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The Plasminogen Activation System Reduces Fibrosis in the Lung by a Hepatocyte Growth Factor-Dependent Mechanism

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Mice deficient in the plasminogen activator inhibitor-1 gene (PAI-$1^{-/-}$ mice) are relatively protected from developing pulmonary fibrosis from bleomycin administration. We hypothesized that one of the protective mechanisms may be the ability of the plasminogen system to enhance hepatocyte growth factor (HGF) effects, which have been reported to be antifibrotic in the lung. HGF is known to be sequestered in tissues by binding to extracellular matrix components. Following bleomycin administration, we found that HGF protein levels were higher in bronchoalveolar lavage fluid from PAI-$1^{-/-}$ mice compared to wild-type (PAI-$1^{+/+}$) mice. This increase could be suppressed by administering tranexamic acid, which inhibits plasmin activity. Conversely, intratracheal instillation of urokinase into bleomycin-injured PAI-$1^{+/+}$ mice to activate plasminogen caused a significant increase in HGF within bronchoalveolar lavage and caused less collagen accumulation in the lungs. Administration of an anti-HGF neutralizing antibody markedly increased collagen accumulation in the lungs of bleomycin-injured PAI-$1^{-/-}$ mice. These results support the hypothesis that increasing the availability of HGF, possibly by enhancing its release from extracellular matrix by a plasmin-dependent mechanism, is an important means by which activation of the plasminogen system can limit pulmonary fibrosis. ***(Am J Pathol 2004, 164:1091–1098)***

Abnormal accumulation of fibrin occurs within the interstitium and alveolar spaces of the lung in a variety of pulmonary diseases in which the integrity of the capillary alveolar barrier is damaged.[1–4] Analysis of bronchoalveolar lavage (BAL) fluid from patients with diseases such as acute respiratory distress syndrome and idiopathic pulmonary fibrosis has revealed that the fibrinolytic activity that is normally present within the alveolar space is inhibited by increased levels of plasminogen activator inhibitor-1 (PAI-1).[5–8] Similar findings have been reported from experiments using animal models of pulmonary fibrosis including that induced by bleomycin.[9–11] The linkage between the plasminogen system and pulmonary fibrosis was first shown using mice having a targeted deletion of the PAI-1 gene (PAI-$1^{-/-}$ mice).[12,13] These mice survived longer and developed less fibrosis following bleomycin administration than PAI-$1^{+/+}$ animals. Furthermore, we found that inhibition of plasmin activity in PAI-$1^{-/-}$ mice with tranexamic acid following bleomycin administration caused an increase in both fibrin accumulation and collagen deposition in the lung.[13] These observations suggested that inhibition of the plasminogen system by increased expression of PAI-1 in lung injury leads to abnormal accumulation of fibrin and progression to pulmonary fibrosis.

The fibrin matrix has been thought to be an important component in the development of pulmonary fibrosis because it can serve as a scaffold onto which fibroblasts migrate and produce interstitial collagens. This perception generated the hypothesis that the protective role of the plasminogen system in pulmonary fibrosis was the plasmin-mediated clearance of fibrin. However, we and others found that mice genetically deficient in fibrinogen developed pulmonary fibrosis to a degree similar to control mice following bleomycin administration.[13,14] This result demonstrated that fibrin is not a prerequisite for the development of pulmonary fibrosis, and therefore clearance of fibrin is not the sole mechanism by which the plasminogen system limits pulmonary fibrosis.

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In addition to fibrinolysis, the plasminogen system is involved in a variety of activities that may influence lung injury and repair.[15–17] For example, it can contribute significantly to the proteolytic activation of matrix metalloproteinases, release of growth factors from extracellular matrices (ECM), and degradation of inflammatory exudate and necrotic tissues. In addition to these activities, we developed a strong interest in the interaction of the plasminogen system with HGF. HGF levels are increased in the BAL fluid of patients with idiopathic pulmonary fibrosis, sarcoidosis, and the interstitial lung disease associated with rheumatoid arthritis.[18] HGF is also up-regulated following bleomycin administration in normal mice.[19] When administered systemically or intratracheally, HGF has been shown to attenuate pulmonary fibrosis following bleomycin-induced injury in mice.[19,20] HGF, which is secreted as an inactive single-chain protein, can be cleaved by urokinase to form the active disulfide-linked heterodimer.[21,22] HGF also induces expression of urokinase,[23,24] thus participating in a positive feedback loop. For these reasons, we hypothesized that some of the protective effect from augmenting the plasminogen system is to increase the availability and activation of HGF.

In the current study, we investigated whether manipulation of the plasminogen system in a bleomycin-induced lung injury model affected HGF expression in lung tissue and the amount of active and total HGF in BAL fluid. We also determined whether inhibition of HGF in PAI-$1^{-/-}$ mice following bleomycin administration could influence the development of pulmonary fibrosis. Furthermore, we evaluated the effect of urokinase administration on collagen accumulation and HGF levels in bleomycin-induced lung injury.

Materials and Methods

Animals

PAI-$1^{-/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type C57BL/6 (PAI-$1^{+/+}$) mice were purchased from CLEA Japan (Tokyo, Japan). Only female mice aged 6 to 8 weeks were used to reduce the variability in animal weights that would occur if both sexes were used.

Bleomycin Exposure

For each experiment, age- and weight-matched groups of mice were used. Mice were anesthetized with intraperitoneal pentobarbital, and the trachea was exposed through a cervical incision. Bleomycin (3 mg/kg body weight; Nippon Kayaku Co., Tokyo, Japan) was dissolved in PBS and then instilled intratracheally with a 27-gauge needle.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was purified from lung homogenates using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed and amplified using the one-step RT-PCR kit (Qiagen, Hilden, Germany) with gene-specific primers. The following sets of primers were used. HGF: forward primer, 5′-TTG GCC ATG AAT TTG ACC TC-3′; reverse primer, 5′-ACA TCA GTC TCA TTC ACA GC-3′. β-actin: forward primer, 5′-GAT ATC GCC GCG CTC GTC GT-3′; reverse primer, 5′-CAG GAA GGA AGG CTG GAA GA-3′. PCR conditions were as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. Twenty-seven cycles were chosen because this level of amplification was found to be within the linear range (data not shown). The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Optical density of each product was determined by Scion Imaging software (Frederick, MD).

Determination of HGF Content in Lung

Mice were sacrificed and the lung vascular bed was perfused with PBS. The lungs were excised and homogenized in 2 ml of buffer composed of 10 mmol/L Tris-HCl (pH 7.5), 2 mol/L NaCl, 0.1% Tween 80, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L ethylenediaminetetra-acetic acid (EDTA). The homogenates were centrifuged at 19,000 $\times$ *g* for 30 minutes at 4°C. The supernatant was used to measure HGF by enzyme-linked immunosorbent assay (ELISA) (Institute of Immunology, Tokyo, Japan) and to analyze molecular forms of HGF by Western blotting.

Tranexamic Acid Treatment

To block the lysine binding sites on plasminogen and plasmin, tranexamic acid (kindly provided by Daiichi Pharmaceutical Co., Tokyo, Japan) was administered by continuous subcutaneous infusion via osmotic pumps (model 2001; ALZA Corp., Palo Alto, CA) implanted under the dorsal skin of mice. The pumps were loaded to deliver 3.6 mg of subcutaneous tranexamic acid per day. In addition, tranexamic acid was added to the drinking water at 20 mg/ml to supplement the parenteral dose. These treatments were started on the day of bleomycin administration and continued until sacrifice of the mice.

Bronchoalveolar Lavage (BAL)

Mice were sacrificed with a lethal dose of pentobarbital, the tracheas were cannulated with an 18-gauge needle, and the lungs were lavaged twice with 1 ml of PBS containing 1 mmol/L PMSF, 1 mmol/L EDTA. The lavage fluids were pooled and were cleared of cells with centrifugation. The concentration of HGF in BAL fluid was determined by ELISA.

Analysis of Molecular Forms of HGF in BAL Fluid and Lung Tissue

One and one-half milliliters of BAL fluid or lung homogenate was applied to a HiTrap heparin HP column (column volume 1 ml, Amersham Pharmacia Biotech, Uppsala, Sweden) that had been pre-equilibrated with binding buffer composed of 10 mmol/L Tris-HCl (pH 7.5), 0.3 mol/L NaCl, 0.01% Tween 80, 1 mmol/L PMSF, 1 mmol/L EDTA. After washing with 3 ml of the same buffer, 2 ml of elution buffer consisting of 10 mmol/L Tris-HCl (pH 7.5), 2 mol/L NaCl, 0.01% Tween 80, 1 mmol/L PMSF, 1 mmol/L EDTA was applied. The first 0.5 ml eluted was discarded and next 1 ml volume was collected. This elutate was dialyzed against PBS and concentrated with a Microcon filter (YM-30, Millipore, Bedford, MA). HGF concentration was determined for each concentrated sample by ELISA and then approximately 100 pg of HGF was loaded into each well of a 10% gel and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 2-mercaptoethanol. Proteins were blotted onto a polyvinylidene difluoride membrane, and the membrane was reacted with an anti-rat HGF α-chain monoclonal antibody (Institute of Immunology), which cross-reacts with mouse HGF. The membrane was then incubated with horseradish peroxidase-conjugated antibodies and developed using enhanced chemiluminescence reagent (SuperSignal West Pico Chemiluminescence Substrate, Pierce, Rockford, IL).

Neutralization of HGF by an Anti-HGF Antibody

Anti-rat HGF neutralizing antibody, which cross-reacts with mouse HGF, was prepared as described previously.[25,26] Specific binding of this antibody to HGF but not other growth factors has previously been confirmed both *in vitro* and *in vivo*.[26,27] In addition, treatment of mice with this antibody has been shown to be effective at neutralizing endogenous HGF present in the lung.[28] Bleomycin was administered intratracheally to PAI-$1^{-/-}$ mice that were then injected intraperitoneally with 250 μg of either neutralizing anti-rat HGF IgG or normal rabbit IgG every 3 days for 9 days starting on the day of bleomycin treatment. The mice were then sacrificed 14 days after bleomycin treatment.

Assessment of Lung Fibrosis

The amount of pulmonary fibrosis was determined by measuring total soluble lung collagen using the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland) as described previously.[29] This method measures newly-synthesized collagen that has not been extensively cross-linked. Previously published articles have demonstrated that measurements performed using the Sircol assay correlate with histological evidence of pulmonary fibrosis in the bleomycin lung injury model in mice.[29,30] Briefly, lungs were homogenized in 5 ml of 0.5 mol/L acetic acid containing 1 mg pepsin (Sigma-Aldrich, St. Louis, MO) per 10 mg tissue residue. Each sample was incubated for 24 hours at 4°C with stirring. After centrifugation, 100 μl of each supernatant was assayed. One ml of Sircol dye reagent that specifically binds to collagen was then added to each sample and mixed for 30 minutes. After centrifugation, the pellet was suspended in 1 ml of alkali reagent (0.5 mol/L NaOH) included in the kit and optical density evaluated at 540 nm with a spectrophotometer. The values in the test samples were compared to the values obtained with collagen standard solutions provided by the manufacturer that were used to construct a standard curve.

Administration of Urokinase into Bleomycin-Injured Mice

Human urokinase purified from urine was kindly provided by Mochida Pharmaceutical Co. (Tokyo, Japan). Six days after bleomycin administration, PAI-$1^{+/+}$ mice were anesthetized with intraperitoneal pentobarbital and the trachea were exposed again. Six thousand units of urokinase or heat-inactivated (100°C for 3 minutes) urokinase that were dissolved in 50 μl of PBS were instilled intratracheally with a 27-gauge needle. Some mice were subjected to BAL on the next day to measure HGF level in BAL fluid, and the remaining mice were sacrificed on day 14 after bleomycin treatment to determine collagen content in the lungs.

Statistics

Results are expressed as mean ± SEM. Differences between treatment groups were analyzed using analysis of variance with Fisher's PLSD test for pairwise comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Expression of HGF in Bleomycin-Injured Lungs

Previous work has demonstrated that HGF expression is markedly increased in the lungs following bleomycin-induced injury.[19] To determine whether enhancement of the plasminogen system would further influence HGF expression in bleomycin-injured lungs, we evaluated the expression of HGF mRNA and protein in the lungs of PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice 7 days after bleomycin administration. Semiquantitative RT-PCR revealed that expression of HGF mRNA was approximately two-fold increased in both PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice after bleomycin exposure ($P < 0.01$, bleomycin *versus* PBS), but the levels did not differ between these two groups (Figure 1) ($P > 0.5$). Similarly, HGF levels in the extracts of bleomycin-injured lungs were significantly higher compared with PBS-treated controls ($P < 0.01$, bleomycin *versus* PBS). However, there again was no difference in HGF levels between PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice ($P > 0.5$). These results indicate that elimination of PAI-1 does

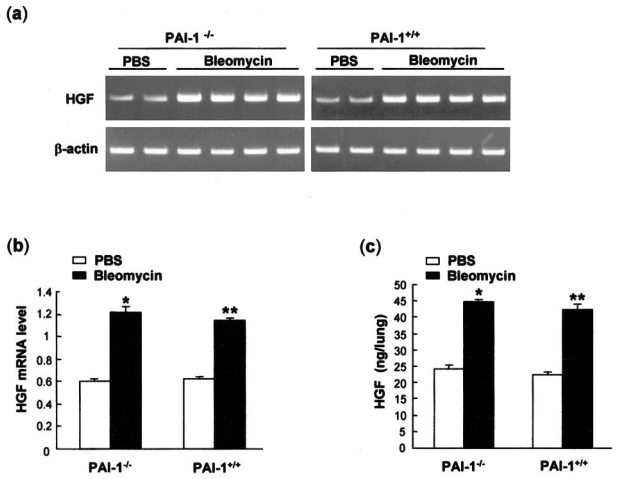

Figure 1. Expression of HGF mRNA and protein in the lungs of bleomycin-injured mice. Bleomycin or PBS was instilled intratracheally into PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice. After 7 days, lungs were harvested and the vasculature perfused with PBS. **a:** Total RNA was extracted from the lungs and examined for HGF and β-actin mRNA by semiquantitative RT-PCR. **b:** Optical density of each PCR product was measured and the HGF mRNA expression level expressed as its ratio to the density of the β-actin band (* $P < 0.01$ for bleomycin PAI-$1^{-/-}$ mice *versus* PBS PAI-$1^{-/-}$ mice, * $P > 0.5$ for bleomycin PAI-$1^{-/-}$ mice *versus* bleomycin PAI-$1^{+/+}$ mice, ** $P < 0.01$ for bleomycin PAI-$1^{+/+}$ mice *versus* PBS PAI-$1^{+/+}$ mice). **c:** Tissue extracts from lungs were prepared as described in Materials and Methods. HGF level in the extract was measured by ELISA. Data are expressed as mean ± SEM; $n = 3$ to 4 mice per group (* $P < 0.01$ for bleomycin PAI-$1^{-/-}$ mice *versus* PBS PAI-$1^{-/-}$ mice, * $P > 0.5$ for bleomycin PAI-$1^{-/-}$ mice *versus* bleomycin PAI-$1^{+/+}$ mice, ** $P < 0.01$ for bleomycin PAI-$1^{+/+}$ mice *versus* PBS PAI-$1^{+/+}$ mice).

not affect the increase in lung HGF expression that follows bleomycin administration.

HGF Levels in BAL Fluids

Adamson et al[31] reported that HGF level in BAL fluid from normal rat lungs was very low, but increased several times soon after bleomycin administration. To confirm this observation and evaluate whether enhancement of plasminogen system would affect HGF levels in BAL fluid, we measured HGF in BAL fluids obtained from PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice 7 days after bleomycin administration. In a previous report,[13] we had shown that fibrinolytic activity in the PAI-$1^{-/-}$ mice lungs was increased compared with PAI-$1^{+/+}$ mice at 7 days after bleomycin administration. As shown in Figure 2, HGF levels in BAL fluids from both control PBS-treated PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice were approximately 0.1 ng/ml. Bleomycin injection resulted in marked increase in HGF level in both PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice, but the levels were significantly higher in PAI-$1^{-/-}$ mice compared to PAI-$1^{+/+}$ mice (2.9 ± 0.4 ng/ml *versus* 1.4 ± 0.1 ng/ml; $P < 0.01$). To determine whether the proteolytic activity of plasmin is involved in the increase in the BAL HGF levels, we administered tranexamic acid, which blocks plasminogen activation and plasmin activity. In the previous study,[13] we had already confirmed that the treatment of bleomycin-injured PAI-$1^{-/-}$ mice with tranexamic acid suppresses fibrinolytic activity in the lungs. Interestingly, tranexamic acid treatment reduced HGF levels in BAL fluids from both bleomycin-injured PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice (1.35 ± 0.2 ng/ml and 0.8 ± 0.2 ng/ml, respectively). These results indicate that the HGF level in BAL fluid following bleomycin-induced lung injury is significantly influenced by the activity of the plasminogen system.

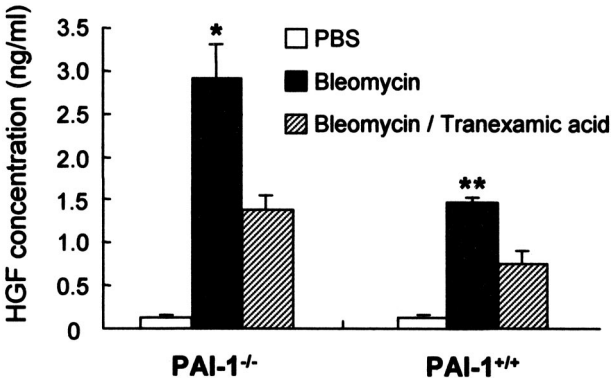

Figure 2. HGF levels in BAL fluids. Bleomycin or PBS was instilled intratracheally into PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice. BAL fluid was obtained 7 days after bleomycin or PBS administration and HGF level was measured by ELISA. Tranexamic acid treatment was performed as described in Methods. Data are expressed as mean ± SEM; $n = 4$ to 6 mice per group (* $P < 0.01$ for bleomycin/tranexamic acid PAI-$1^{-/-}$ mice *versus* bleomycin PAI-$1^{+/+}$ mice; ** $P < 0.05$ for bleomycin/tranexamic acid PAI-$1^{-/-}$ mice *versus* bleomycin/tranexamic acid PAI-$1^{+/+}$).

Evidence of Active Forms of HGF in BAL Fluid and Lung Tissue

To determine whether HGF is in its activated form, we performed Western blot analysis on BAL fluids and lung homogenates obtained from PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice 7 days after bleomycin administration. Activated HGF is a heterodimeric protein consisting of a heavy α-chain and a light β-chain held together by a disulfide bond. The two chains are generated from an inactive, single-chain precursor by proteolytic processing. Western blot analysis under reducing conditions using an anti-α-chain antibody can distinguish the two-chain form of HGF from the single-chain HGF (approximately 97 kd) by demonstrating the presence of the cleaved lower molecular weigh α-chain (approximately 69 kd).[32] Equal amounts of HGF from BAL fluid and lung tissue, as determined by ELISA, were separated by SDS-PAGE and probed with the anti-α-chain antibody. As shown in Figure 3, single-chain, pro-HGF was only found in the lung homogenates but not found in the BAL fluid from bleomycin-injured PAI-$1^{-/-}$ or PAI-$1^{+/+}$ mice, indicating that all of the HGF present in BAL fluid from these mice was activated. An additional lower weight band at approximately 54 kd was detected in BAL fluid from both bleomycin-injured PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice, but its amount tended to be greater in PAI-$1^{-/-}$ mice than in PAI-$1^{+/+}$ mice. This band corresponds in molecular weight to a non-glycosylated form of the α-chain of HGF.[33]

Neutralization of HGF in Bleomycin-Injured PAI-$1^{-/-}$ Mice

Previous reports have shown that exogenously administered HGF attenuates bleomycin-induced pulmonary fi-

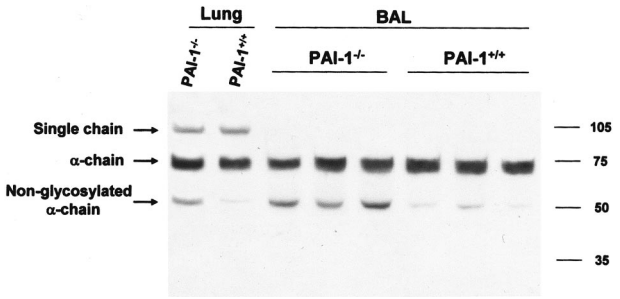

Figure 3. Molecular forms of HGF in BAL fluid and lung tissue. Bleomycin or PBS was instilled intratracheally into PAI-1$^{-/-}$ and PAI-1$^{+/+}$ mice. The heparin-binding fraction was prepared from BAL fluid and lung homogenate obtained 7 days after bleomycin administration. After dialyzing against PBS, this fraction was concentrated and the HGF level was determined by ELISA. Approximately 100 pg of HGF in each concentrated fraction was subjected to SDS-PAGE under reducing condition and immunoblotted with an anti-HGF α-chain antibody. Bands corresponding in molecular weights to the single chain of HGF (97 kd), the α-chain of HGF (69 kd), and a non-glycosylated α-chain (54 kd) were detected.

brosis.[19,20] These observations indicated that HGF has an anti-fibrotic effect on the development of pulmonary fibrosis. As described above, HGF recovered in BAL fluid was in its active form and more abundant in PAI-1$^{-/-}$ mice after bleomycin injury. We next questioned if endogenous HGF is related to the protection from bleomycin-induced pulmonary fibrosis in PAI-1$^{-/-}$ mice. To determine this, we administered neutralizing anti-HGF rabbit IgG or normal rabbit IgG to PAI-1$^{-/-}$ mice following bleomycin administration and compared collagen accumulation in the lungs. As shown in Figure 4, neutralization of HGF caused a marked increase in collagen content in the lungs of bleomycin-injured PAI-1$^{-/-}$ mice (control IgG, 275 ± 38 μg/lung; anti-HGF IgG, 867 ± 169 μg/lung; $P <$ 0.01).

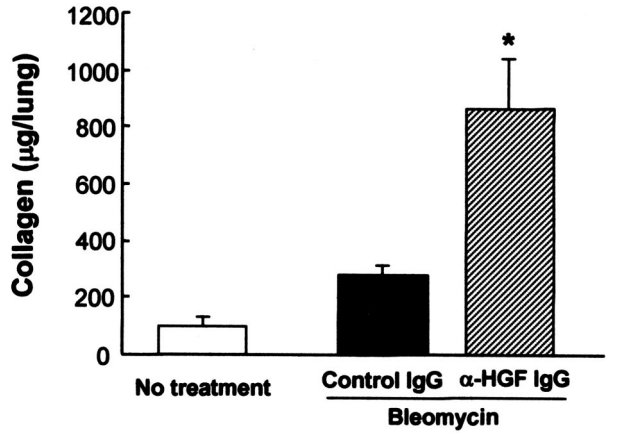

Figure 4. Effect of neutralizing antibody against HGF on lung collagen accumulation in bleomycin-injured PAI-1$^{-/-}$ mice. Bleomycin was instilled intratracheally into PAI-1$^{-/-}$ mice, and anti-rat HGF rabbit serum (αHGF IgG) or normal rabbit serum (control IgG) was administered intraperitoneally as described in Materials and Methods. After 14 days, lungs were harvested and analyzed for collagen content using the Sircol biochemical assay. To evaluate baseline collagen content in lungs, lungs from PAI-1$^{-/-}$ mice without any treatment were also assayed. Data are expressed as mean ± SEM; n = 5 to 6 mice per group (* $P < 0.01$ αHGF *versus* control IgG).

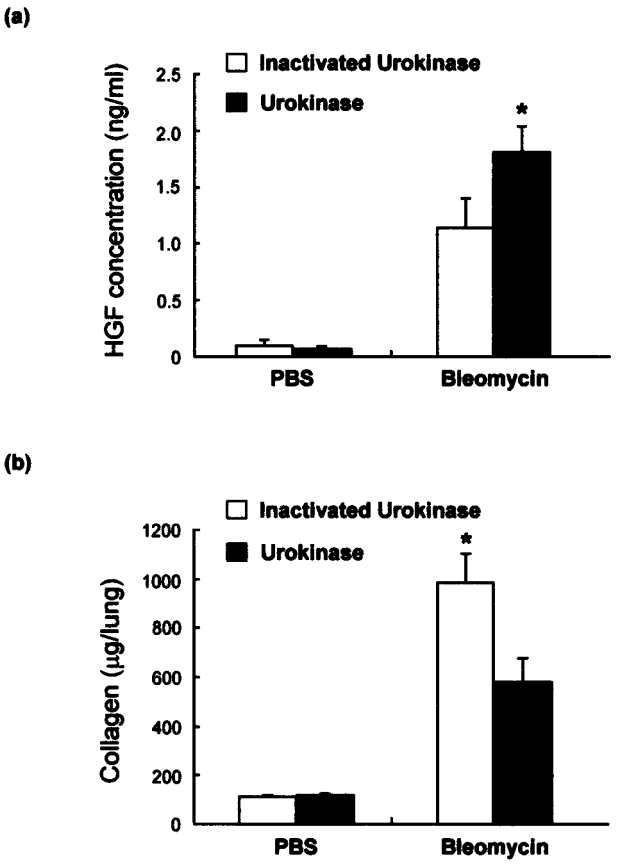

Figure 5. Effect of urokinase on HGF level in BAL fluid and lung collagen accumulation in bleomycin-injured PAI-1$^{+/+}$ mice. Bleomycin or PBS was instilled intratracheally into PAI-1$^{+/+}$ mice. On day 6 after administration, urokinase or heat-inactivated urokinase was injected via exposed trachea. **a:** On day 7 after bleomycin treatment, BAL fluid was obtained and analyzed for HGF level by ELISA (n = 4 to 5 mice per group) (* $P < 0.05$ urokinase *versus* inactivated urokinase). **b:** The remaining mice (n = 6 to 8 mice per group) were sacrificed on day 14 after bleomycin administration to determine collagen content in the lungs. Data are expressed as mean ± SEM (* $P < 0.05$ for urokinase *versus* inactivated urokinase).

Effect of Urokinase Administration into Bleomycin-Injured Lungs

Hart and colleagues[34] reported that intratracheal instillation of recombinant human urokinase into rats was effective in diminishing collagen content of established bleomycin-induced pulmonary fibrosis. Sisson and colleagues[35] have also reported that intratracheal administration of an adenoviral vector containing a urokinase gene into mice with bleomycin-induced pulmonary fibrosis reduces the amount of collagen accumulation. We therefore questioned if urokinase treatment of bleomycin-injured mice would influence the HGF level in BAL fluid and possibly prevent the development of pulmonary fibrosis. To examine this, PAI-1$^{+/+}$ mice were injected intratracheally with bleomycin on day 0, and 6000 IU of urokinase or heat-inactivated urokinase was instilled intratracheally on day 6. On day 7, at least four mice from each group were sacrificed and BAL fluids were collected. The remaining mice were maintained until day 14 after which the lungs were harvested for collagen assay. As shown in Figure 5, intratracheal administration of

urokinase caused an increase in HGF level in BAL fluid (inactive urokinase, 1.14 ± 0.25 ng/ml; active urokinase 1.81 ± 0.23 ng/ml; $P < 0.05$) and a reduction in collagen accumulation in the lungs (inactive urokinase, 981 ± 117 μg/lung; active urokinase, 578 ± 100 μg/lung; $P < 0.05$).

Discussion

A relationship between the plasminogen system and the development of pulmonary fibrosis after lung inflammation is firmly established. Mice with impaired fibrinolysis caused by either overexpression of a PAI-1 transgene[12] or by targeted deletion of the plasminogen gene develop increased lung fibrosis after inflammation.[36] Conversely, increasing the activity of the fibrinolytic system by targeted deletion of PAI-1 genes or increasing urokinase protects the lung from fibrosis induced by inflammation. A key mechanism for this protection was hypothesized to be the accelerated clearance of fibrin, thus preventing the establishment of a provisional matrix onto which fibroblasts migrate and form collagenous scars. However, experiments using mice genetically deficient in fibrinogen have shown that fibrin was not required for fibrosis to occur in the lungs after inflammation.[13,14] This indicates that the fibrinolytic activity of the plasminogen system cannot be considered the sole mechanism for this protection. Seeking another mechanism, we investigated the role of HGF because it is activated by urokinase and previous studies have demonstrated a protective effect of HGF on the development of pulmonary fibrosis. In the present study, we have demonstrated that enhancement of plasminogen activation increases HGF levels in BAL fluids collected during lung inflammation. Neutralization of endogenous HGF in bleomycin-injured PAI-1$^{-/-}$ mice resulted in increased collagen accumulation in the lungs. Furthermore, intratracheal administration of urokinase into bleomycin-injured PAI-1$^{+/+}$ mice was shown both to increase HGF in BAL fluid and to reduce collagen accumulation in the lungs. These results indicate that the protective effect against fibrosis of the plasminogen activation system is mediated, at least in part, by its effects on HGF.

HGF is a multipotent growth factor that has mitogenic, motogenic, and morphogenic properties.[37,38] It stimulates DNA synthesis[39,40] and proliferation of alveolar type II epithelial cells,[41] thus contributing to regeneration of alveolar structures following injury. Previous studies have also shown that administration of exogenous HGF via systemic[20] or intratracheal route[19] into bleomycin-injured mice resulted in the reduction of pulmonary fibrosis. This anti-fibrotic effect is thought to be mediated by its facilitation of epithelial repair that follows lung injury. Inhibition of the fibrotic process by HGF has been reported in other organs such as liver,[42] heart,[43] and kidney.[44]

The major sources of HGF in the alveolar space are thought to be alveolar macrophages and interstitial fibroblasts, and the cellular target is the alveolar epithelial cells.[45] HGF that is secreted by these cells is likely to become bound to the ECM through its strong affinity to various ECM components,[46] particularly heparan sulfate-containing proteoglycans.[47] The presence of ECM-bound HGF is well documented,[21] and release of HGF from matrix-bound stores has been thought to increase the bioavailability of HGF to distant target cells. Indeed, release of HGF sequestrated in the ECM by heparin was reported to elevate plasma HGF levels and accelerate liver regeneration.[48] We speculate that the release of HGF from matrix-bound stores may be particularly important for the bioavailability of HGF in the alveolar spaces. In the current study, we have shown that bleomycin-induced injury caused a marked increase in HGF level in BAL fluids. However, bleomycin did not cause an increase in HGF mRNA or protein levels in lung tissue from PAI-1$^{+/+}$or PAI-1$^{-/-}$mice. Given the known ability of plasmin to digest proteoglycans, a plausible explanation for these results is that the enhanced generation of plasmin in PAI-1$^{-/-}$ mice was responsible for increasing the release of HGF from ECM leading to its increased detection in BAL fluid. In support of this is our previous finding that fibrinolytic activity within the alveolus of PAI-1$^{-/-}$ mice is increased compared to PAI-1$^{+/+}$ mice.[13] To confirm that plasmin was involved in the increase in BAL HGF levels, we administered tranexamic acid, which blocks plasminogen activation and plasmin activity. We previously reported that tranexamic acid decreases fibrinolytic activity within the lung of bleomycin-treated mice.[13] In support of our hypothesis, we found that tranexamic acid treatment reduced BAL HGF levels in both groups of bleomycin-treated mice. These findings further support the supposition that plasmin-mediated breakdown of ECM is involved in releasing HGF into the alveolar spaces.

Biologically active HGF is a heterodimeric protein that is produced from a single-chain precursor by proteolytic processing. Four proteases are reported to activate HGF *in vitro*, namely, HGF activator, blood coagulation factor XIIa,[49] urokinase, and tissue-type plasminogen activator.[21] Because the activity of urokinase is retained in the airways of PAI-1$^{-/-}$ mice following bleomycin-induced lung injury due to the absence of its major inhibitor, we initially hypothesized that HGF activation would be more efficient in PAI-1$^{-/-}$ mice. However, in the current study, we found that HGF recovered in BAL fluid from both PAI-1$^{-/-}$ and PAI-1$^{+/+}$ mice following bleomycin administration was all in its activated form. This finding indicates that, despite the presence of a major inhibitor of plasminogen activators in PAI-1$^{+/+}$ mice, HGF can be activated in the alveolar spaces.

A previous study reported the detection of a non-glycosylated form of the α-chain of HGF in biological samples, eg, in rat serum.[33] *In vitro* studies demonstrated that deglycosylated HGF retains its full receptor binding and activating properties.[50] Using Western blot analysis, we also detected an immunoreactive band at the same molecular weight as the non-glycosylated HGF α-chain. This band was more abundant in the BAL fluids from PAI-1$^{-/-}$ mice compared to PAI-1$^{+/+}$ mice following bleomycin. The underlining mechanism of its generation and why it is higher in PAI-1$^{-/-}$ mice are uncertain.

Because of embryonic lethality, mice genetically deficient in HGF cannot be used to study loss of HGF func-

tion in disease models. Therefore, strategies to neutralize endogenous HGF with antibodies have been used. Previous reports using an inhibitory anti-HGF antibody have shown that HGF is essential for protection of the heart[27] and lungs[51] from reperfusion injury, maintenance of kidney function following obstructive nephropathy,[52] and wound healing in the skin.[53] In the current study, we showed that treatment of bleomycin-injured PAI-$1^{-/-}$ mice with this inhibitory anti-HGF antibody worsened pulmonary fibrosis while a control antibody did not. This result suggests that *in vivo* neutralization of HGF lessens the protection that PAI-1 deficiency confers on bleomycin-induced pulmonary fibrosis. Although this is the most likely interpretation of the data, we cannot exclude the possibility that a nonspecific inflammatory effect from forming antigen/antibody complexes (HGF/anti-HGF) might have contributed to the worsened fibrosis.

Of particular interest in the present study, intratracheal administration of exogenous urokinase reduced collagen accumulation following bleomycin-induced lung injury. This finding is in agreement with previous work demonstrating that increasing urokinase by any of several different methods reduced fibrosis following bleomycin delivery. Increased urokinase levels were achieved by directly instilling urokinase intratracheally,[34] by systemic administration,[54] by delivery of a urokinase gene to the lung using an adenoviral vector,[35] and by genetically engineering a mouse to have an inducible, lung-specific urokinase gene.[55] These observations support the potential benefits of increasing plasminogen activation within the lung for the treatment and/or prevention of pulmonary fibrosis.

Our results demonstrate that an important part of the mechanism by which urokinase protects the lung from bleomycin-induced fibrosis is via its effects on HGF. This conclusion does not exclude other protective effects including the benefits from generating higher levels of plasmin. Past reports have supported a protective role for plasmin in that plasminogen-deficient mice have been shown to develop worse pulmonary fibrosis following bleomycin-induced injury.[36] In a liver injury model, these mice have a marked impairment in the reparative removal of necrotic liver tissue and this impediment persisted in the absence of fibrin deposition.[56] Deficiency of plasminogen was also reported to impair wound healing in the skin,[57] large arteries,[58] and heart,[59] suggesting a significant involvement of plasmin in tissue repair. Considering that plasminogen activation is retarded during lung injury due to overexpression of PAI-1, restoration of plasmin action by urokinase treatment may provide a favorable environment for tissue repair in the lung, and consequently prevent the development of pulmonary fibrosis.

In conclusion, our results demonstrate a strong linkage between HGF and the plasminogen system in the model of bleomycin-induced lung injury and fibrosis. Enhanced plasminogen activation leads to increased levels of active HGF within the alveolar spaces and contributes to reducing the extent of subsequent fibrosis.

References

1. Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, Crystal RG: Intraluminal fibrosis in interstitial lung disorders. Am J Pathol 1986, 122:443–461
2. Fukuda Y, Ishizaki M, Masuda Y, Kimura G, Kawanami O, Masugi Y: The role of intraalveolar fibrosis in the process of pulmonary structural remodeling in patients with diffuse alveolar damage. Am J Pathol 1987, 126:171–182
3. Kuhn C 3rd, Boldt J, King TE Jr, Crouch E, Vartio T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 1989, 140:1693–1703
4. Kuhn C: The pathogenesis of pulmonary fibrosis. Monogr Pathol 1993, 78–92
5. Idell S, James KK, Levin EG, Schwartz BS, Manchanda N, Maunder RJ, Martin TR, McLarty J, Fair DS: Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. J Clin Invest 1989, 84:695–705
6. Bertozzi P, Astedt B, Zenzius L, Lynch K, LeMaire F, Zapol W, Chapman HA Jr: Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. N Engl J Med 1990, 322: 890–897
7. Chapman HA, Allen CL, Stone OL: Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. Am Rev Respir Dis 1986, 133:437–443
8. Kotani I, Sato A, Hayakawa H, Urano T, Takada Y, Takada A: Increased procoagulant and antifibrinolytic activities in the lungs with idiopathic pulmonary fibrosis. Thromb Res 1995, 77:493–504
9. Idell S, Gonzalez KK, MacArthur CK, Gillies C, Walsh PN, McLarty J, Thrall RS: Bronchoalveolar lavage procoagulant activity in bleomycin-induced lung injury in marmosets: characterization and relationship to fibrin deposition and fibrosis. Am Rev Respir Dis 1987, 136:124–133
10. Idell S, James KK, Gillies C, Fair DS, Thrall RS: Abnormalities of pathways of fibrin turnover in lung lavage of rats with oleic acid and bleomycin-induced lung injury support alveolar fibrin deposition. Am J Pathol 1989, 135:387–399
11. Olman MA, Mackman N, Gladson CL, Moser KM, Loskutoff DJ: Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. J Clin Invest 1995, 96:1621–1630
12. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH: Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest 1996, 97:232–237
13. Hattori N, Degen JL, Sisson TH, Liu H, Moore BB, Pandrangi RG, Simon RH, Drew AF: Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. J Clin Invest 2000, 106:1341–1350
14. Ploplis VA, Wilberding J, McLennan L, Liang Z, Cornelissen I, DeFord ME, Rosen ED, Castellino FJ: A total fibrinogen deficiency is compatible with the development of pulmonary fibrosis in mice. Am J Pathol 2000, 157:703–708
15. Chapman HA: Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 1997, 9:714–724
16. Carmeliet P, Collen D: Development and disease in proteinase-deficient mice: role of the plasminogen, matrix metalloproteinase and coagulation system. Thromb Res 1998, 91:255–285
17. Rifkin DB, Mazzieri R, Munger JS, Noguera I, Sung J: Proteolytic control of growth factor availability. APMIS 1999, 107:80–85
18. Sakai T, Satoh K, Matsushima K, Shindo S, Abe S, Abe T, Motomiya M, Kawamoto T, Kawabata Y, Nakamura T, Nukiwa T: Hepatocyte growth factor in bronchoalveolar lavage fluids and cells in patients with inflammatory chest diseases of the lower respiratory tract: detection by RIA and in situ hybridization. Am J Respir Cell Mol Biol 1997, 16:388–397
19. Dohi M, Hasegawa T, Yamamoto K, Marshall BC: Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med 2000, 162:2302–2307
20. Yaekashiwa M, Nakayama S, Ohnuma K, Sakai T, Abe T, Satoh K, Matsumoto K, Nakamura T, Takahashi T, Nukiwa T: Simultaneous or delayed administration of hepatocyte growth factor equally represses

the fibrotic changes in murine lung injury induced by bleomycin: a morphologic study. Am J Respir Crit Care Med 1997, 156:1937–1944
21. Naldini L, Tamagnone L, Vigna E, Sachs M, Hartmann G, Birchmeier W, Daikuhara Y, Tsubouchi H, Blasi F, Comoglio PM: Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO J 1992, 11:4825–4833
22. Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F, Comoglio PM: Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. J Biol Chem 1995, 270:603–611
23. Pepper MS, Matsumoto K, Nakamura T, Orci L, Montesano R: Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. J Biol Chem 1992, 267:20493–20496
24. Jeffers M, Rong S, Vande Woude GF: Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol 1996, 16:1115–1125
25. Santos OF, Barros EJ, Yang XM, Matsumoto K, Nakamura T, Park M, Nigam SK: Involvement of hepatocyte growth factor in kidney development. Dev Biol 1994, 163:525–529
26. Ohmichi H, Koshimizu U, Matsumoto K, Nakamura T: Hepatocyte growth factor (HGF) acts as a mesenchyme-derived morphogenic factor during fetal lung development. Development 1998, 125:1315–1324
27. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T: Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest 2000, 106:1511–1519
28. Sakamaki Y, Matsumoto K, Mizuno S, Miyoshi S, Matsuda H, Nakamura T: Hepatocyte growth factor stimulates proliferation of respiratory epithelial cells during postpneumonectomy compensatory lung growth in mice. Am J Respir Cell Mol Biol 2002, 26:525–533
29. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA: Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor β (1). J Exp Med 2001, 194:809–821
30. Chung MP, Monick MM, Hamzeh NY, Butler NS, Powers LS, Hunninghake GW: Role of repeated lung injury and genetic background in bleomycin-induced fibrosis. Am J Respir Cell Mol Biol 2003, 29:375–380
31. Adamson IY, Bakowska J: Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. Am J Pathol 1999, 155:949–954
32. Nakamura T, Nawa K, Ichihara A, Kaise N, Nishino T: Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Lett 1987, 224:311–316
33. Wang SN, Lapage J, Hirschberg R: Glomerular ultrafiltration of IGF-I may contribute to increased renal sodium retention in diabetic nephropathy. J Lab Clin Med 1999, 134:154–160
34. Hart DA, Whidden P, Green F, Henkin J, Woods DE: Partial reversal of established bleomycin-induced pulmonary fibrosis by rh-urokinase in a rat model. Clin Invest Med 1994, 17:69–76
35. Sisson TH, Hattori N, Xu Y, Simon RH: Treatment of bleomycin-induced pulmonary fibrosis by transfer of urokinase-type plasminogen activator genes. Hum Gene Ther 1999, 10:2315–2323
36. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA: The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. Am J Pathol 2000, 157:177–187
37. Zarnegar R, Michalopoulos GK: The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. J Cell Biol 1995, 129:1177–1180
38. Matsumoto K, Nakamura T: Emerging multipotent aspects of hepatocyte growth factor. J Biochem (Tokyo) 1996, 119:591–600
39. Shiratori M, Michalopoulos G, Shinozuka H, Singh G, Ogasawara H, Katyal SL: Hepatocyte growth factor stimulates DNA synthesis in alveolar epithelial type II cells in vitro. Am J Respir Cell Mol Biol 1995, 12:171–180
40. Ohmichi H, Matsumoto K, Nakamura T: In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. Am J Physiol 1996, 270:L1031–L1039
41. Panos RJ, Rubin JS, Csaky KG, Aaronson SA, Mason RJ: Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for alveolar type II cells in fibroblast-conditioned medium. J Clin Invest 1993, 92:969–977
42. Matsuda Y, Matsumoto K, Yamada A, Ichida T, Asakura H, Komoriya Y, Nishiyama E, Nakamura T: Preventive and therapeutic effects in rats of hepatocyte growth factor infusion on liver fibrosis/cirrhosis. Hepatology 1997, 26:81–89
43. Taniyama Y, Morishita R, Nakagami H, Moriguchi A, Sakonjo H, Shokei K, Matsumoto K, Nakamura T, Higaki J, Ogihara T: Potential contribution of a novel antifibrotic factor, hepatocyte growth factor, to prevention of myocardial fibrosis by angiotensin II blockade in cardiomyopathic hamsters. Circulation 2000, 102:246–252
44. Mizuno S, Kurosawa T, Matsumoto K, Mizuno-Horikawa Y, Okamoto M, Nakamura T: Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. J Clin Invest 1998, 101:1827–1834
45. Ware LB, Matthay MA: Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. Am J Physiol Lung Cell Mol Physiol 2002, 282:L924–L940
46. Lamszus K, Joseph A, Jin L, Yao Y, Chowdhury S, Fuchs A, Polverini PJ, Goldberg ID, Rosen EM: Scatter factor binds to thrombospondin and other extracellular matrix components. Am J Pathol 1996, 149:805–819
47. Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT: Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J Biol Chem 1994, 269:11216–11223
48. Nakao T, Arii S, Kaido T, Mori A, Murata T, Matsumori A, Imamura M: Heparin accelerates liver regeneration following portal branch ligation in normal and cirrhotic rats with increased plasma hepatocyte growth factor levels. J Hepatol 2002, 37:87–92
49. Shimomura T, Miyazawa K, Komiyama Y, Hiraoka H, Naka D, Morimoto Y, Kitamura N: Activation of hepatocyte growth factor by two homologous proteases, blood-coagulation factor XIIa and hepatocyte growth factor activator. Eur J Biochem 1995, 229:257–261
50. Hofmann R, Joseph A, Bhargava MM, Rosen EM, Goldberg I: Scatter factor is a glycoprotein but glycosylation is not required for its activity. Biochim Biophys Acta 1992, 1120:343–350
51. Yamada T, Hisanaga M, Nakajima Y, Mizuno S, Matsumoto K, Nakamura T, Nakano H: Enhanced expression of hepatocyte growth factor by pulmonary ischemia-reperfusion injury in the rat. Am J Respir Crit Care Med 2000, 162:707–715
52. Mizuno S, Matsumoto K, Nakamura T: Hepatocyte growth factor suppresses interstitial fibrosis in a mouse model of obstructive nephropathy. Kidney Int 2001, 59:1304–1314
53. Yoshida S, Yamaguchi Y, Itami S, Yoshikawa K, Tabata Y, Matsumoto K, Nakamura T: Neutralization of hepatocyte growth factor leads to retarded cutaneous wound healing associated with decreased neovascularization and granulation tissue formation. J Invest Dermatol 2003, 120:335–343
54. Ikeda T, Hirose N, Koto H, Hirano H, Shigematsu N: Fibrin deposition and fibrinolysis in the pathogenesis of pulmonary fibrosis. Nihon Kyobu Shikkan Gakkai Zasshi 1989, 27:448–451
55. Sisson TH, Hanson KE, Subbotina N, Patwardhan A, Hattori N, Simon RH: Inducible lung-specific urokinase expression reduces fibrosis and mortality after lung injury in mice. Am J Physiol Lung Cell Mol Physiol 2002, 283:L1023–L1032
56. Bezerra JA, Bugge TH, Melin-Aldana H, Sabla G, Kombrinck KW, Witte DP, Degen JL: Plasminogen deficiency leads to impaired remodeling after a toxic injury to the liver. Proc Natl Acad Sci USA 1999, 96:15143–15148
57. Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, Dano K: Impaired wound healing in mice with a disrupted plasminogen gene. Nat Med 1996, 2:287–292
58. Drew AF, Tucker HL, Kombrinck KW, Simon DI, Bugge TH, Degen JL: Plasminogen is a critical determinant of vascular remodeling in mice. Circ Res 2000, 87:133–139
59. Creemers E, Cleutjens J, Smits J, Heymans S, Moons L, Collen D, Daemen M, Carmeliet P: Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. Am J Pathol 2000, 156:1865–1873