activation in response to epidermal growth factor [32], raising a possibility that ellagic acid might directly inhibit these kinases. In contrast, despite of its anti-oxidant property, ellagic acid did not inhibit the activation of NF-κB. 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 [33,34]. In human aortic smooth muscle cells, hydrogen peroxide failed to activate NF-κB or induce degradation of IκB-α [34]. IL-1β did not increase intracellular oxidative stress and IL-1β-induced NF-κB activation was not inhibited by an anti-oxidant *N*-acetylcysteine, excluding a role of oxidative stress in IL-1β-induced activation of NF-κB at least in human aortic smooth muscle cells [34]. Li and Karin [33] 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.

We have previously shown that specific inhibitors of p38 MAP kinase and JNK inhibited cell functions of PSCs [6,8]. Specific inhibitors of p38 MAP kinase inhibited PDGF-induced proliferation, α-SMA expression, α_1(I)procollagen gene expression and IL-1-induced MCP-1 production [6]. Compared with the effect of ellagic acid, inhibition of MCP-1 production was less potent. A JNK inhibitor inhibited PDGF-induced proliferation, type I collagen production and inducible MCP-1 production, but not α-SMA expression or transformation to myofibroblast-like cells [8]. Because ellagic acid inhibited all three classes of MAP kinases, it is not surprising that ellagic acid inhibited more broad range of cell functions of PSCs in a more potent manner. Very recently, we have found that another polyphenolic anti-oxidant, EGCG, blocks PDGF-induced proliferation and migration, but not collagen gene expression in PSCs [35]. Because EGCG is one of the most potent anti-oxidant [36], some unique activity other than its anti-oxidant property of ellagic acid might play a role in its inhibitory actions on PSCs.

α-SMA expression has been accepted as a marker of PSC activation [1]. Ellagic acid here decreased α-SMA gene expression in 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. Other polyphenols such as resveratrol and quercetin decreased α-SMA expression in hepatic stellate cells [37]. Regulation of α-SMA expression in PSCs remains largely unknown, but activation of p38 MAP kinase [5] and Rho-Rho kinase [6] 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 [38]. Transfection of c-*myb* anti-sense RNA inhibited both expression of the endogenous α-SMA gene and myofibroblastic transformation of quiescent cells, whereas transfection of c-*myb* stimulated α-SMA expression in quiescent hepatic stellate cells [39]. 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 [4]. Along this line, another polyphenol compound curcumin activated PPAR-γ and inhibited proliferation and extracellular matrix synthesis in hepatic stellate cells [40]. We previously reported that troglitazone, a ligand of the PPAR-γ, blocked activation of rat PSCs in vitro in a similar manner to ellagic acid [4]. Shimizu et al. [41] have reported that troglitazone

prevented the progression of pancreatic inflammatory process and fibrosis in an animal model of chronic pancreatitis, suggesting that PSCs are conceivable targets of anti-fibrogenic and anti-inflammatory strategies in vivo. Although little is known about the effective concentrations of ellagic acid required to modulate PSC functions in vivo, it would be of interest to see whether ellagic acid inhibits the development of pancreatic fibrosis. Experiments to test this hypothesis are under way in our laboratory.

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