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Immunopathology and Infectious Diseases

α2-Antiplasmin Is Associated with the Progression of Fibrosis

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Systemic sclerosis results in tissue fibrosis due to the activation of fibroblasts and the ensuing overproduction of the extracellular matrix. We previously reported that the absence of α2-antiplasmin (α2AP) attenuated the process of dermal fibrosis; however, the detailed mechanism of how α2AP affects the progression of fibrosis remained unclear. The goal of the present study was to examine the role of α2AP in fibrotic change. We observed significantly higher levels of α2AP expression in the skin of bleomycin-injected systemic sclerosis model mice in comparison with the levels seen in control mice. We also demonstrated that α2AP induced myofibroblast differentiation, and the absence of α2AP attenuated the induction of myofibroblast differentiation. Moreover, we found that connective tissue growth factor induced the expression of α2AP through both the extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) pathways in fibroblasts. Interestingly, α2AP also induced transforming growth factor-β expression through the same pathways, and the inhibition of ERK1/2 and JNK slowed the progression of bleomycin-induced fibrosis. Our findings suggest that α2AP is associated with the progression of fibrosis, and regulation of α2AP expression by the ERK1/2 and JNK pathways may be an effective antifibrotic therapy for the treatment of systemic sclerosis. ***(Am J Pathol 2010, 176:238–245; DOI: 10.2353/ajpath.2010.090150)***

Systemic sclerosis (SSc) affects the skin and the internal organs, resulting in tissue fibrosis. Although the disease process involves immunological mechanisms, vascular damage, and activation of fibroblasts, the pathogenesis of SSc remains to be further elucidated. Fibrotic diseases are characterized by excessive scarring due to excessive production, deposition, and contraction of the extracellular matrix (ECM). This process usually occurs over many months and years, and can lead to organ dysfunction or death. Connective tissue growth factor (CTGF) is constitutively overexpressed in fibrotic lesions such as in scleroderma,[1] liver,[2] renal,[3,4] lung,[5] and pancreatic fibrosis.[5] CTGF acts as a downstream effecter of at least some of the profibrotic effects of transforming growth factor-β (TGF-ß),[6] and promotes fibroblast proliferation, myofibroblasts differentiation, matrix production, and granulation tissue formation.[7,8]

Human and murine α2-antiplasmin (α2AP) are serpins (serine protease inhibitors) with a molecular weight of 65 to 70 kd,[9] which rapidly inactivate plasmin, resulting in the formation of a stable inactive complex, plasmin-α2AP.[10] Tissue fibrosis is generally considered to arise due to a failure of the normal wound healing response to terminate.[11] Previous our studies show that α2AP is associated with the wound healing and the fibrosis.[12,13] In addition, it has been reported that the level of plasmin-α2AP complex in plasma is elevated in SSc patients.[14] These findings suggest that α2AP may be associated with the progression of fibrotic disease, but the physiological roles of α2AP are not precisely understood. We herein report that α2AP plays an important role in the progression of fibrosis.

Materials and Methods

Animals

Deficient mice were generated by homologous recombination using embryonic stem cells, as described previ-

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ously.[15,16] All experiments were performed in accordance with institutional guidelines.

Reagents

α2AP was purchased from Calbiochem (San Diego, CA). Other chemical substances were obtained from Sigma Chemical Co. (St. Louis, MO).

Induction of Dermal Fibrosis

We induced dermal fibrosis in mice as previously described.[13] We induced dermal fibrosis in mice by injection of phosphate-buffered saline (PBS) or bleomycin or bleomycin and 2 mg/kg JNK specific inhibitor (SP600125; Calbiochem) or MEK specific inhibitor (PD98059; Calbiochem) (male 8-week-old C57BL/6J mice, $n = 7$). In another study, we induced dermal fibrosis in α2AP-deficient ($\alpha2AP^{-/-}$) and wild-type mice ($\alpha2AP^{+/+}$) by injection of PBS or bleomycin (male 8 weeks old, $n = 4$). Bleomycin was dissolved in PBS at 1 mg/ml. PBS or bleomycin, 100 μl, was injected subcutaneously into the shaved back of the mice. Injection in the same site was performed daily for up to 3 weeks. At the end of different observation period, the mice were sacrificed at the indicated times using an overdose of pentobarbital, and a skin sample was carefully collected from each mouse. These samples were used for the RNA and protein preparations. For the extraction of the RNA and protein, skin samples, including the scab and the complete epithelial margins, were trimmed to 7- to 8-mm diameter specimens, placed immediately in liquid nitrogen, and stored at −80°C until use.

Assay for α2AP or TGF-β Expression

Fibroblasts were obtained as previously described.[12,13] The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO_2/95% air. After 3 days, the medium was replaced with serum-free Dulbecco's modified Eagle's medium. The cells were stimulated by either CTGF (Peprotech Inc, NJ) or α2AP for 24 hours. In some experiments, a pretreatment with PD98059 (Calbiochem), SB203580 (Calbiochem), or SP600125 (Calbiochem) was performed for 60 minutes. The cells were subsequently stimulated by either CTGF or α2AP for 24 hours. These samples were used for the RNA and protein preparations.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We studied RT-PCR as previously described.[17] We used the following primer sequence: α2AP, 5′-TTCTCTTTGGTGGCCCAAACATCTACCAGC-3′ and 5′-AGGAAATCGTCTTTGATGGGAAATCCTTTC-3′; CTGF, 5′-CGCAAGATTGGAGTGTGCAC-3′ and 5′-ACTCCTTGCAGCATTTCCCA-3′; TGF-β_1, 5′-TGCTAAAGAGGTCACCCGCG-3′ and 5′-TAACGCCAGGAATTGTTGCT-3′; GAPDH, 5′-TTCATTGACCTCAACTACATG-3′ and 5′-GTGGCAGTGATGGCATGGAC-3′ PCR amplification of cDNA for 35 cycles was included at 94°C denaturation (60 seconds), 60°C annealing (60 seconds), and 72°C extension (60 seconds). Following PCR amplification, the amplified cDNAs were further extended by additional incubation at 72°C for 10 minutes. An equal amount of each reaction was separated by electrophoresis on 1% agarose gels and visualized with ethidium bromide, and the signal strengths were quantified using a densitometric program. After normalizing versus GAPDH intensity, percent increase was determined for each gene ($n = 4$). Each experiment was repeated at least three times.

Western Blot Analysis

We studied Western blot analysis as previously described.[18] We detected α2AP, α-smooth muscle actin (α-SMA), CTGF, TGF-β, phospho-ERK1/2, phospho-p38 MAPK, phospho-SAPK/JNK, ERK1/2, p38 MAPK, and JNK by incubation with anti-α2AP antibody (R&D Systems, Minneapolis, MN), anti-α-SMA antibody (clone 1A4; DakoCytomation, CA), anti-CTGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-TGF-β antibody (R&D Systems), anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Danvers, MA), anti-phospho-p38 MAPK antibody (Cell Signaling Technology), anti-phospho-JNK antibody (Cell Signaling Technology), anti-ERK1/2 antibody (Cell Signaling Technology), anti-p38 MAPK antibody (Cell Signaling Technology), and anti-JNK antibody (Cell Signaling Technology) followed incubation with horseradish peroxidase-conjugated antibody to rabbit IgG (Amersham Pharmacia Biotech, Uppsala, Sweden). The quantitative representations of expression levels were calculated by scanning the serial tracing into an image analysis program ($n = 4$).

Visualization of Stress Fibers

Actin stress fibers were visualized with phalloidin-fluorescein isothiocyanate (Sigma-Aldrich, Steinheim, Germany). Fibroblasts were fixed with methanol-free 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 10 minutes and permeabilized for 5 minutes with 0.1% Triton X-100/PBS. Then, cells were incubated with 50 μg/ml phalloidin-fluorescein isothiocyanate for 40 minutes at room temperature. The signals were then detected using a laser-scanning microscope.

JNK siRNA Study

NIH3T3 cells were transfected with JNK siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A nonspecific siRNA was used as the control. At 3 days after transfection, the cells were stimulated by CTGF, and then they were studied using Western blot analysis and RT-PCR.

Measurement of Dermal Thickness

The dermal thickness (distance from the epidermal-dermal junction to dermal-muscle junction) was measured in

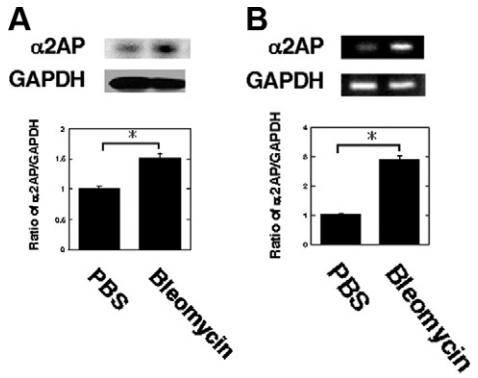

Figure 1. The expression of α2AP in fibrotic lesions. The expression of α2AP in the skin of PBS or bleomycin injected wild-type mice was measured by Western blot analysis (**A**) and RT-PCR (**B**). The histogram on the bottom panels showed quantitative representations of α2AP expression obtained from densitometry analysis ($n = 4$). The data represent the mean ± SEM; $^{*}P < 0.01$.

skin sections from PBS, bleomycin alone, bleomycin and SP600125, or PD98059-injected mice ($n = 7$).

Collagen Content in Skin (Sircol Biochemical Assay)

The collagen content was measured as previously described.[17] The collagen content was assessed using Sirius red staining. This approach was chosen because it accurately reflects the collagen content assessed with hydroxyproline assay and allows areas of localized collagen accumulation to be specifically evaluated. In these assays, sections of skin are stained with Sirius red as described by Junqueira et al.[19] After deparaffinization, the skin sections are treated in 0.2% phosphomolybdic acid for 5 minutes. Next, the skin section stained in 0.1% Sirius red for 90 minutes and 0.01 N HCl for 2 minutes. The red stain was then detected using a laser-scanning microscope. In each section, Sirius red positive area was measured in seven randomly chosen fields. Sirius red positive area was expressed as a percentage of the observed with PBS-injected mice.

Statistical Analysis

All data are expressed as the mean ± SEM. The statistical significance of the effect of each treatment ($P < 0.01$) was determined by the analysis of variance followed by the Student Newman-Keuls test.

Results

Expression of α2AP in Fibrotic Lesions

Fibrosis can be induced in mice by subcutaneous injection of bleomycin.[20] In this mouse model, the sequence of

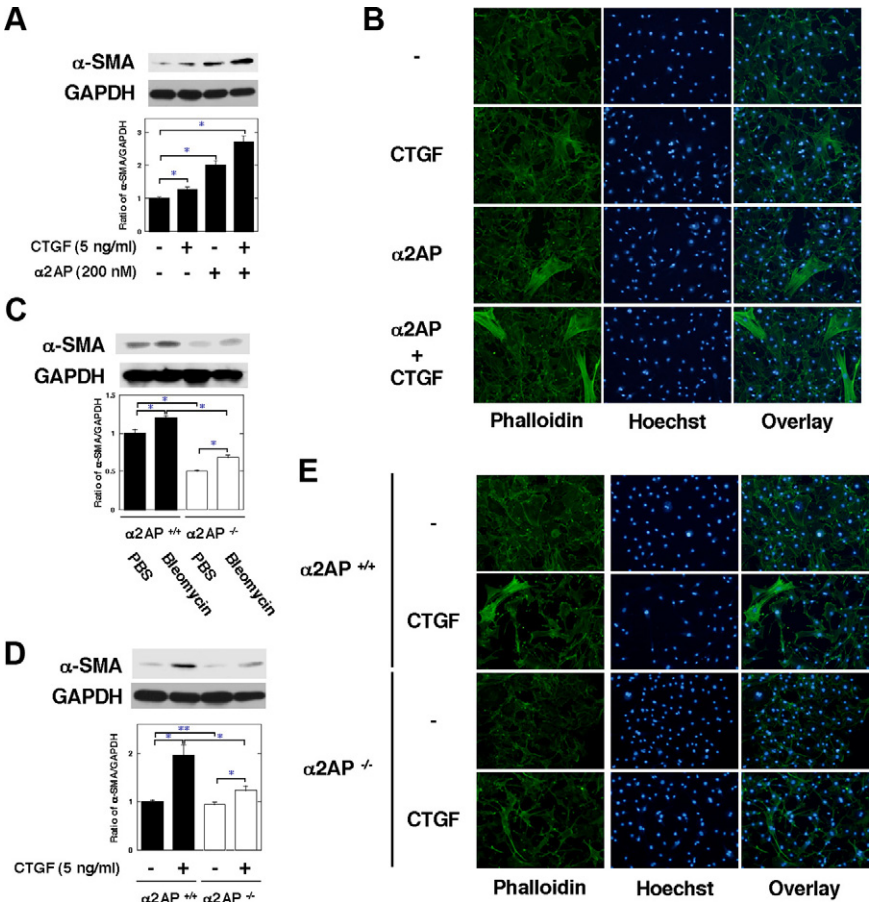

Figure 2. Effects of α2AP on myofibroblast differentiation. **A:** The dermal fibroblasts were stimulated with 5 ng/ml CTGF alone or 5 ng/ml CTGF and 200 nmol/L α2AP for 24 hours. The expression of α-SMA was measured by Western blot analysis. The histogram on the bottom panels shows quantitative representations of α-SMA expression obtained from densitometry analysis ($n = 4$). **B:** The dermal fibroblasts were stimulated with 5 ng/ml CTGF alone or 5 ng/ml CTGF and 200 nmol/L α2AP for 24 hours. Then, we examined Phalloidin staining of stress fibers in fibroblasts. **C:** The expression of α-SMA in the skin of PBS or bleomycin injected $\alpha2AP^{+/+}$ and $\alpha2AP^{-/-}$ mice was measured by Western blot analysis. The histogram on the bottom panels shows quantitative representations of α-SMA expression obtained from densitometry analysis ($n = 4$). **D:** The primary cultured fibroblasts from $\alpha2AP^{+/+}$ and $\alpha2AP^{-/-}$ mice were stimulated with 5 ng/ml CTGF for 24 hours. The expression of α-SMA was measured by Western blot analysis. The histogram on the bottom panels shows quantitative representations of α-SMA expression obtained from densitometry analysis ($n = 4$). **E:** The primary cultured fibroblasts from $\alpha2AP^{+/+}$ and $\alpha2AP^{-/-}$ mice were stimulated with 5 ng/ml CTGF for 24 hours. Then, we examined Phalloidin staining of stress fibers in fibroblasts. The data represent the mean ± SEM; $^{*}P < 0.01$, $^{**}P < 0.05$. NS, not significant.

histopathological changes in the skin closely resembles the changes observed in SSc. To clarify the mechanism behind the progression of fibrosis, we examined bleomycin-induced dermal fibrosis in a murine model of SSc. To determine the role of α2AP in fibrosis, we examined the expression of α2AP in bleomycin-induced fibrotic lesions by Western blot analysis. The expression of α2AP in bleomycin-injected skin was significantly higher than that in PBS-injected skin (Figure 1A). In addition, we examined the α2AP mRNA expression in bleomycin-induced fibrotic lesions by RT-PCR analysis. The α2AP mRNA expression in bleomycin-injected skin increased significantly in comparison with that in PBS-injected skin (Figure 1B). TGF-β and CTGF are crucial molecules in the progression of fibrosis.[7,21] RT-PCR and Western blot analysis confirmed the levels of expression of TGF-β and CTGF to have increased in the bleomycin-induced fibrotic lesions (See Supplemental Figure S1, A and B, at *http://ajp.amjpathol.org*).

Effects of α2AP on Myofibroblast Differentiation

Myofibroblasts synthesize large amounts of ECM protein and are thought to play a central role in fibrotic disorders such as SSc. To clarify the role of α2AP in the differentiation of myofibroblasts from fibroblasts, we examined the expression of α-SMA (a hallmark of the myofibroblast phenotype) by Western blot analysis. α2AP alone induced myofibroblast differentiation from fibroblasts (Figure 2A). It has previously been reported that CTGF plays an important role in myofibroblast differentiation[8], and we examined the expression of α-SMA to determine the specific effect of CTGF on α2AP-induced myofibroblast differentiation. Our results demonstrated that CTGF enhanced the α2AP-induced α-SMA expression (Figure 2A). Stress fibers are a pathological hallmark of myofibroblasts. To confirm the role of α2AP on an additional experiment in myofibroblast differentiation, we examined whether α2AP increases the number of stress fibers. Stress fibers were visualized with phalloidin. α2AP increased the number of stress fibers, and CTGF enhanced α2AP-induced the stress fibers (Figure 2B). Moreover, to verify the role of α2AP on myofibroblast differentiation, we examined the expression of α-SMA in the skin of bleomycin-injected $\alpha2AP^{+/+}$ mice and $\alpha2AP^{-/-}$ mice using Western blot analysis. The degree of bleomycin-induced α-SMA expression in the skin of $\alpha2AP^{-/-}$ mice was less than that in $\alpha2AP^{+/+}$ mice, although the injection of bleomycin did indeed induce α-SMA expression in the skin of $\alpha2AP^{-/-}$ mice (Figure

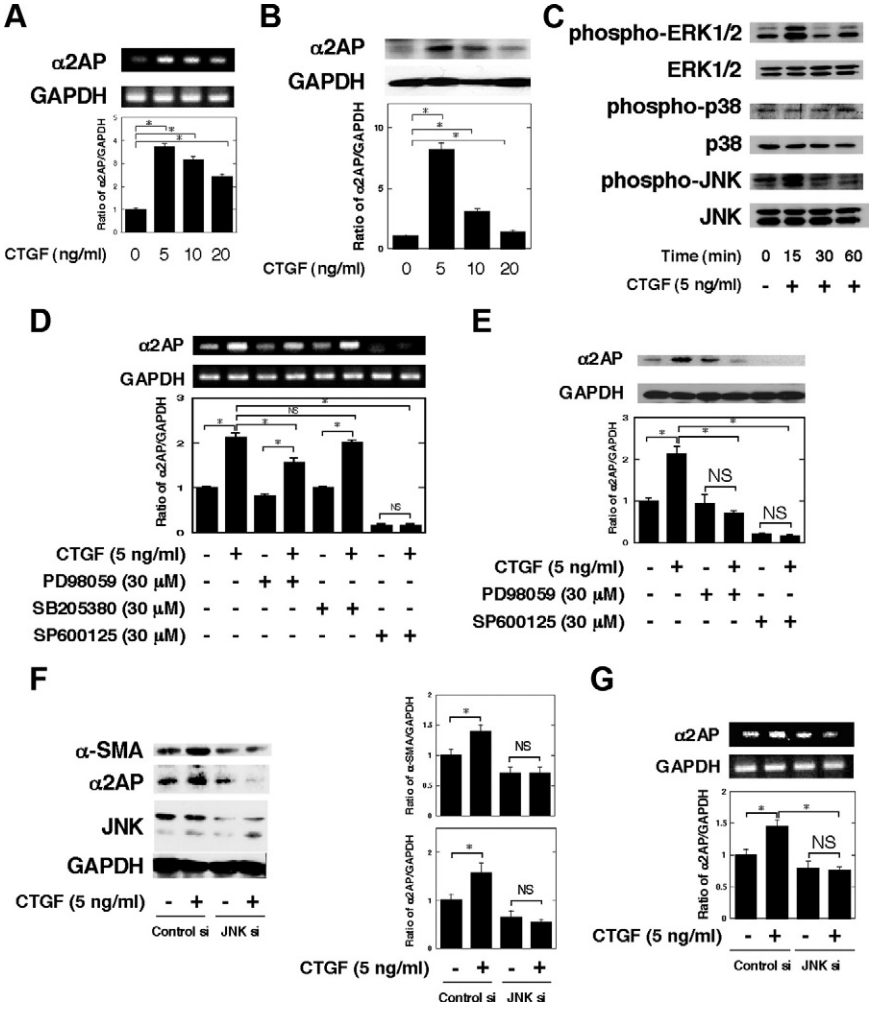

Figure 3. CTGF induced the expression of α2AP via the ERK1/2 and JNK pathways in fibroblasts. The dermal fibroblasts were stimulated by CTGF (5, 10, 20 ng/ml) for 24 hours. The expression of α2AP in dermal fibroblasts was determined by RT-PCR (**A**) and Western blot analysis (**B**). The histogram in the **bottom panels** shows quantitative representations of α2AP expression obtained from densitometry analysis ($n = 4$). **C:** The dermal fibroblasts were stimulated with 5 ng/ml CTGF for the indicated periods. Phosphorylation of ERK1/2, p38 MAPK and JNK were measured by Western blot analysis using an antibody to ERK1/2, p38 MAPK and JNK. **D:** The cultured cells were pretreated with dimethyl sulfoxide, 30 μmol/L PD98059, 30 μmol/L SB203580, and 30 μmol/L SP600125 for 60 minutes and then stimulated with 5 ng/ml CTGF for 24 hours. Levels of mRNA for α2AP in dermal fibroblasts were determined by RT-PCR. The histogram in the **bottom panels** shows quantitative representations of mRNA expression levels of α2AP obtained from densitometry analysis ($n = 4$). **E:** The cultured cells were pretreated with dimethyl sulfoxide, 30 μmol/L PD98059, and 30 μmol/L SP600125 for 60 minutes and then stimulated with 5 ng/ml CTGF for 24 hours. The expression of α2AP in dermal fibroblasts was determined by Western blot analysis. The histogram in the **bottom panels** shows quantitative representations of α2AP expression obtained from densitometry analysis ($n = 4$). The transfection of NIH-3T3 cells with control or JNK siRNA confirms the specific depletion of JNK, and then stimulated with 5 ng/ml CTGF for 24 hours. The expression of α2AP was determined by Western blot analysis (**F**) and RT-PCR (**G**). The histogram on the **right** shows quantitative representations of α2AP expression obtained from densitometry analysis ($n = 4$). The data represent the mean ± SEM; $^*P < 0.01$. NS, not significant.

2C). To clarify the effect of α2AP on CTGF-induced myofibroblast differentiation from the fibroblasts, we examined the expression of α-SMA in CTGF-stimulated fibroblasts from both $\alpha2AP^{+/+}$ and $\alpha2AP^{-/-}$ mice. The absence of α2AP attenuated CTGF-induced α-SMA expression (Figure 2D). In addition, the absence of α2AP attenuated CTGF-induced the stress fibers (Figure 2E). These data suggest that the absence of α2AP attenuated CTGF-induced myofibroblast differentiation.

CTGF Induced the Expression of α2AP via the ERK1/2 and JNK Pathways in Fibroblasts

To verify the role of CTGF on α2AP-induced myofibroblast differentiation, we examined whether CTGF induces the expression of α2AP in fibroblasts by RT-PCR and Western blot analysis. Our results demonstrated that CTGF induced the expression of α2AP in fibroblasts (Figure 3, A and B). We thereafter examined the CTGF-

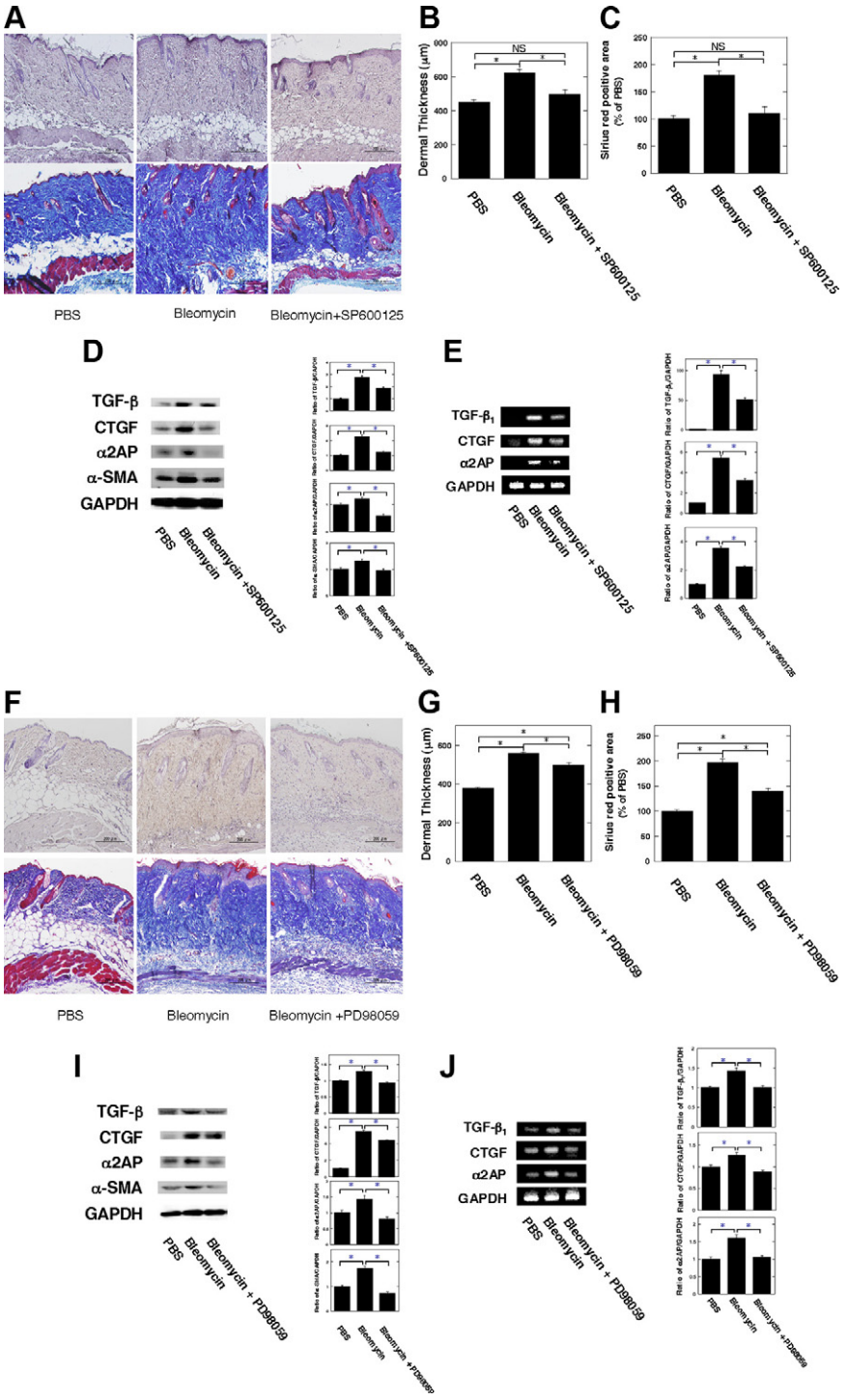

Figure 4. Prevention of bleomycin-induced fibrosis via inhibition of the ERK1/2 and JNK pathways. **A:** Representative skin section from PBS or bleomycin alone or bleomycin and SP600125 injected wild-type mice (hematoxylin and eosin stain and Masson's trichrome stain). **B:** The dermal thickness (distance from the epidermal-dermal junction to dermal-muscle junction) was measured in the skin sections from PBS or bleomycin alone or bleomycin and SP600125 injected wild-type mice ($n = 7$). **C:** The collagen content measured by Sirius red stain in the skin of PBS or bleomycin alone or bleomycin and SP600125 injected wild-type mice ($n = 7$). Sirius red positive area was expressed as a percentage of the observed with PBS-injected wild-type mice. The expression of TGF-β, CTGF, α-SMA, and α2AP in the skin of PBS or bleomycin alone or bleomycin and SP600125 injected wild-type mice were determined by and Western blot analysis (**D**) and RT-PCR (**E**). The histogram on the **right** shows quantitative representations of TGF-β, CTGF, α-SMA, and α2AP expression of obtained from densitometry analysis ($n = 4$). **F:** Representative skin section from PBS or bleomycin alone or bleomycin and PD98059 injected wild-type mice (hematoxylin and eosin stain and Masson's trichrome stain). **G:** The dermal thickness (distance from the epidermal-dermal junction to dermal-muscle junction) was measured in the skin sections from PBS or bleomycin or bleomycin alone and PD98059 injected wild-type mice ($n = 7$). **H:** The collagen content measured by Sirius red stain in the skin of PBS or bleomycin alone or bleomycin and PD98059 injected wild-type mice ($n = 7$). Sirius red positive area was expressed as a percentage of the observed with PBS-injected wild-type mice. The expression of TGF-β, CTGF, α-SMA and α2AP in the skin of PBS or bleomycin alone or bleomycin and PD98059 injected wild-type mice were determined by Western blot analysis (**I**) and RT-PCR (**J**). The histogram on the **right** shows quantitative representations of TGF-β, CTGF, α-SMA, and α2AP expression obtained from densitometry analysis ($n = 4$). The data represent the mean ± SEM; $^*P < 0.01$. NS, not significant.

stimulated phosphorylation of ERK1/2, p38 MAPK and JNK to determine whether CTGF activates extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) in fibroblasts. CTGF activated ERK1/2 and JNK, although not p38 MAPK, in fibroblasts (Figure 3C). In addition, we examined whether the ERK1/2, p38 MAPK, and JNK pathways are associated with the CTGF-induced expression of α2AP mRNA in fibroblasts by using mitogen-activated protein kinase kinase (MEK), p38 MAPK, and JNK specific inhibitors (PD98059, SB203580, SP600125). PD98059 and SP600125 attenuated CTGF-induced expression of α2AP in fibroblasts. However, SB203580 showed no influence on CTGF-induced α2AP expression in fibroblasts (Figure 3D). We subsequently examined the effects of PD98059 and SP600125 on CTGF-induced expression of α2AP by Western blot analysis. The results revealed that PD98059 and SP600125 attenuated CTGF-induced expression of α2AP in fibroblasts, although SP600125 had a somewhat stronger effect (Figure 3E). We also examined CTGF-induced α2AP expression on the forced reduction of JNK by siRNA and confirmed that the reduction of JNK inhibited CTGF-induced α2AP and α-SMA expression (Figure 3, F and G). These data suggest the CTGF-induced α2AP expression to be associated with the ERK1/2 and JNK pathways.

Prevention of Bleomycin-Induced Fibrosis via Inhibition of the ERK1/2 and JNK Pathways

The inhibition of JNK significantly reduced CTGF-induced expression of α2AP. To verify the potential of JNK as a novel target for antifibrotic therapies *in vivo*, we examined the effect of the JNK inhibitor SP600125 on bleomycin-induced fibrosis. The injection of SP600125 attenuated the progression of bleomycin-induced fibrotic changes such as increased collagen production and increased dermal thickness (Figure 4, A–C). In addition, we demonstrated that SP600125 prevented bleomycin-induced expression of α-SMA, α2AP, TGF-β, and CTGF (Figure 4, D and E). These data suggest that the inhibition of JNK prevented bleomycin-induced fibrosis by attenuating myofibroblast differentiation and ECM production. We thereafter examined the effect of PD98059 on bleomycin-induced fibrosis and observed that it also inhibited any increase in dermal thickness and collagen synthesis (Figure 4, F–H). PD98059 also repressed bleomycin-induced expression of TGF-β, CTGF, α-SMA and α2AP (Figure 4, I and J).

α2AP Induced the Expression of TGF-β via the ERK1/2 and JNK Pathways in Fibroblasts

To determine whether α2AP activates ERK1/2, p38 MAPK, and JNK in fibroblasts, we examined whether α2AP stimulated the phosphorylation of ERK1/2, p38 MAPK and JNK. Our findings revealed that α2AP activated ERK1/2 and JNK in fibroblasts (Figure 5A). In a previous study, we demonstrated that α2AP induces the production of TGF-β_1.[13] To explore whether the ERK1/2 and JNK pathways are associated with α2AP-induced TGF-β expression in fibroblasts, we examined the effects of PD98059 and SP600125 on α2AP-induced TGF-β expression by Western blot analysis and RT-PCR. The presence of PD98059 or SP600125 significantly decreased α2AP-induced TGF-β expression in fibroblasts (Figure 5, B and C). Furthermore, the reduction of JNK by siRNA also attenuated α2AP-induced TGF-β expression (See Supplemental Figure S2 at *http://ajp.amjpathol.org*). These results suggest that α2AP-induced TGF-β expression is associated with the ERK1/2 and JNK pathways. To clarify the relationship between α2AP, CTGF and TGF-β, we examined the effect of TGF-β-neutralizing antibody on bleomycin-induced fibrosis. The inhibition of TGF-β attenuated the bleomycin-induced expression of α2AP, CTGF and TGF-β (See Supplemental Figure S3 at *http://ajp.amjpathol.org*).

Discussion

The pathogenesis of SSc is initiated by microvascular injury. This injury induces inflammation and autoimmunity, which have both direct and indirect roles in inducing fibroblast activation and myofibroblast differentiation.

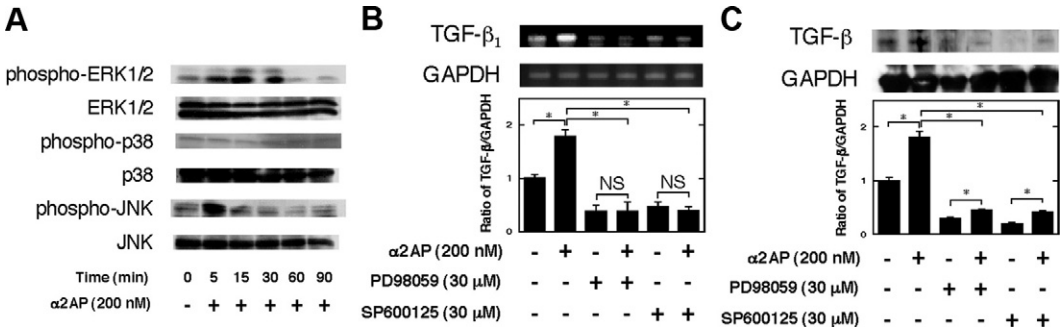

Figure 5. α2AP induced the expression of TGF-β via the ERK1/2 and JNK pathways in fibroblasts. **A:** The dermal fibroblasts were stimulated with 200 nmol/L α2AP for the indicated periods. Phosphorylation of ERK1/2, p38 MAPK and JNK were measured by Western blot analysis. The dermal fibroblasts were pretreated with dimethyl sulfoxide or 30 μmol/L PD98059 or 30 μmol/L SP600125 for 60 minutes and then were stimulated with 200 nmol/L α2AP for 24 hours. The expression of TGF-β in dermal fibroblasts were determined by RT-PCR (**B**) and Western blot analysis (**C**). The histogram in the **bottom panels** shows quantitative representations of TGF-β expression obtained from densitometry analysis ($n = 4$). The data represent the mean ± SEM; $^*P < 0.01$. NS, not significant.

Activated fibroblasts in the lesions perform a series of functions that culminate in the development of myofibroblasts.[22] Our previous studies demonstrated that α2AP is involved in vascular injury, wound healing, and fibrosis.[12,13,23] In addition, the level of plasma plasmin-α2AP complex was elevated in SSc patients.[14] We observed that the expression of α2AP in fibrotic skin tissue was significantly higher than that in normal skin. We also observed that α2AP induced myofibroblast differentiation. These data suggest that α2AP is associated with the progression of fibrosis and may function as a local regulator of fibrotic changes.

As observed in many other autoimmune diseases, disturbances in cytokine and growth factor profiles have been noted in SSc.[24] In the murine model studied herein, bleomycin was shown to promote dermal fibrosis by enhancing the expression of CTGF.[25] In the bleomycin-induced model, CTGF expression was induced in various cell types in the affected areas before the fibrosis progression.[26] An increase in CTGF expression is believed to induce a profibrotic environment.[27] We confirmed the expression of CTGF to be induced in bleomycin-injected mice. CTGF promotes fibroblast proliferation and myofibroblast differentiation, thereby inducing ECM production and fibrosis.[8,28,29] In addition, CTGF maintains these changes in various fibrotic disorders.[30] CTGF-neutralizing antibody has been shown to ameliorate fibrosis.[31] We showed that CTGF enhanced α2AP-induced myofibroblast differentiation, and the absence of α2AP attenuated CTGF-induced myofibroblast differentiation. These data suggest that α2AP might act as a downstream effector of CTGF. Therefore, we examined whether CTGF induces the expression of α2AP and observed that it does indeed stimulate the α2AP expression in fibroblasts. Moreover, we confirmed that CTGF activates ERK1/2 and JNK and the inhibition of the ERK1/2 and JNK pathways attenuated CTGF-induced α2AP expression. These data suggest that CTGF-induced the expression of α2AP is associated with the ERK1/2 and JNK pathways. However, it is not clear that CTGF directly induces α2AP expression. Other components may be involved in directly induction of α2AP expression. Further investigations would be required to clarify the details. Increased CTGF expression in fibrotic regions promotes the expression of α2AP, and CTGF-induced α2AP enhances myofibroblast differentiation, which might be associated with the progression of fibrotic disorders.

ERK1/2[32–34] and JNK[35–38] are involved in fibrotic changes such as collagen synthesis and the induction of TGF-β and CTGF expression. To clarify the effect of ERK1/2 and JNK inhibition on the progression of fibrosis, we administered PD98059 and SP600125 in bleomycin-induced fibrosis. The inhibition of the ERK1/2 and JNK pathways repressed bleomycin-induced α2AP, CTGF, and TGF-β expression as well as the progression of fibrotic changes such as increased collagen synthesis and increased dermal thickness. We have previously shown that α2AP induces the production of TGF-β.[13] Interestingly, α2AP-induced TGF-β expression was also attenuated by the inhibition of the ERK1/2 and JNK pathways. CTGF is transcriptionally activated by TGF-β and has been identified as a downstream mediator of TGF-β mediated profibrotic activity.[39] To clarify the relationship between α2AP, CTGF, and TGF-β, we demonstrated that the inhibition of TGF-β by TGF-β-neutralizing antibody attenuated bleomycin-induced α2AP, CTGF and TGF-β expression. These data suggest that CTGF-induced α2AP increased TGF-β expression, and TGF-β in turn stimulates ECM and CTGF expression. Furthermore, our previous study demonstrated that α2AP inhibits the plasmin activity, and the inhibition of plasmin slows ECM degradation.[40] These data suggest that α2AP expression increases ECM production as well as the inhibition of ECM degradation, which can result in the progression of fibrosis. The reduction of both CTGF-induced α2AP expression and α2AP-induced TGF-β expression by the inhibition of the ERK1/2 and JNK pathways might prevent bleomycin-induced fibrosis.

Recent studies have shown that the accumulation of fibroblasts arises from an epithelial-mesenchymal transition of cells at injury sites. This process is associated with the progression of fibrotic disease,[41] and the epithelial-mesenchymal transition has been reported in several models of tissue fibrosis.[42,43] It is known that TGF-β plays a critical role in the induction of epithelial-mesenchymal transition in fibrosis,[44] and α2AP or α2AP-induced TGF-β may be involved in epithelial-mesenchymal transition induction. Further investigations are necessary to clarify the mechanism of this process.

In conclusion, significant levels of α2AP are synthesized in fibrotic tissue, and α2AP is involved in myofibroblast differentiation. In addition, CTGF induces the expression of α2AP through the ERK1/2 and JNK pathways. The inhibition of these pathways attenuates the bleomycin-induced expression of α2AP and prevents the progression of fibrosis. It is quite likely that α2AP plays a critical role in the progression of fibrosis. Our findings may provide new insight into this process, which could eventually lead to the development of new clinical therapies for the prevention of fibrosis.

References

1. Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Fujimoto M, Grotendorst GR, Takehara K: Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. J Invest Dermatol 1996, 106:729–773
2. Paradis V, Dargere D, Vidaud M, De Gouville AC, Huet S, Martinez V, Gauthier JM, Ba N, Sobesky R, Ratziu V, Bedossa P: Expression of connective tissue growth factor in experimental rat and human liver fibrosis. Hepatology 1999, 30:968–976
3. Ito Y, Aten J, Bende RJ, Oemar BS, Rabelink TJ, Weening JJ, Goldschmeding R: Expression of connective tissue growth factor in human renal fibrosis. Kidney Int 1998, 53:853–861
4. Wang S, Denichilo M, Brubaker C, Hirschberg R: Connective tissue growth factor in tubulointerstitial injury of diabetic nephropathy. Kidney Int 2001, 60:96–105
5. Allen JT, Knight RA, Bloor CA, Spiteri MA: Enhanced insulin-like growth factor binding protein-related protein 2 (Connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am J Respir Cell Mol Biol 1999, 21:693–700
6. Grotendorst GR: Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. Cytokine Growth Factor Rev 1997, 8:171–179

7. Leask A, Abraham DJ: TGF-beta signaling and the fibrotic response. FASEB J 2004, 18:816–827
8. Grotendorst GR, Duncan MR: Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. FASEB J 2005, 19:729–738
9. Collen D: Identification and some properties of a new fast-reacting plasmin inhibitor in human plasma. Eur J Biochem 1976, 69:209–216
10. Lijnen HR, De Cock F, Van Hoef B, Schlott B, Collen D: Characterization of the interaction between plasminogen and staphylokinase. Eur J Biochem 1994, 224:143–149
11. Eckes B, Zigrino P, Kessler D, Holtkötter O, Shephard P, Mauch C, Krieg T: Fibroblast-matrix interactions in wound healing and fibrosis. Matrix Biol 2000, 19:325–332
12. Kanno Y, Hirade K, Ishisaki A, Nakajima K, Suga H, Into T, Matsushita K, Okada K, Matsuo O, Matsuno H: Lack of alpha2-antiplasmin improves cutaneous wound healing via over-released vascular endothelial growth factor-induced angiogenesis in wound lesions. J Thromb Haemost 2006, 4:1602–1610
13. Kanno Y, Kuroki A, Okada K, Tomogane K, Ueshima S, Matsuo O, Matsuno H: alpha2-Antiplasmin is involved in the production of transforming growth factor beta1 and fibrosis. J Thromb Haemost 2007, 5:2266–2273
14. Jinnin M, Ihn H, Yamane K, Asano Y, Yazawa N, Tamaki K: Plasma plasmin-alpha2-plasmin inhibitor complex levels are increased in systemic sclerosis patients with pulmonary hypertension. Rheumatology (Oxford) 2003, 42:240–243
15. Okada K, Lijnen HR, Dewerchin M, Belayew A, Matsuo O, Collen D, Bernaerts R: Characterization and targeting of the murine alpha2-antiplasmin gene. Thromb Haemost 1997, 78:1104–1110
16. Lijnen HR, Okada K, Matsuo O, Collen D, Dewerchin M: Alpha2-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 1999, 93:2274–2281
17. Kanno Y, Kaneiwa A, Minamida M, Kanno M, Tomogane K, Takeuchi K, Okada K, Ueshima S, Matsuo O, Matsuno H: The absence of uPAR is associated with the progression of dermal fibrosis. J Invest Dermatol 2008, 128:2792–2797
18. Kanno Y, Into T, Lowenstein CJ, Matsushita K: Nitric oxide regulates vascular calcification by interfering with TGF-signalling. Cardiovasc Res 2008, 77:221–230
19. Junqueira LC, Bignolas G, Brentani RR: Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979, 11:447–455
20. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, Nishioka K: Animal model of sclerotic skin. I: local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 1999, 112:456–462
21. Perbal B: NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 2001, 54:57–79
22. Varga J, Abraham D: Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007, 117:557–567
23. Matsuno H, Ishisaki A., Nakajima K, Okada K, Ueshima S, Matsuo O, Kozawa O: Lack of alpha 2-antiplasmin promotes re-endothelialization via over-release of VEGF after vascular injury in mice. Blood 2003, 102:3621–3628
24. Varga J: Antifibrotic therapy in scleroderma: extracellular or intracellular targeting of activated fibroblasts? Curr Rheumatol Rep 2004, 6:164–170
25. Yamamoto T, Eckes B, Krieg T: Bleomycin increases steady-state levels of type I collagen, fibronectin and decorin mRNAs in human skin fibroblasts. Arch Dermatol Res 2000, 292:556–561
26. Higashiyama H, Yoshimoto D, Okamoto Y, Kikkawa H, Asano S, Kinoshita M: Receptor-activated Smad localisation in bleomycin-induced pulmonary fibrosis. J Clin Pathol 2007, 60:283–289
27. Bonniaud P, Martin G, Margetts PJ, Ask K, Robertson J, Gauldie J, Kolb M: Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. Am J Respir Cell Mol Biol 2004, 31:510–516
28. Leask A, Abraham DJ: The role of connective tissue growth factor, a multifunctional matricellular protein, in fibroblast biology. Biochem Cell Biol 2003, 81:355–363
29. Perbal B: CCN proteins: multifunctional signalling regulators. Lancet 2004, 363:62–64
30. Takehara K: Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol 2003, 30:755–759
31. Ikawa Y, Ng PS, Endo K, Kondo M, Chujo S, Ishida W, Shirasaki F, Fujimoto M, Takehara K: Neutralizing monoclonal antibody to human connective tissue growth factor ameliorates transforming growth factor-beta-induced mouse fibrosis. J Cell Physiol 2008, 216:680–687
32. Chen Y, Shi-Wen X, van Beek J, Kennedy L, McLeod M, Renzoni EA, Bou-Gharios G, Wilcox-Adelman S, Goetinck PF, Eastwood M, Black CM, Abraham DJ, Leask A: Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. Am J Pathol 2005, 167:1699–1711
33. Rodríguez-Barbero A, Obreo J, Alvarez-Munoz P, Pandiella A, Bernabéu C, López-Novoa JM: Endoglin modulation of TGF-beta1-induced collagen synthesis is dependent on ERK1/2 MAPK activation. Cell Physiol Biochem 2006, 18:135–142
34. Sullivan DE, Ferris M, Pociask D, Brody AR: Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal-regulated kinase pathway. Am J Respir Cell Mol Biol 2005, 32:342–349
35. Alcorn JF, van der Velden J, Brown AL, McElhinney B, Irvin CG, Janssen-Heininger YM: c-Jun N-terminal kinase 1 is required for the development of pulmonary fibrosis. Am J Respir Cell Mol Biol 2009, 40:422–432
36. Shi-Wen X, Rodríguez-Pascual F, Lamas S, Holmes A, Howat S, Pearson JD, Dashwood MR, du Bois RM, Denton CP, Black CM, Abraham DJ, Leask A: Constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. Mol Cell Biol 2006, 26:5518–5527
37. Utsugi M, Dobashi K, Ishizuka T, Masubuchi K, Shimizu Y, Nakazawa T, Mori M: C-Jun-NH2-terminal kinase mediates expression of connective tissue growth factor induced by transforming growth factor-beta1 in human lung fibroblasts. Am J Respir Cell Mol Biol 2003, 28:754–761
38. Wang XM, Zhang Y, Kim HP, Zhou Z, Feghali-Bostwick CA, Liu F, Ifedigbo E, Xu X, Oury TD, Kaminski N, Choi AM: Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. J Exp Med 2006, 203:2895–2906
39. Duncan MR, Frazier KS, Abramson S, Williams S, Klapper H, Huang X, Grotendorst GR: Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. FASEB J 1999, 13:1774–1786
40. Okada K, Ueshima S, Imano M, Kataoka K, Matsuo O: The regulation of liver regeneration by the plasmin/alpha 2-antiplasmin system. J Hepatol 2004, 40:110–116
41. Kalluri R, Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003, 112:1776–1784
42. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA: Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA 2006, 103:13180–13185
43. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002, 110:341–350
44. Nawshad A, Lagamba D, Polad A, Hay ED: Transforming growth factor-beta signaling during epithelial-mesenchymal transformation: implications for embryogenesis and tumor metastasis. Cells Tissues Organs 2005, 179:11–23