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Reduction of Bleomycin-Induced Pulmonary Fibrosis by Serum Amyloid P

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Fibrotic diseases such as scleroderma, severe chronic asthma, pulmonary fibrosis, and cardiac fibrosis kill tens of thousands of people each year in the U.S. alone. Growing evidence suggests that in fibrotic lesions, a subset of blood monocytes enters the tissue and differentiates into fibroblast-like cells called fibrocytes, causing tissue dysfunction. We previously found that a plasma protein called serum amyloid P (SAP) inhibits fibrocyte differentiation in vitro. Bleomycin treatment is a standard model for pulmonary fibrosis, and causes an increase in collagen, fibrocytes, and leukocytes in the lungs, and a decrease in peripheral blood hemoglobin oxygen saturation. We find that injections of rat SAP in rats reduce all of the above bleomycin-induced changes, suggesting that the SAP injections reduced the bleomycin-induced pulmonary fibrosis. We repeated these studies in mice, and find that injections of murine SAP decrease bleomycin-induced pulmonary fibrosis. To confirm the efficacy of SAP treatment, we used a delayed treatment protocol using SAP from day 7 to 13 only, and then measured fibrosis at day 21. Delayed SAP injections also reduce the bleomycin-induced decrease in peripheral blood hemoglobin oxygen saturation, and an increase in lung collagen, leukocyte infiltration, and fibrosis. Our data suggest the possibility that SAP may be useful as a therapy for pulmonary fibrosis in humans. The Journal of Immunology, 2007, 179: 4035–4044.

Pulmonary fibrosis is a common problem of end-stage lung disease caused by environmental toxins, radiation, or chemotherapy treatments for cancer or many chronic inflammatory diseases (1–3). Fibrosis leads to reduced lung function and has a high mortality rate (4). The processes that drive fibrosis are dynamic, involving infiltrating leukocytes, activation and proliferation of fibroblast-like cells, destruction of the alveolar structures, and the deposition of extracellular matrix proteins. These events appear to be due to repeated and/or aberrant repair events (1, 5). There is currently no Food and Drug Administration-approved therapy for pulmonary fibrosis or other fibrosing diseases (6).

Bleomycin is the primary chemotherapeutic drug for testicular cancer, but lung fibrosis is a side effect in ~10% of patients (2). Bleomycin-induced lung fibrosis is the most frequently used rodent model of lung fibrosis, and produces inflammatory and fibrotic events similar to those seen in human pulmonary fibrosis (7). Bleomycin administration into rodents leads to the accumulation of leukocytes, especially macrophages, followed by the activation of fibroblasts and fibroblast-like cells and the deposition of collagen (8–10).

Despite impressive advances in the field, much remains to be understood about the source of the fibroblast-like cells that are thought to be responsible for lung fibrosis. One hypothesis is that local interstitial lung fibroblasts migrate into the affected area and produce extracellular matrix proteins, leading to fibrosis (1). An alternative hypothesis is that in addition to migration of local fibroblasts, circulating bone marrow-derived fibroblast precursors present within the blood are attracted to sites of injury, where they differentiate into spindle-shaped fibroblast-like cells called fibrocytes and at least in part mediate tissue repair (11, 12). Fibrocytes express markers of both hematopoietic cells (CD45, MHC class II, CD34) and stromal cells (collagen-I, collagen-III, and fibronectin) (13, 14). Fibrocyte precursors appear to differentiate from a subpopulation of CD14+ peripheral blood monocytes (14–16).

In irradiated mice engrafted with GFP-expressing bone marrow cells, bleomycin installation into the trachea causes fibrosis in the lungs, and the fibrotic lesions contained GFP-expressing cells (17). Very few GFP-expressing cells were found in the lungs of control mice. The GFP-expressing cells in the lungs of the bleomycin-treated mice expressed collagen-I and the chemokine receptors CCR7 and CXCR4, which are also expressed by fibrocytes (14, 15, 17). When cultured in vitro, the GFP-expressing cells also had the spindle-shaped morphology of fibrocytes (17). Installation of FITC into the lung also causes pulmonary fibrosis in mice, and this fibrosis also involves the recruitment of bone marrow-derived fibrocytes (18). Injections of mature fibrocytes into bleomycin- or FITC-treated mice augment pulmonary fibrosis (12, 19). These observations indicate that bone marrow-derived fibrocytes participate in pulmonary fibrosis.

We previously found that fibrocyte differentiation is inhibited by the plasma protein serum amyloid P (SAP) (3, 15, 20, 21). SAP is a member of the pentraxin family of proteins that include C-reactive protein (CRP). SAP is not related to serum amyloid A, serum amyloid protein, or amyloid precursor protein (15, 20, 22). SAP is a 27-kDa protein that is produced by the liver, secreted into
the blood, and circulates as stable 135-kDa pentamers (23, 24). SAP binds to apoptotic cells, DNA, and certain microorganisms, and is cleared by macrophage-like cells through FcγRs (25–28). In humans and rats, the levels of SAP in the serum are maintained at relatively constant levels, with CRP acting as an acute-phase response protein (23). In mice, SAP acts as an acute-phase protein, and CRP is at low steady-state levels (23, 29). However, human, rat, and mouse SAP bind to the same molecules, although with differing affinities, indicating a functional similarity (30, 31). Injections of SAP into humans, mice, and rats appear to have no toxic effects (32, 33).

The ability of SAP to inhibit fibrocyte differentiation in vitro, and the observation that fibrocytes play a role in animal models of lung fibrosis, suggested that SAP might be able to reduce fibrocyte accumulation or fibrocyte differentiation in vivo, and thus reduce lung fibrosis. We report in this work that SAP injections in rats and mice diminish bleomycin-induced lung fibrosis.

Materials and Methods

Production of murine and rat SAP

Native rat or murine SAP was prepared from commercially available serum (Sigma-Aldrich or Gemini Bio-Products) using calcium-dependent binding to phosphoethanolamine-conjugated agarose, following standard purification techniques (33–35). Rat SAP was then repurified using pneumococcal C-polysaccharide-coated beads to remove any excess CRP (35). The purified rat or mouse SAP was diluted with 20 mM sodium phosphate buffer (pH 7.4) and concentrated with a 15-ml centrifugal filter device (UFV2BGC40; Millipore). This procedure was repeated to obtain 500 μg/ml SAP in 20 mM sodium phosphate buffer (pH 7.4). Endotoxin levels were tested using the Limulus amebocyte lysate assay (E-Toxate; Sigma-Aldrich) and were also tested using HEK293 cells transfected with the LPS receptors CD14 and TLR4 (HEK-Blue LPS detection kit; InvivoGen). Endotoxin levels were always below detectable levels. Analysis of the SAP by Coomassie- and silver-stained polyacrylamide gels (Bio-Rad) revealed only one band at 25–27 kDa under reducing conditions, and a single 130- to 135-kDa band under nonreducing conditions (Fig. 1 and data not shown). The identity of the protein (either rat or mouse SAP) was confirmed by Western blotting using goat anti-rat SAP or sheep anti-mouse SAP polyclonal Abs (R&D Systems), as described previously (15) (Fig. 1). BenchMark protein and MagicMark XP m.w. markers (Invitrogen Life Technologies) were used for reducing and Western blot gels, respectively. BSA (Pierce), commercially purified human CRP, human SAP, and murine SAP (EMD Biosciences-Calbiochem), and rat CRP (R&D Systems) were used as loading controls.

Animal models of pulmonary fibrosis

Lung fibrosis was induced in 150-g male Sprague-Dawley rats (Charles River Laboratories) with an intratracheal instillation of 100 μl of 10 U/ml bleomycin (EMD-Calbiochem), as previously described (36). Lung fibrosis was induced in 20-g male C57BL/6 mice (The Jackson Laboratory) with an intratracheal instillation of 60 μl of bleomycin (3 U/kg). All animals were used in accordance with rules published by the National Institutes of Health. The Rice University Institutional Animal Use and Care Committee approved the rat procedures (which were performed at Rice University), and the M.D. Anderson Cancer Center Institutional Animal Use and Care Committee approved the mouse procedures (which were performed at M.D. Anderson Cancer Center).

Injections of SAP

Following bleomycin instillation, rats were injected with an i.p. injection of 240 μg of rat SAP or an equal volume of sodium phosphate buffer the day after bleomycin treatment and again on days 3, 5, 7, and 9. Alternatively, rats were injected with SAP on days 7, 9, 11, and 13. Mice were treated daily, starting the day after bleomycin injection, with an i.p. injection of either 50 μg of murine SAP or an equal volume of sodium phosphate buffer. Intrapriseonel injections of SAP reach the blood within 1 h (30, 37).

Measurement of peripheral blood oxygen content

To assess peripheral blood oxygen content in vivo, rats were monitored for the percentage of hemoglobin saturated with oxygen (pulse Ox). Rats were briefly sedated with 4% isoflurane in 4 L/min oxygen. Rats were then removed to room air, and a peripheral pulse Ox sensor (9847V monitor with a 2000SL sensor; Nonin Medical) was attached to the left rear paw. Pulse Ox readings were taken as the animal regained consciousness.

Quantification of collagen

Rats and mice were euthanized at day 14 or 21 after bleomycin instillation, and the lungs were perfused by injections of PBS into the right ventricle of the heart to remove blood. For rats, the whole right lung was removed, weighed, and minced into small pieces, and whole lung collagen content was assessed by the Sircol collagen assay (Biocolor), according to the manufacturer’s instructions (12). For mice, collagen was assessed on formalin-fixed paraffin-embedded sections of whole lung, as described previously (38, 39). Briefly, 15-μm sagittal (longitudinal from top to bottom) sections were cut, and 10 sections from across the lung were used to quantify collagen content. Sections were deparaffinized, and then incubated for 30 min at room temperature with a saturated solution of picric acid in distilled water containing 0.1% fast green FCF and 0.1% Sirius red F3BA (Polysciences). Sections were then rinsed with distilled water, and the dye was eluted with a mixture of 0.1 N NaOH and absolute methanol (1:1, v/v). Spectrophotometer readings were taken at 540 and 605 nm (corresponding to the maximum absorbance of Sirius red and fast green, respectively). The absorbances were used to calculate the amount of collagen and noncollagenous protein in the samples. Collagen content is expressed as a percentage of total protein.

Immunohistochemistry

For rats, the left lung was inflated in prewarmed OCT (VWR) and then embedded in OCT, frozen on dry ice, and then stored at –80°C. Immunohistochemistry was performed, as described previously (40, 41). Briefly, 10-μm cryosections were mounted on Superfrost Plus microscope slides (VWR). Sections were fixed in acetone for 30 min at room temperature. Non-specific binding was then blocked by incubation in 4% BSA (fraction V, globulin free; Sigma-Aldrich) in PBS for 60 min. Slides were then incubated with 5 μg/ml Primary Abs in PBS containing 4% BSA for 60
FIGURE 2. SAP injections reduce bleomycin-induced histological changes in rat lungs. A, Cryosections from day 14 rat lungs were stained with H&E to show cellular inflammation, or picrosirius red to show total collagen deposition. Sections were also stained with anti-collagen-I or anti-α-SMA Abs. Bars are 200 μm. **Bottom row**, Sections were also dual labeled with anti-α-SMA Abs (green) and the nuclear counterstain DAPI (blue), at higher magnification to show α-SMA expression in individual cells. Bar in bottom row is 50 μm. Photomicrographs are representative sections of three to eight animals per group. Saline, intratracheal instillation of saline and then injections of phosphate buffer; Bleo, intratracheal instillation of bleomycin and then injections of buffer; Bleo + SAP, intratracheal instillation of bleomycin and then SAP injections; Saline + SAP, intratracheal instillation of saline and then SAP injections. **B–D**, Low magnification images of cryosections from day 14 rat lungs. Sections were stained with picrosirius red to show collagen deposition. **B**, Intratracheal instillation of saline; **C**, intratracheal instillation of bleomycin, and arrows point to areas of fibrosis. **D**, Intratracheal instillation of bleomycin and then SAP injections. Bar is 2 mm.

For immunofluorescence staining, following incubation with primary Abs, as described above, or α-SMA directly conjugated to Cy3 (clone 1A4; Sigma-Aldrich), slides were incubated with biotinylated rat F(ab')2 anti-mouse IgG (Jackson ImmunoResearch Laboratories) or biotinylated goat F(ab')2 anti-rabbit IgG (Southern Biotechnology Associates) in PBS containing 4% BSA. Sections were then fixed for 30 min in 70% ethanol containing 0.1% Sudan Black B to quench tissue autofluorescence (42). Slides were then washed in PBS and mounted with VectaShield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) for 10 min. Sections were then counterstained for 10 s with Gill’s hematoxylin No. 3 diluted 1/5 with water (Sigma-Aldrich), and were then rinsed in water. Slides were dehydrated through 70, 95, and 100% ethanol; cleared with xylene; and mounted with Permount (VWR).

For immunofluorescence staining, following incubation with primary Abs, as described above, or α-SMA directly conjugated to Cy3 (clone 1A4; Sigma-Aldrich), slides were incubated with biotinylated rat F(ab')2 anti-mouse IgG or Rhodamine Red-X donkey F(ab')2 anti-rabbit IgG (Jackson ImmunoResearch Laboratories). After washing, the biotinylated Abs were detected with streptavidin-Alexa 488 (Invitrogen Life Technologies-Molecular Probes) in PBS containing 4% BSA. Sections were then fixed for 30 min in 70% ethanol containing 0.1% Sudan Black B to quench tissue autofluorescence (42). Slides were then washed in PBS and mounted with VectaShield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). All procedures were at room temperature.

The number of positive cells was assessed by point counting 10 selected fields per section with a random start position, as described previously (40, 43–47). Sections were analyzed from the top, middle, and lower portions of each lung, generating 30 regions of analysis per lung. Results were obtained from at least two individuals blinded to the identity of the sections. Analysis of sections stained with Abs to α-SMA or collagen was analyzed with ImageJ software using standard algorithms to define the areas of staining, after first excluding areas containing vessels that contain α-SMA or collagen (Rasband, W. S., ImageJ, U.S. National Institutes of Health, Bethesda, MD).

Fibrosis scoring in mice

Following euthanasia of mice, the lungs were perfused with 10% neutral buffered formalin, fixed in formalin overnight, and then embedded in paraffin. For each mouse, the lung lobes were separated before being embedded together in paraffin to facilitate analysis. Sectioning, staining with H&E, and video imaging to identify fibrosis in the lung were performed, as previously described (48–51). The fibrosis score from this technique correlates with the score from noninvasive microcomputed tomography scans (52). Results were obtained from two individuals blinded to the identity of the sections.

Statistics

Statistical analysis was performed using GraphPad Prism software. Differences between two groups were assessed by Student’s t test. Differences between multiple groups were assessed by ANOVA using Tukey’s posttest. Significance was defined as p < 0.05. In the figures, * indicates p < 0.05; ** indicates p < 0.01; and *** indicates p < 0.001.

Results

SAP injections reduce bleomycin-induced pulmonary fibrosis in rats

To test the ability of SAP to reduce fibrosis, rats were given an intratracheal instillation of bleomycin (day 0) to induce pulmonary fibrosis. To reduce the possible effects of SAP interacting with bleomycin, we used a single injection of bleomycin rather than a 2-wk continuous dose via osmotic pumps (48). Bleomycin is rapidly cleared from the body of mammals with a t1/2 of ~60 min, so only residual levels of bleomycin were likely to be present in the animals when the SAP injections were started on day 1 (53–56). Rats were then treated with either five injections of 240 μg of rat SAP or phosphate buffer every 2 days (days 1, 3, 5, 7, and 9).
Other rats were given an intratracheal instillation of saline, and then received either SAP or buffer injections. As previously observed, bleomycin instillation led to a significant elevation in collagen levels as determined by Student’s t test. Compared with bleomycin instillation, intratracheal instillation of bleomycin and then SAP injections had a significant reduction in collagen levels as determined by Student’s t test. B and C. Sections were stained with anti-collagen-I (B) or anti-α-SMA Abs (C), and the percentage area stained was quantified as a percentage of the total area of lung. Values are means ± SEM (n = 3–5 per group). Significance was determined by ANOVA. D. Rats (n = 6 per group) were assessed for peripheral blood oxygen content (pulse Ox) at day 14, and compared with values from 25 healthy age-matched male rats (baseline). Values are means ± SEM. Significance was determined by ANOVA.

A key marker of bleomycin-induced lung fibrosis is excessive collagen deposition. As expected, as compared with the saline instillation control, bleomycin instillation led to an elevation in collagen content in the lungs as assessed by picrosirius red staining, whole lung collagen levels, and immunohistochemical staining for collagen-I (Figs. 2 and 3, A and B). Collagen levels in the lungs of rats injected with SAP following bleomycin instillation were significantly lower than collagen levels in the lungs of rats injected

FIGURE 3. SAP injections reduce bleomycin-induced changes in rat lungs. A. Whole right lungs were removed at day 14, and assessed for collagen content. Values are means ± SEM (n = 4–5 per group). Compared with saline, bleomycin instillation led to a significant elevation in collagen levels as determined by Student’s t test. Compared with bleomycin instillation, intratracheal instillation of bleomycin and then SAP injections had a significant reduction in collagen levels as determined by Student’s t test. B and C. Sections were stained with anti-collagen-I (B) or anti-α-SMA Abs (C), and the percentage area stained was quantified as a percentage of the total area of lung. Values are means ± SEM (n = 3–5 per group). Significance was determined by ANOVA. D. Rats (n = 6 per group) were assessed for peripheral blood oxygen content (pulse Ox) at day 14, and compared with values from 25 healthy age-matched male rats (baseline). Values are means ± SEM. Significance was determined by ANOVA.

FIGURE 4. SAP injections reduce bleomycin-induced leukocyte infiltration in rat lungs. Cryosections of rat lung were labeled with Abs against CD32 (FcyRII-bearing B cells, neutrophils, and macrophages), CD45 (total leukocytes), and CD68 (macrophages). A, Representative photomicrographs of lungs at day 14. Bars are 200 μm. B–D, Quantification of the numbers of cells stained for CD32 (B), CD45 (C), and CD68 (D). Values are means ± SEM (n = 3–6 rats per group). Significance was determined by ANOVA.
with saline following bleomycin instillation (Figs. 2 and 3, A and B). Injections of SAP in saline-instilled rats had no obvious effect on collagen levels in the lungs.

Another marker of lung fibrosis is the increased expression of $\alpha$-SMA, a marker for activated fibroblasts and fibrocytes (14, 20, 57). As expected, intratracheal instillation of bleomycin led to an increase in the expression of $\alpha$-SMA compared with the saline instillation control (Figs. 2 and 3 C). There was an increase in the number of individual $\alpha$-SMA-positive cells in the alveoli of rats following instillation of bleomycin (Fig. 2). Levels of $\alpha$-SMA in the lungs of rats injected with SAP following bleomycin instillation were significantly lower than the levels in bleomycin/saline rat lungs (Figs. 2 and 3 C). Injections of SAP in saline-instilled rats had no obvious effect on $\alpha$-SMA in the lungs.

Pulse oximetry is a simple, noninvasive method to determine peripheral blood oxygen content, and is widely used in the

FIGURE 5. SAP injections reduce the number of bleomycin-induced fibrocytes in rat lungs. A, Representative photomicrographs of lungs at day 14. Cryosections of rat lungs were labeled with Abs to collagen-I (red) and CD45 (green), and were counterstained with DAPI (blue). Bar is 200 $\mu$m. B, Regions from A at higher magnification show individual cells dual labeled (yellow) for collagen-I and CD45. Bar is 50 $\mu$m. C and D, Quantification of the numbers of cells stained for collagen-I and CD45 (C), or $\alpha$-SMA and CD45 (D). Values are means ± SEM, n = 3–6 per group. Significance was determined by ANOVA.

FIGURE 6. SAP injections reduce bleomycin-induced pulmonary fibrosis in mice. A, Percentage change in body weight in mice given intratracheal bleomycin alone (Bleo) or intratracheal bleomycin plus SAP (Bleo + SAP), compared with age-matched saline controls (Saline). B and C, SAP injections decrease pulmonary fibrosis (B) and percentage collagen content (C) in lungs of bleomycin-treated mice. Values are means ± SEM, n = 6. Significance was determined by ANOVA. D, Photomicrographs of H&E-stained sections of whole lungs from individual mice instilled with saline, bleomycin, or bleomycin, and then injected with murine SAP. Arrows point to areas of fibrosis. Each lung lobe was separated before being embedded in paraffin to facilitate analysis. Bars are 2 mm.
management of critically ill patients. To assess peripheral blood oxygen content in vivo, rats were monitored for the percentage of hemoglobin saturated with oxygen (pulse Ox). Intratracheal instillation of bleomycin led to a reduction in pulse Ox readings as compared with saline controls (Fig. 3D). Pulse Ox readings of rats injected with SAP following bleomycin instillation were similar to both the levels in saline control rats and baseline measurements from 25 healthy age-matched rats. Together, our data indicate that SAP injections reduce the severity of bleomycin-induced pulmonary fibrosis.

**SAP injections reduce bleomycin-induced leukocyte infiltration into rat lungs**

Besides collagen deposition, bleomycin instillation induces a profound leukocyte infiltration into lungs (9, 12, 17, 58). Therefore, we assessed whether SAP injections led to an alteration of leukocyte numbers in rat lungs 14 days after bleomycin instillation. Bleomycin instillation induced significant increases in the number of CD32-, CD45-, and CD68-positive leukocytes, compared with saline controls (Fig. 4). The increased numbers of CD32-, CD45-, and CD68-positive cells were present not only in areas of collapsed alveoli and fibrosis, but also in the interstitial space between alveoli. Compared with bleomycin/saline, there were decreased numbers of CD32-, CD45-, or CD68-positive cells in lungs from rats injected with SAP following bleomycin instillation (Fig. 4). Injections of SAP in saline-instilled rats had no obvious effect on leukocyte infiltration in the lungs. These data indicate that SAP injections reduce bleomycin-induced CD32-, CD45-, and CD68-positive leukocyte infiltration in rat lungs.

**SAP injections reduce the number of bleomycin-induced fibrocytes in rat lungs**

We also assessed whether fibrocyte numbers were altered following SAP injections. CD45 and collagen-I dual-positive cells (fibrocytes) were elevated following bleomycin instillation (Fig. 5, A–C), as described previously (12, 19). Compared with bleomycin/saline, there was a significant decrease in the number of CD45/collagen-I dual-positive cells in lungs from rats injected with SAP following bleomycin instillation (Fig. 5, A–C). Injections of SAP in saline-instilled rats had no obvious effect on the number of CD45/collagen-I-positive fibrocytes.

Double labeling with α-SMA and CD45 to identify activated fibrocytes indicated that bleomycin instillation led to a significant increase in the number of activated fibrocytes compared with saline controls (Fig. 5D). As above, compared with bleomycin/saline, there was a significant decrease in the number of activated fibrocytes in lungs from rats injected with SAP following bleomycin instillation (Fig. 5D). Compared with saline controls, injections of SAP in saline-instilled rats had no statistically significant effect on activated fibrocytes in the lungs. Together our data indicate that SAP injections reduce the bleomycin-induced increase in the number of fibrocytes and activated fibrocytes in rat lungs.

**SAP injections reduce bleomycin-induced pulmonary fibrosis in mice**

To test whether SAP injections were effective in another species, we repeated these studies in mice. Compared with bleomycin-treated mice not given SAP, mice injected with murine SAP after bleomycin instillation had significantly less weight loss (Fig. 6A). The pulmonary fibrosis induced by bleomycin instillation in mice injected with SAP was significantly lower compared with bleomycin-treated mice not injected with SAP (Fig. 6B). Collagen levels in the lungs of mice injected with murine SAP following bleomycin instillation were similar to the levels in control rat lungs (Fig. 6C). Analysis of whole lung sections indicates that there was no apparent fibrosis in the saline-treated mice (Fig. 6D, top panels). The mice instilled with bleomycin had profound fibrosis, both adjacent to the bronchi, and at distal sites (Fig. 6D, middle panels). The mice instilled with bleomycin and then injected with SAP had a much reduced level of fibrosis, which was usually restricted to areas near the bronchi (Fig. 6D, lower panels). Together, these data indicate that SAP injections decrease bleomycin-induced lung fibrosis in mice as well as rats.

**Delayed SAP injections reduce bleomycin-induced pulmonary fibrosis in rats**

To test the efficacy of SAP treatment on an ongoing fibrotic response, rats were given an intratracheal instillation of bleomycin (day 0) to induce pulmonary fibrosis, as described above. We then...
delayed treatment with SAP until day 7, which would likely reduce the possible effects of SAP interacting with apoptotic material generated by bleomycin instillation during the first 12–48 h (59–61).

Rats were treated with injections of 240 μg of rat SAP on days 7, 9, 11, and 13. Intratracheal instillation of bleomycin led to a reduction in pulse Ox readings at days 7, 14, and 21, as compared with saline control (Fig. 7B–F). Cryosections from day 21 rat lungs were stained with picrosirius red to show collagen deposition. B. Intratracheal instillation of saline; C and D, intratracheal instillation of bleomycin; E and F, intratracheal instillation of bleomycin and then delayed SAP injections. Asterisks indicate areas of fibrosis. Bars are 2 mm.

**FIGURE 8.** Delayed SAP injections reduce bleomycin-induced fibrosis in rat lungs. A. Whole right lungs were removed at day 21 and assessed for lung collagen content. Values are means ± SEM (n = 6–7 per group). B–F. Cryosections from day 21 rat lungs were stained with picrosirius red to show collagen deposition. B. Intratracheal instillation of saline; C and D, intratracheal instillation of bleomycin; E and F, intratracheal instillation of bleomycin and then delayed SAP injections. Asterisks indicate areas of fibrosis. Bars are 2 mm.

**FIGURE 9.** Delayed SAP injections reduce bleomycin-induced inflammation in rat lungs. A–C, Cryosections from day 21 rat lungs were stained with CD45 to show cellular inflammation. A, Intratracheal instillation of saline; B, intratracheal instillation of bleomycin; C, intratracheal instillation of bleomycin and then delayed SAP injections. Bars are 200 μm. D–F, Cryosections of rat lung were labeled with Abs against CD32, CD45, and CD68. Quantification of the numbers of cells stained for CD32 (D), CD45 (E), and CD68 (F). Values are means ± SEM (n = 4 for controls, n = 6 for bleo and bleo + SAP).
with saline controls (Fig. 7). Pulse Ox readings of rats that were to be injected with SAP on days 7, 9, 11, and 13 following bleomycin instillation had reduced pulse Ox readings at day 7 (before SAP treatment began), indicating that these animals received a similar insult to the bleomycin-untreated rats (Fig. 7A). However, at days 14 and 21, the pulse Ox readings of rats treated with delayed SAP injections were significantly higher than the bleomycin/saline readings (Fig. 7, B and C). These data indicate that delayed SAP injections reduce bleomycin-induced lung dysfunction.

As seen with rats at day 14, rats at day 21 had an elevation in collagen content in the lungs as assessed by whole lung collagen levels (Fig. 8A) and picrosirius red staining (Fig. 8, B–F). Collagen levels in the lungs of rats injected with SAP from days 7 to 13 following bleomycin instillation were similar to the levels in control rat lungs (Fig. 8A). Analysis of low power images of lung sections indicated that there was no apparent fibrosis in the saline-treated rats (Fig. 8B). The rats instilled with bleomycin had extensive fibrosis (Fig. 8, C and D). The rats instilled with bleomycin and then injected with SAP had a much reduced level of fibrosis (Fig. 8, E and F).

We also assessed whether delayed SAP injections affect leukocyte infiltration in rats following bleomycin instillation. At day 21, bleomycin instillation induced significant increases in the number of CD32-CD45, and CD68-positive leukocytes, compared with saline controls (Fig. 9). Compared with bleomycin/saline, delayed injections of SAP caused a significant decrease in the numbers of CD32-, CD45-, and CD68-positive cells in the lungs (Fig. 9, D–F). These data indicate that the delayed SAP injection protocol was also able to reduce bleomycin-induced CD32-, CD45-, and CD68-positive leukocyte infiltration in rat lungs.

Discussion
We have shown that injections of a plasma protein, SAP, are able to decrease bleomycin-induced lung fibrosis in both rats and mice. In rats, SAP injections significantly reduced the increase in numbers of leukocytes, fibrocytes, and activated fibrocytes associated with bleomycin-induced lung fibrosis. SAP injections not only reduced pathological changes in lung morphology, but also maintained peripheral blood oxygen content, as assessed by pulse Ox measurements.

The concentrations of SAP injected into rats and mice were designed to only double the normal physiological dose of SAP. Rats have ~30 μg/ml circulating SAP levels, and C56BL/6 mice have ~10–20 μg/ml SAP (29, 35, 62). Assuming that a 150-g rat has ~8 ml of serum and the serum concentration of SAP in a rat is ~30 μg/ml, 240 μg of SAP will approximately double the serum level of SAP (35). Similarly, a 20-g mouse has ~1.4 ml of blood, and the serum concentration of SAP in C57BL/6 mice is ~15 μg/ml, so 50 μg of murine SAP will approximately double the serum level of SAP (62).

We previously found that SAP inhibits fibrocyte differentiation (15, 20, 21). Fibrosis involves fibrocytes, so the ability of SAP injections to reduce bleomycin-induced pulmonary fibrosis may be due to SAP inhibiting fibrocyte differentiation. Alternatively, SAP may reduce fibrosis by reducing the accumulation of mature fibrocytes or their precursors. However, we have also observed that SAP reduces the bleomycin-induced increase in the number of leukocytes in lungs. Because fibrocytes secrete cytokines that promote leukocyte recruitment (63), and SAP decreases the accumulation of fibrocytes, SAP may reduce levels of these inflammatory cytokines, and thus reduce leukocyte recruitment. A second possibility is that SAP may directly inhibit the recruitment of leukocytes into lungs following bleomycin instillation. A third possibility is that because SAP binds apoptotic material, treatment with SAP may accelerate clearance of apoptotic material following instillation of bleomycin or during fibrosis (61). As the presence of apoptotic material recruits leukocytes, the rapid removal of this material by SAP may decrease leukocyte recruitment after bleomycin instillation or during fibrosis (64).

The reduced fibrosis in rats treated with the delayed SAP treatment protocol indicates that SAP may be able to affect an ongoing fibrosis. The treatment of SAP from days 7 to 13 would most likely preclude the effect of SAP binding to either bleomycin or apoptotic material generated during the acute stage of bleomycin toxicity (53–56, 59–61). The peak of leukocyte and fibrocyte accumulation in lungs following bleomycin or FITC instillation occurs between days 7 and 14 (12, 13, 18, 65). Our data indicate that treatment with SAP during this time either inhibits the differentiation of fibrocyte precursors into fibrocytes in situ, or prevents their initial accumulation in the lung.

Besides pulmonary fibrosis, there is now clear evidence that fibrocytes are involved in other fibrosing diseases. In humans, fibrocytes have been detected in tumors, skin wounds following either burns or incisions, hypertrophic scars, bronchial asthma, and nephrogenic fibrosing dermopathy (11, 65–67). In animal models, fibrocytes are associated with experimental fibrosis induced by irradiation damage, bleomycin injections into the skin, intimal hyperplasia of the carotid artery, systemic acetaminophen (paracetamol) administration, unilateral ureteral obstruction, bile duct ligation, chronic granuloma formation following Schistosoma japonicum infection, and skin wounding (11, 68–72). We have also recently found that fibrocytes are involved in cardiac fibrosis following ischemia-reperfusion injury in mice, and that SAP inhibits this fibrosis (21). These studies clearly indicate that bone marrow-derived fibrocytes are involved in many forms of fibrosis. We have shown in this study that SAP injections reduce bleomycin-induced pulmonary fibrosis in both rats and mice. These results suggest that SAP may be useful as an anti-fibrotic therapy for pulmonary fibrosis.

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Disclosures
Rice University has patent applications on the use of SAP to inhibit fibrosis, and this intellectual property has been licensed to Promedior. D. Pilling and R.H. Gomer are founding members of, have equity in, and receive royalties from Promedior.

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