Red blood cells increase secretion of matrix metalloproteinases from human lung fibroblasts in vitro

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Fredriksson, K., X. D. Liu, J. Lundahl, J. Klominek, S. I. Rennard, and C. M. Skold. Red blood cells increase secretion of metalloproteinases from human lung fibroblasts in vitro. Am J Physiol Lung Cell Mol Physiol 290: L326–L333, 2006; doi:10.1152/ajplung.00057.2005.—Tissue remodeling is an important process in many inflammatory and fibrotic lung disorders. RBC may in these conditions interact with extracellular matrix (ECM). Fibroblasts can produce and secrete matrix components, matrix-degrading enzymes (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Imbalance in matrix synthesis/degradation may result in rearrangement of tissue architecture and lead to diseases such as emphysema or fibrosis. Neutrophil elastase (NE), a protease released by neutrophils, is known to activate MMP. We hypothesized that RBC can stimulate secretion of MMPs from human lung fibroblasts and that NE can augment this effect. Human fetal lung fibroblasts were cultured in floating collagen gels with or without RBC. After 4 days, the culture medium was analyzed with gelatin zymography, Western blot, and ELISA for MMP-1, -2, -3, and TIMP-1, -2. RBC augmented NE-induced fibroblast-mediated collagen gel contraction compared with NE alone (18.4 ± 1.6%; 23.7 ± 1.4% of initial gel area, respectively). A pan-MMP inhibitor (GM-6001) completely abolished the stimulating effect of NE. Gelatin zymography showed that RBC stimulated MMP-2 activity and that NE enhanced conversion to the active form. Addition of GM-6001 completely inhibited MMP-2 activity in controls, whereas it only partially altered RBC-induced MMP activity. Western blot confirmed the presence of MMP-1 and MMP-3 in fibroblasts stimulated with RBC, and ELISA confirmed increased concentrations of pro-MMP-1. We conclude that stimulation of MMP secretion by fibroblasts may explain the ability of RBC to augment fibroblast-mediated collagen gel contraction. This might be a potential mechanism by which hemorrhage in inflammatory conditions leads to ECM remodeling.

tissue inhibitors of metalloproteinases; neutrophil elastase; erythrocyte; remodeling; emphysema

MATRIX METALLOPROTEINASES (MMPs) constitute a family of zinc endoproteases that share structural domains and are capable of degrading extracellular matrix (ECM) components (5). At present, the human MMP family comprises 23 members that can be divided into several subgroups based on structure and substrate specificity including collagenases, gelatinases, stromelysins, and membrane-type (MT) MMPs. MMPs are synthesized as inactive zymogens in which activation can occur through intracellular, extracellular, or cell surface-mediated proteolytic mechanisms (43). Catalytic functions of MMPs are regulated by a family of tissue inhibitors of metalloproteinases (TIMPs). There are currently four members of the TIMP family (TIMP-1 to -4) that, besides their MMP inhibitory action, can affect cell growth-promoting activity, cell survival-promoting activity, and apoptosis (1, 21, 23). Importantly, MMPs and TIMPs are involved in the turnover of ECM; imbalance in matrix synthesis/degradation may lead to tissue destruction as in emphysema, or fibrotic lesions as in idiopathic pulmonary fibrosis (IPF) (2, 4, 36, 37, 44).

Neutrophils have been associated with a variety of fibrotic disorders. Thus increased numbers of neutrophils have been detected both in bronchoalveolar lavage fluid (BALF) and tissue specimens from patients with pulmonary fibrosis (24, 34) as well as in animal models of the disease (20). Neutrophil elastase (NE), a serine protease released by neutrophils, has also been shown to be elevated in BALF and tissue specimens from patients with pulmonary fibrosis (25, 51). Furthermore, NE-deficient mice are resistant to bleomycin-induced fibrosis (13). In addition, NE stimulate fibroblast-mediated collagen gel contraction, an in vitro model for tissue remodeling (47, 55, 56). Together, these data imply a role of NE as a profibrotic mediator important in the remodeling process.

In a variety of injurious and inflammatory lung disorders, red blood cells (RBC) may interact with the ECM, as illustrated by idiopathic pulmonary hemosiderosis, a disease characterized by lung hemorrhages and where patients frequently develop fibrosis (35). Lines of evidence suggest that RBC have the potential to participate in the inflammatory response and in tissue repair. For example, RBC can efficiently bind inflammatory mediators such as interleukin-8 (IL-8); regulated on activation, normal T-expressed and secreted (RANTES); and monocyte chemotactic protein 1. These mediators can also be released from the RBC, indicating a role for RBC in the regulation of inflammation (8, 9, 12, 33, 53). In line with these data, we have recently demonstrated that RBC can stimulate fibroblast-mediated collagen gel contraction in a time- and concentration-dependent manner and that coculture results in increased release of fibronectin (16). We also showed that RBC can stimulate fibroblast secretion of IL-8 and increase neutrophil migration in vitro (19). Together, the data propose a role for RBC in the remodeling process.

Given a potential role for RBC to coexist with neutrophils, NE, fibroblasts, and ECM in inflammatory lung diseases, we...
hypothesized that RBC can stimulate human lung fibroblast MMP secretion. Moreover, we also tested the hypothesis that NE can stimulate this effect.

MATERIALS AND METHODS

Materials. Type I collagen (rat tail tendon collagen, RTTC) was extracted according to a previously published method (14). In brief, tendons were excised from rat tails, and the tendon sheath and other connective tissues were carefully removed. After repeated washing with Tris-buffered saline (0.9% NaCl, 10 mM Tris, pH 7.5), the tendons were washed in increasing concentrations of ethanol for 24 h. Type I collagen was then extracted in 6 M acetic acid. Protein concentration was determined by weighing a lyophilized aliquot from each lot of collagen.

α1-Antitrypsin (αPI) was purchased from Sigma-Aldrich (St. Louis, MO), and the broad-spectrum inhibitor of MMP (GM-6001) was purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). Human sputum elastase was purchased from Elastin Products (Owensville, MO).

Cell culture. Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured on 100-mm tissue culture dishes (FALCON, Franklin Lakes, NJ) with Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL/Life Technologies, Rockville, MD), 50 μM penicillin G sodium, 50 μg/ml streptomycin sulfate (penicillin-streptomycin, Sigma-Aldrich), and 1 μg/ml amphotericin B (Sigma-Aldrich). The fibroblasts were passaged weekly and were used between the 15th and 20th passage. Confluent fibroblasts were trypsinized (trypsin-EDTA; Sigma-Aldrich, 0.05% trypsin 0.53 mM EDTA) and resuspended in DMEM without serum containing 0.2% of soybean trypsin inhibitor (Sigma-Aldrich).

Leukocyte-depleted RBC concentrates were obtained from healthy blood donors at the blood bank at Karolinska Hospital (Stockholm, Sweden). Blood was collected in blood bags (Terumo Imuflex; TERUMO, Tokyo, Japan) containing 63 ml of CPD (Acidum citrum, Natrii citras, Mononatri phras, Dextrosum, Aq ad) and 100 ml SAGM (Natri chloridum, Adenine, Dextrosum, Mannitolum, Aq ad). The bags were centrifuged, and the plasma anduffy coat were removed. The erythrocyte bag was thereafter stored for 24 h in +4°C, and the erythrocytes were filtered through a leukocyte removal filter to obtain leukocyte-reduced RBC concentrate. Five milliliters of the filtered RBC concentrate were removed from the bag with a sterile technique routinely used for quality controls. This aliquot was washed by centrifugation (1,700 g, for 10 min) four times in 40 ml of sterile phosphate-buffered saline (PBS), to remove SAGM and plasma. The RBC was resuspended in DMEM without FBS and then counted in a hemocytometer to determine the RBC concentration. The leukocyte-depleted erythrocyte concentrates are routinely quality controlled at the Karolinska Hospital blood bank by manual counting of leukocytes in a Nageotte chamber. Each bag (300 ml) contains an average of 270 leukocytes, which gives a concentration of <1 leukocyte/ml gel. The RBC concentrate did not contain any significant amount of platelets (<350 platelets/5 × 10^6 RBC) (16).

**RBC-conditioned medium.** RBC-conditioned medium (RBC-CM) was prepared by culturing washed RBC in serum-free DMEM (5 × 10^7 RBC/ml) for 48 h (5% CO₂, +37°C). The RBC were removed by centrifugation (1,700 g, 10 min), and the RBC-CM was stored at −70°C until use. The number of RBC did not change during the culture period, indicating no significant disruption or hemolysis of RBC. In addition, free hemoglobin was not detected in the conditioned medium, measured by a spectrophotometric assay, routinely used at the Clinical Chemistry Laboratory, Karolinska University Hospital Huddinge (Stockholm, Sweden).

**Collagen gels.** Collagen gels were prepared as described previously by Mio et al. (32). Briefly, distilled water, RTTC, 4 × DMEM, and fibroblast and RBC suspensions were mixed together to a final concentration of 0.75 mg/mL of collagen, 3 × 10^5 fibroblasts/ml, and physiological ionic strength of 1× DMEM. The RBC were added in a density of 5 × 10^7 RBC/ml gel. Fibroblasts and RBC were added after all the above ingredients had been mixed. Finally, NE, αPI, and GM-6001 were added to the gel solution. The gels were kept on ice during the preparation. Gel solution (550 μl) was then cast into each well of a 24-well tissue culture plate with a 2-cm² growth area (FALCON). Gelation occurred within 30 min at +37°C. After gelation, the gels were released from the surface of the culture well with a sterile spatula, and the gels were cascad into 60-mm culture plates (FALCON) containing 5 ml of serum-free DMEM.

Measurement of gel area. The area of the collagen gels was measured using an image analyzer system (Leica Microsystems, Wetzlar, Germany). The images were recorded by a video camera comprising a zoom lens mounted ~15 cm above a lighted stage. The 60-mm culture dishes rested on this stage, and the gels were observed directly. No special mounting was required for stability. The area of the gels was then captured and processed by computer software (Leica Microsystems), and gel size in the horizontal axis was determined. Vertical dimension was not assessed. With the described method, the area of each gel could be measured with preserved sterility.

Gelatin zymography. Gelatin zymography was performed with a modification of a previously published procedure (29, 54). Culture supernatants from the fibroblast collagen gel cultures were collected and frozen in −70°C until analysis. After thawing, the supernatants were precipitated with 50% ethanol (vol/vol). After centrifugation the supernatants were discarded, and the samples were dried at room temperature. Samples were then dissolved in 50 μl of H2O, and 20 μl of each sample was mixed with equal volume of 2× electrophoresis sample buffer (0.5 M Tris·HCl, pH 6.8, 10% SDS, 0.1% bromphenol blue, and 20% glycerol) and heated for 5 min at 95°C. Forty microliters of each sample were then loaded into each lane, and electrophoresis was performed at 45 mA/gel. After electrophoresis, the gels were soaked with 2.5% (vol/vol) Triton X-100 and gently shaken at 20°C for 30 min. After this, the gels were incubated in the metalloproteinase buffer (0.06 M Tris·HCl, pH 7.5 containing 5 mM CaCl₂ and 1 μM ZnCl₂) for 18 h at 37°C. The gels were then stained with 0.4% Coomassie blue (wt/vol) and rapidly destained with 30% methanol (vol/vol) and 10% acetic acid (vol/vol). The relative density of gelatinolytic bands was determined from scanned images of gels using Scion Image software (Scion, Frederick, MD).

Western blot. To confirm the identity of the MMPs in the culture system, Western blot analysis was performed. The supernatants from three-dimensional cultures were precipitated with 50% ethanol (vol/vol) resuspended in equal volumes of distilled water and 2× sample buffer (0.5 M Tris·HCl, pH 6.8, 10% SDS, 0.1% bromphenol blue, and 20% glycerol). After being heated for 3 min at 95°C, 30 μl of each sample were loaded into wells for electrophoresis. The proteins were transferred in electroblotting buffer (20 mM Tris, pH 8.0, 150 mM glycine, and 20% methanol (vol/vol)) at 20 V for 35 min. The blots were blocked in 5% nonfat milk in PBS-Tween at room temperature for 1 h and were then exposed to the primary antibodies (MMP-1 and MMP-3; Calbiochem, Cambridge, MA) for 1 h and subsequently developed with the use of rabbit anti-mouse IgG horseradish peroxidase (Rockland, Gilbertsville, PA) in conjunction with an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK).

**ELISA.** The amount of pro-MMP-1, MMP-3, and TIMP-1 in the cultures was determined by ELISA. Floating collagen gels containing fibroblasts (3 × 10^5 /ml) or fibroblasts together with RBC (5 × 10^7 /ml) or RBC-CM were cultured with or without NE, GM-6001, and αPI for 4 days. After incubation, the supernatants surrounding the gels were collected and analyzed for pro-MMP-1 and MMP-3 by ELISA kits, following the manufacturer’s instructions.
(R&D Systems, Minneapolis, MN). Briefly, pro-MMP-1 or MMP-3 in the sample was captured by the antibody on the microtiter plate. Subsequently, enzyme-linked monoclonal antibody specific for pro-MMP-1 or MMP-3 was added, followed by the addition of substrate solution. Color development was terminated by the addition of a stop solution, and absorbance was measured in a microtiter plate reader (Bio-Rad) at 450 nm with correction wavelength set at 540 nm.

The concentration of TIMP-1 and TIMP-2 in culture media was also determined by ELISA technique. Ninety-six-well ELISA plates were coated overnight at 4°C with 100 μl of anti-human TIMP-1 or TIMP-2 antibodies (R&D Systems) diluted in Voler’s buffer (pH 9.6). Plates were then washed three times in PBS with 0.05% Tween 20 (pH 7.2–7.4) and 100 μl of recombinant human TIMP-1 standards (31.25–4,000 pg/ml) or TIMP-2 standards (15.62–2,000 pg/ml) were added in duplicate. Samples (diluted 1:100 in PBS for TIMP-1 and 1:20–1:50 for TIMP-2) were added in duplicate to individual wells and incubated at room temperature for 2 h. After three washes, 100 μl of biotinylated anti-human TIMP-1 antibody (R&D Systems) or biotinylated anti human TIMP-2 (R&D Systems) diluted in PBS-Tween were added for 1 h. After another three washes, 100 μl of horseradish peroxidase-avidin conjugate (Zymed, San Francisco, CA), diluted 1:20,000 in PBS-Tween, were added and incubated for 1 h at room temperature. After the final three washes, 200 μl of tetrathiom benzidine substrate were added, and color was developed for 30 min at room temperature. The reaction was stopped by adding 50 μl of stop solution (1 M H2SO4), and the degree of color generated was determined by measuring the optical density at 450 nm in a microplate reader (Bio-Rad).

**Statistical analysis.** Results are presented as means ± SE. Samples with multiple comparisons were analyzed for significance by analysis of variance followed by a post hoc analysis (Tukey). Data were considered as significantly different when \( P < 0.05 \).

**RESULTS**

Fibroblast-mediated collagen gel contraction. Both RBC and NE, added separately, stimulated fibroblast-mediated collagen gel contraction. When cultured together, NE and RBC had an additive stimulatory effect that was present during the whole culture period (Fig. 1A). The pan-MMP inhibitor GM-6001 attenuated basal- and inhibited RBC-induced fibroblast-mediated collagen gel contraction (Fig. 1B). The inhibitor completely abolished the stimulatory effect of RBC and NE when they were added together. Both GM-6001 and the protease inhibitor αPI attenuated the effect of NE on collagen gel contraction. In contrast to GM-6001, αPI only inhibited the effect of NE alone on fibroblast-mediated collagen gel contraction and not by NE and RBC together (Fig. 1C). αPI alone did not affect fibroblast-mediated collagen gel contraction (data not shown). In all experiments RBC-CM affected fibroblast-mediated gel contraction in a similar manner as whole RBC (data not shown).

Gelatin zymography. To evaluate whether RBC and RBC-CM could induce gelatinase activity, gelatin zymography was performed on gel culture supernatants. MMP-2, both active and proform, was elevated in supernatants from fibroblasts cultured with RBC or RBC-CM (Fig. 2A), whereas MMP-9 was not detectable in any of the samples. NE alone and incubated at room temperature for 2 h. After three washes, 100 μl of biotinylated anti-human TIMP-1 antibody (R&D Systems) or biotinylated anti human TIMP-2 (R&D Systems) diluted in PBS-Tween were added for 1 h. After another three washes, 100 μl of horseradish peroxidase-avidin conjugate (Zymed, San Francisco, CA), diluted 1:20,000 in PBS-Tween, were added and incubated for 1 h in room temperature. After the final three washes, 200 μl of tetrathiom benzidine substrate were added, and color was developed for 30 min at room temperature. The reaction was stopped by adding 50 μl of stop solution (1 M H2SO4), and the degree of color generated was determined by measuring the optical density at 450 nm in a microplate reader (Bio-Rad).
increased the active form of MMP-2. Furthermore, NE augmented RBC- and RBC-CM-induced MMP-2 activity. αPI inhibited the stimulatory effect of NE-induced MMP-2 activity, both in control samples and in coculture with RBC and RBC-CM (Fig. 2A).

The pan-MMP inhibitor GM-6001 attenuated the active form of MMP-2 from fibroblasts in control samples and inhibited the effect of NE. In addition, GM-6001 also decreased active MMP-2 release from fibroblasts cocultured with RBC, as well as NE/RBC-induced active form of MMP-2 (Fig. 2B).

Western blot. Western blot analysis for MMP-1 and MMP-3 was performed on the cell culture supernatants surrounding the fibroblast three-dimensional collagen gel cultures. Both MMP-1 and MMP-3 were identified in cultures with fibroblasts cultured together with both RBC and RBC-CM but not in any of the control samples (Fig. 3, A and B). The Western blot analysis did not show any difference in the levels of MMPs in coculture with either NE or GM-6001.

ELISA. Pro-MMP-1 and MMP-3 ELISA was performed to determine the protein concentration of the two MMPs identified with Western blot in the cell culture medium. RBC augmented fibroblast secretion of pro-MMP-1, and addition of NE to gel cultures further stimulated pro-MMP-1 release from fibroblasts (Fig. 4A). GM-6001 attenuated secretion of pro-MMP-1 from fibroblasts stimulated with NE in control samples but not from samples with both RBC and NE. However, GM-6001 did not alter the concentration of pro-MMP-1 from cultures where RBC were present (Fig. 4A). αPI inhibited NE-stimulated release of pro-MMP-1 in all conditions (data not shown).

MMP-3 ELISA demonstrated a tendency toward increased MMP-3 secretion in coculture with RBC. When GM-6001 was added to the cultures there were significantly higher levels of MMP-3 in coculture with RBC and RBC/NE compared with each control respectively (Fig. 4B). RBC-CM had similar effects as RBC on the pro-MMP-1 and MMP-3 release (data not shown).

MMP activity can be regulated by the presence of inhibitors, predominately TIMP-1 and TIMP-2; therefore, TIMP-1 and TIMP-2 were assayed in the culture supernatants by ELISA technique. Detectable TIMP-1 was released from fibroblasts cultured in three-dimensional collagen gel cultures under control conditions, and NE alone did not change the amount of TIMP-1 released from fibroblasts. TIMP-1 was increased in coculture with RBC both with and without NE compared with the control (Fig. 5). GM-6001 and αPI had no effect on TIMP-1 secretion by fibroblasts in this system (data not shown). RBC-CM had similar effects as RBC on the TIMP-1 secretion (data not shown). In line with these data, TIMP-2 was significantly increased in coculture with RBC compared with the control (326 ± 41.8, mean ± SD, vs. 258.5 ± 39.2) (P < 0.01). There was no significant difference in TIMP-2 secretion between fibroblasts cultured with only NE and fibroblasts cocultured with RBC and NE (291.1 ± 34.7 vs. 301.3 ± 41.7).

DISCUSSION

The current study demonstrates that RBC augment fibroblast-mediated contraction of three-dimensional collagen gels induced by the inflammatory mediator NE. Furthermore, it was shown that the release of MMP-1, -2, and -3 and TIMP-1 and -2 from human lung fibroblasts was increased by the presence of RBC. Together these findings indicate that RBC are able to stimulate fibroblasts to increased production and release of matrix-degrading enzymes, leading to augmented contraction of the extracellular collagen matrix.

The culture of fibroblasts in three-dimensional collagen gels has been utilized as an in vitro system to evaluate tissue repair
and remodeling (3, 22). Both fibroblast proliferation and protein production in the gels differ markedly from those in monolayer cultures (3). If the gels are floating in cell culture medium the fibroblasts will contract the gels, and this contraction can be modified by a variety of exogenous agents, which either simulate or inhibit the collagen gel contraction (11, 48, 54, 57). In vivo, tissue remodeling is a complex process and can play an important role in many pathophysiological processes in which altered tissue structure leads to loss of function. In the lung, tissue remodeling plays a prominent role in many disorders, including pulmonary fibrosis, pulmonary emphysema, chronic bronchitis, and asthma. Interestingly, a role for both MMPs and neutrophils has also been suggested in these conditions (10, 15, 41, 45, 49). Patients with idiopathic pulmonary hemosiderosis frequently develop fibrosis in the lung parenchyma (7, 31), suggesting a possible role of RBC in ECM remodeling. Although the mechanisms that regulate the pathological ECM turnover in different lung diseases are incompletely understood, it is likely that MMPs play an important role. The MMPs are proteolytic enzymes released by various cell types (5) that are able to degrade all components of the ECM (5, 46). Our study shows that both NE and RBC augment fibroblast-mediated collagen gel contraction. This contraction could be inhibited by the pan-MMP-inhibitor GM-6001, indicating that RBC and NE stimulation of fibroblasts results in augmented collagen gel matrix degradation by increased levels of MMPs. Under control conditions fibroblast-mediated collagen gel contraction was inhibited by GM-6001, suggesting that MMPs are involved also in the basal contraction of collagen gels. The protease inhibitor αPI inhibited the NE-induced fibroblast-mediated collagen gel contraction both in the control, with only fibroblasts, and in coculture with RBC. However, αPI did not inhibit basal or RBC-induced fibroblast-mediated collagen gel contraction. Together, these results show that inhibiting active forms of MMPs will attenuate fibroblast-mediated collagen gel contraction in all conditions studied, indicating an important role of MMPs in the RBC- and NE-induced contraction.

The MMPs are generally released as latent forms and are activated by a variety of mechanisms, including proteolytic cleavage. A crucial step in the regulation of MMP activity is the generation of active enzyme from latent precursors. Several proteases can activate the proteolytic cascade. In this regard, several MMPs, including MT-MMPs, are capable of activating MMP-2 (38). In addition, MMP-3, when activated, can activate MMP-2 and -9 (40). Serine proteases are also capable of activating several of the MMPs as shown in the present study. Thus NE could stimulate conversion of pro-MMP-2 to the active form of MMP-2 both in the control and in coculture with RBC. Because collagen is abundantly occurring in the ECM of the lung, and the gel matrix used in the experiments consisted of type I collagen, we have in our study focused on MMPs that have the capacity to degrade collagen (e.g., collagenases). In addition, type I collagen is also the most fibrous form of collagen and comprises ~84% of the collagen synthesized by fibroblasts. MMP-3 was analyzed due to its ability to activate fibroblasts. MMP-3 was analyzed due to its ability to activate fibroblasts. MMP-3 was analyzed due to its ability to activate fibroblasts. MMP-3 was analyzed due to its ability to activate fibroblasts.

**Fig. 3.** A: Western blot for MMP-1. Fibroblasts were cultured in floating collagen gels in the presence or absence of NE (10 nM) and GM-6001 (1 μM), added separately or together with RBC (5 × 10⁷/ml) or RBC-CM. Cell culture supernatants were harvested after 4 days of culture and subjected to SDS-PAGE, followed by Western blotting with MMP-1 antibodies. The data shown are taken from 1 representative experiment repeated on multiple occasions. Control, HFL-1 alone.

B: Western blot for MMP-3. Fibroblasts were cultured in floating collagen gels in the presence or absence of NE (10 nM) and GM-6001 (1 μM), added separately or together with RBC (5 × 10⁷/ml) or RBC-CM. Cell culture supernatants were harvested after 4 days of culture and subjected to SDS-PAGE, followed by Western blotting with MMP-3 antibodies. The data shown are taken from 1 representative experiment repeated on multiple occasions. Control, HFL-1 alone.
response to RBC stimulation were not able to inhibit the MMPs released. In agreement with previous studies (56) we could not show any effect on fibroblast TIMP-1 release in response to NE stimulation. Because a strict regulation of MMP and TIMP activity is important in tissue remodeling, our findings may pinpoint a potential mechanism by which RBC interfere with this complex process.

Others have shown that RBC can induce fibrosis in an animal model (27), and in previous studies we have demonstrated that both whole RBC and cell-free RBC-CM have the capacity to stimulate fibroblast-mediated collagen gel contraction (16) and fibroblast secretion of IL-8 (19). In the present study we show that both RBC and RBC-CM can induce MMP secretion and activity from human lung fibroblasts, indicating a role of intact RBC as well as soluble factors released. The findings were confirmed by three independent methods (e.g., gelatin zymography, Western blot, and ELISA). The present study, however, does not define which factor/factors released from the RBC stimulate fibroblast MMP and TIMP production. Additional studies, including analysis of RBC-CM by two-dimensional gel electrophoresis or high-pressure liquid chromatography followed by mass spectrometry, may answer this question. One possible candidate is the phospholipid sphingosine-1-phosphate, which can be released from RBC (52) and has been suggested to stimulate fibroblast functions related to remodeling in vitro (50).

Tissue injury and inflammation can be followed by effective repair with restoration of normal organ function or by abnormal repair with consequent remodeling and loss of organ function. The interplay between inflammatory and mesenchymal cells in the repair or remodeling process is crucially important in lung disorders. In IPF and asthma, diseases associated with inflammation and fibrosis, imbalance between MMPs and TIMPs can result in fibrosis (28, 30, 39, 42), and patients with idiopathic pulmonary hemosiderosis frequently develop pulmonary fibrosis (7, 31). In contrast, patients with chronic obstructive pulmonary disease and emphysema, diseases characterized by inflammation and increased matrix degradation, also have altered synthesis of MMPs and decreased TIMP synthesis (4, 26). Our data show that RBC and NE can stimulate fibroblast secretion of MMP-1, -2, and -3 as well as augment fibroblast-mediated contraction of collagen gels in

The MMPs can be downregulated by the presence of TIMPs (6). Four TIMPs have been identified and described. The current study demonstrates an increased production of both TIMP-1 and -2 by fibroblasts in coculture with RBC compared with the control. However, the increased TIMP-1 and -2 in

Fig. 4. A: ELISA for pro-MMP-1. Fibroblasts were cultured in floating collagen gels in the presence or absence of NE (10 nM) and GM-6001 (1 μM), added separately or together with RBC (5 × 10⁶/ml) or RBC-CM. Pro-MMP-1 in the surrounding culture media was analyzed with ELISA technique after 4 days of culture. y-Axis, pro-MMP-1 (ng/ml); x-axis, samples. Data presented are means ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control. B: ELISA for MMP-3. Fibroblasts were cultured in floating collagen gels in the presence or absence of NE (10 nM) and GM-6001 (1 μM), added separately or together with RBC (5 × 10⁶/ml) or RBC-CM. MMP-3 in the surrounding media was analyzed with ELISA technique after 4 days of culture. y-Axis, MMP-3 (ng/ml); x-axis, samples. Data presented are means ± SE from 3 independent experiments. **P < 0.01, ***P < 0.001 compared with the control.

Fig. 5. ELISA for tissue inhibitor of metalloprotease (TIMP)-1. Fibroblasts were cultured in floating collagen gels in the presence or absence of NE (10 nM) and GM-6001 (1 μM), added separately or together with RBC (5 × 10⁶/ml). TIMP-1 in the surrounding culture media was analyzed with ELISA technique after 4 days of culture. y-Axis, TIMP-1 release (ng/ml); x-axis, samples. Data presented are means ± SE from 3 separate experiments. *P < 0.05 compared with the control.
additive interactions may be a contributing mechanism in the regulation and fibrotic conditions may lead to interactions between fibroblasts, myofibroblasts, and wound contraction. These additive interactions may be a contributing mechanism in the tissue remodeling in inflammatory lung diseases.

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GRANTS
The current study demonstrates that interactions between RBC and the inflammatory mediator NE can lead to increased MMP release and inhibit the collagen gel contraction, indicating that RBC-

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