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Regulatory Effects of Endogenous Protease Inhibitors in Acute Lung Inflammatory Injury

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Inflammatory lung injury is probably regulated by the balance between proteases and protease inhibitors together with oxidants and antioxidants, and proinflammatory and anti-inflammatory cytokines. Rat tissue inhibitor of metalloprotease-2 (TIMP-2) and secreted leukoprotease inhibitor (SLPI) were cloned, expressed, and shown to be up-regulated at the levels of mRNA and protein during lung inflammation in rats induced by deposition of IgG immune complexes. Using immunoaffinity techniques, endogenous TIMP-2 in the inflamed lung was shown to exist as a complex with 72- and 92-kDa metalloproteinases (MMP-2 and MMP-9). In inflamed lung both TIMP-2 and SLPI appeared to exist as enzyme inhibitor complexes. Lung expression of both TIMP-2 and SLPI appeared to involve endothelial and epithelial cells as well as macrophages. To assess how these endogenous inhibitors might affect the lung inflammatory response, animals were treated with polyclonal rabbit Abs to rat TIMP-2 or SLPI. This intervention resulted in significant intensification of lung injury (as revealed by extravascular leak of albumin) and substantially increased neutrophil accumulation, as determined by cell content in bronchoalveolar lavage (BAL) fluids. These events were correlated with increased levels of C5a-related chemotactic activity in BAL fluids, while BAL levels of TNF-α and chemokines were not affected by treatment with anti-TIMP-2 or anti-SLPI. The data suggest that endogenous TIMP-2 and SLPI dynamically regulate the intensity of lung inflammatory injury, doing so at least in part by affecting the generation of the inflammatory mediator, C5a. The Journal of Immunology, 1999, 162: 3653–3662.

Evidence suggests that matrix metalloproteases (MMPs), including interstitial and neutrophil-derived collagenases, gelatinase A and B, stromelysins, metalloelastase, matrilysin, and membrane-associated MMPs, have the ability to bring about hydrolysis of connective tissue matrix proteins (1). It is less clear what role MMPs play in promoting the tissue damage that occurs during the course of an inflammatory response. It is known that MMPs are regulated by at least four tissue inhibitors of MMPs (TIMP-1, -2, -3, and -4) (2). These inhibitors are constitutively expressed by a variety of cell types and can be transcriptionally up-regulated by numerous agonists (2–4). TIMPs have been detected in body fluids (5, 6) and have a common molecular size, ranging from 21–28 kDa. TIMP-2, a nonglycosylated form that inhibits the 72-kDa gelatinase/type IV collagenase (MMP-2, gelatinase A) as well as other MMPs (7–9), has been cloned from human, bovine, mouse, and rat sources (9–14). For TIMPs, there is considerable homology at the nucleotide and amino acid levels (10–14). Recent studies have suggested that TIMP-2 is present at high levels in the lung (2).

Another naturally occurring protease inhibitor, secretory leukocyte protease inhibitor (SLPI), is a constitutively expressed, up-regulatable inhibitor of serine proteases. SLPI was originally isolated from porcine saliva (15). Human SLPI exists as a nonglycosylated 12-kDa protein (16). Murine SLPI has also been cloned recently and exhibits a high content of cysteine residues, 16 in all (17, 18). SLPI is found in extrapulmonary secretory fluids (15, 19) and in skin and articular cartilage (20). By immunostaining, SLPI has also been detected in serous cells of salivary glands, in airway surface epithelial cells, in Clara cells of the bronchial epithelium, and in association with elastin fibers of the lung interstitium (21–23). Serine proteases inhibited by SLPI include neutrophil elastase, cathepsin G, chymotrypsin, chymase, and trypsin (22, 24). SLPI has been shown recently to inhibit replication of HIV-1 in cultured human monocytes (25). In addition, it has been reported that transgene-induced production of SLPI reduces TNF-α production in LPS-stimulated phagocytic cells (17). Other local anti-proteases include anti-trypsin, Elafin, and anti-chymotrypsin, to name but a few.

The balance between oxidants and antioxidants and between proinflammatory and anti-inflammatory cytokines together with proteases and antiproteases in the inflamed lung probably determines the outcome of an inflammatory insult (24, 26, 27). It is known that the breakdown of elastin and collagen can result in the appearance of peptides that are chemotactic for neutrophils (28–30). It is also likely that MMPs and serine proteases directly attack connective tissue matrix proteins, causing structural damage in the inflamed tissue. In experimental studies, exogenously administered recombinant human SLPI and TIMP-2 were shown to reduce the
intensity of lung inflammatory injury after deposition of IgG immune complexes in a manner associated with reduced accumulation of neutrophils, implying that TIMP-2 and SLPI somehow regulate neutrophil influx (31). Recently, there have been suggestions that SLPI regulates the intensity of elastase-induced pulmonary emphysema (32).

Because it is likely that MMPs and serine proteases play a determinative role in the outcome of lung inflammatory injury, we developed cDNA probes and Abs to rat TIMP-2 and SLPI and employed these in studies of IgG immune complex-induced alveolitis. Our results suggest that both endogenous TIMP-2 and SLPI are up-regulated during lung inflammation and govern the intensity of inflammatory lung injury. The administration of polyclonal Abs to rat TIMP-2 or SLPI resulted in intensified lung injury associated with enhanced recruitment of neutrophils. It appears that MMPs and serine proteases in lung may contribute to the generation of C5a, the production of which enhances the recruitment of neutrophils and intensifies the attendant lung injury.

Materials and Methods

Reagents

Except where noted, all reagents were purchased from Sigma (St. Louis, MO).

Rat model of acute lung injury

Lung injury was induced in rats as previously described, using a well-characterized model of IgG immune complex-mediated alveolitis (33, 34). Briefly, 275- to 300-g male Long-Evans rats (specific pathogen free, Harlan Industries, Chesterfield, MO) were anesthetized with i.p. administered ketamine (60–80 mg/kg). This model of injury, which is associated with structural damage of endothelial and alveolar epithelial cells, has been described in detail previously (33). Injury was induced by the intratracheal instillation of rabbit polyclonal IgG containing precipitating Ab to BSA (anti-BSA) in a volume of 300 μl of PBS, pH 7.4. Immediately thereafter, 10 mg of BSA together with trace amounts of [125I]-BSA (as a quantitative marker of permeability) were injected i.v. Rats were sacrificed 4 h later, and the pulmonary arterial circulation was flushed with 10 ml of PBS. The lungs were surgically dissected, and the amount of remaining radioactivity ([125I]-labeled BSA) was determined to assess the permeability index. Negative control animals received anti-BSA intratracheally without i.v. administration. For calculation of the permeability index, the amount of radioactive [125I]-BSA remaining in PBS-perfused lungs was compared with the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice.

Bronchoalveolar lavage (BAL) fluid analysis

At the indicated times, lungs were lavaged thrice with 8 ml of PBS via an intratracheal cannula. BAL fluids were collected, and neutrophil content was determined by conventional microcytometry. BAL fluid supernatants were evaluated for TFN-α activity using a standard WEHI cell cytotoxicity bioassay (34) and for chemokine content and C5a as described below.

Cloning of rat TIMP-2

IgG immune complex-induced injury in rats was accomplished as described above. At the indicated time points, whole lungs were immediately dissected and frozen in liquid nitrogen. Total RNA was extracted from whole lung homogenates using a guanidinium isothiocyanate/chloroform-disruption were performed as described above. First-strand cDNAs were constructed from 1 μg of mRNA with a RT reaction primed with poly(dT) primers (cDNA Cycle Kit, Invitrogen). Rat SLPI was PCR amplified using the RT reaction product as template together with a 5’ oligonucleotide primer (5’-ATG AAG TCC AGC GCC CTC TTC-3’) constructed from the human SLPI sequence and a 3’ oligonucleotide primer that was degenerate for the human sequence (5’-CTT(CT/CT)/A (A/G/C/T)/G (G/C)TTT (A/C/G/T)/AC (A/C/G/T)/GG (A/C/G/T)/GC (A/T) (A/C/G/T)/AC (A/G/C)-3’; residues in parentheses indicate nucleotide degeneracy). Additional cDNA nucleotide degeneracy. Additional cDNA nucleotide primers were constructed to sequences within the rat SLPI open reading frame (5’-AAG AGA AGA TGG TGT CCT GAC ACT-3’ and 5’-GCA GGA TTT CCC ACA CAT GCC-3’) and used for internal sequence confirmation. After ligation into a pCRII vector (TA Cloning Kit, Invitrogen), the inserted PCR product was sequenced bidirectionally from the vector’s T7 and SP6 promoter regions and compared with the human sequence (15, 16). This procedure was performed in triplicate, producing three independent clones of the SLPI sequence from IgG-immune complex-injured rat lungs, with identical sequence results.

Rabbit polyclonal anti-TIMP-2 and anti-SLPI IgG

Polyclonal rabbit Ab was raised against recombinant rat TIMP-2 or against a BAL fluid recognized by anti-human SLPI. Because recombinant rat SLPI was extremely resistant to solubilization procedures, we used BAL fluids from IgG immune complex-injured lungs. Western blot analysis with polyclonal anti-human SLPI recognized a band from BAL fluids very close to 18.5-kDa marker (data not shown).

Expression and purification of rat TIMP-2

EcoRI (5’) and BamHI (3’) restriction enzyme sites were added by PCR to the 5’ and 3’ ends of the cDNA encoding the mature protein for TIMP-2 for further ligation into the pET15b expression vector (Novagen, Madison, WI). After ligation, pET-TIMP-2 plasmids were transformed into competent Nova Blue Escherichia coli. The pET-TIMP-2 plasmids were purified, sequenced, and then used for transformation of BL21 (DE3) or E. coli strain E. coli. These bacterial cultures were used for optimization of isopropyl β-D-thiogalactoside-stimulated overexpression of recombinant TIMP-2 according to a previously described procedure (35). Recombinant rat TIMP-2 was purified to homogeneity using an immobilized metal ion (nickel) affinity chromatography column (Novagen) under nonnaturating conditions.

Characterization of TIMP-2 expression products from E. coli

Proteins expressed by pET15b-transformed E. coli recombinants were subjected to SDS-PAGE (17%) under reducing conditions according to the method of Laemmli (36). The separated proteins were blotted onto nitrocellulose (0.45 μm; Bio-Rad, Hercules, CA) for 2 h at 12 V. Following transfer, the membrane was blocked with 5% BSA in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% Tween-20 (v/v) for 2 h at room temperature and developed with anti-rat TIMP-2 Ab. Recombinant rat TIMP-2 contained an N-terminal His tag. The tag was isolated by elution from a nickel affinity column with imidazole and analyzed by Western blot analysis using anti-rat TIMP-2 Abs. The Ab reacted with recombinant human TIMP-2 and defined a single band near the 18.5-kDa marker (data not shown).

Measurement of metalloproteinase activity by gel zymography

BAL fluids were obtained 0 and 4 h after initiation of lung injury. They were then preclarified of any residual IgG that was used to initiate lung injury by incubation with Gammabind Sepharose beads (Amersham, Arlington Heights, IL). BAL fluids were then incubated with 10 μg of either anti-rat TIMP-2 IgG or control nonspecific IgG. These were then further incubated with Gammabind Sepharose beads. The beads were then washed with PBS before the addition of SDS-PAGE sample buffer in the absence of 2-ME. SDS-PAGE substrate-embedded enzymography (zymography) was performed using modifications of the procedure of Heusen and Dowd (37, 38). Electrophoresis was conducted in 7.5% acrylamide gels containing 1 mg/ml gelatin. Nonreduced samples and standards were run at a constant voltage (150 V) until the dye front reached approximately 0.5 cm from the bottom of the gels. The gels were then subjected to the following washing protocol: twice for 15 min in 50 mM Tris buffer, pH 7.6, containing 2.5% Triton X-100, and once for 5 min in 50 mM Tris buffer, pH 7.6, alone, then incubated overnight at 37°C in 50 mM Tris buffer, pH 7.6, containing 1.0% Triton X-100. Gels were stained with Coomassie brilliant blue 250-R for several hours and then destained to reveal zones of enzyme activity, which appeared as bands of clearing.

Cloning of rat SLPI cDNA

IgG immune complex-induced injury in rats and RNA extraction and isolation were performed as described above. First-strand cDNAs were constructed from 1 μg of mRNA with a RT reaction primed with poly(dT) primers (cDNA Cycle Kit, Invitrogen). Rat SLPI was PCR amplified using the RT reaction product as template together with a 5’ oligonucleotide primer (5’-ATG AAG TCC AGC GCC CTC TTC-3’) constructed from the human SLPI sequence and a 3’ oligonucleotide primer that was degenerate for the human sequence (5’-CTT(CT/CT)/A (A/G/C/T)/G (G/C)TTT (A/C/G/T)/AC (A/C/G/T)/GG (A/C/G/T)/GC (A/T) (A/C/G/T)/AC (A/G/C)-3’; residues in parentheses indicate nucleotide degeneracy). Additional cDNA nucleotide degeneracy. Additional cDNA nucleotide primers were constructed to sequences within the rat SLPI open reading frame (5’-AAG AGA AGA TGG TGT CCT GAC ACT-3’ and 5’-GCA GGA TTT CCC ACA CAT GCC-3’) and used for internal sequence confirmation. After ligation into a pCRII vector (TA Cloning Kit, Invitrogen), the inserted PCR product was sequenced bidirectionally from the vector’s T7 and SP6 promoter regions and compared with the human sequence (15, 16). This procedure was performed in triplicate, producing three independent clones of the SLPI sequence from IgG-immune complex-injured rat lungs, with identical sequence results.
to the 12-kDa position. New Zealand White rabbits were repeatedly immunized with approximately 10 μg of purified rat TIMP-2 or rat SLPI extracted from gel slices. The gel slices or purified protein were emulsified in CFA and subsequently in IFA. The resulting antisera was purified with a protein G-Sepharose column (Pharmacia, Piscataway, NJ). Immune serum was diluted 1/1 with PBS and applied to the column. After 1-h incubation at 4°C and extensive washing with PBS, the column was stripped by addition of 100 mM glycine-HCl, pH 2.7. Eluted fractions (1 ml) were collected in tubes containing 200 μl of 1 M Tris buffer, pH 8.0. The purified IgG fraction was then dialyzed against PBS. For determination of serum titers of Ab, an indirect ELISA was used. Briefly, a 96-well Immulon-4 ELISA plate (Dynatech, Chantilly, VA) was coated with soluble recombinant rat TIMP-2 (or human SLPI [5 mg/ml]) overnight at 4°C. Plates were blocked with 2% BSA in PBS for 1 h before addition of serial dilutions of rabbit anti-TIMP-2 or anti-SPLI serum. The plate was washed, and 100 μl/well horseradish peroxidase-conjugated goat anti-rabbit Abs (1/3000 dilution; Bio-Rad) were incubated with samples. The reaction was developed with orthophenylenediamine dihydrochloride substrate and was stopped with 3 M H2SO4. Titers, which were determined by measuring absorbance at 490 nm, were approximately 104.

**Detection of TIMP-2 and SLPI proteins in BAL samples**

Aliquots of the BAL samples obtained at various time points from lung-injured rats were analyzed by Western blot for protein immunoreactive with rabbit anti-rat TIMP-2 and/or anti-rat (or goat anti-human) SLPI. Two milliliters of BAL fluid were concentrated 10-fold using Centricron-3 microconcentrators (Amicon, Beverly, MA), with centrifugation at 7500 × g for 6–10 h. Five microliters of ammonium bicarbonate-laced buffer was added to 20 μl of each concentrated sample and analyzed by SDS-PAGE in adjacent lanes, then transferred to nitrocellulose (Bio-Rad). When nonreducing conditions were used, sample buffer without SDS or 2-ME was added to the concentrated BAL samples and analyzed on polyacrylamide gels before transfer to nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline/Tween-20. It was then incubated with anti-rat TIMP-2 or anti-SLPI diluted 1/10000. After incubation with a 1/10000 dilution of a secondary Ab (goat anti-rabbit or rabbit anti-goat conjugated to alkaline phosphate), the blot was developed with alkaline phosphate color reagents A and B (Bio-Rad).

**TIMP-2 and SLPI mRNA expression in lung homogenates**

Whole lungs of rats following IgG immune complex deposition were obtained between 0 and 8 h following initiation of injury and frozen in liquid nitrogen for RNA extraction. RNA was fractionated electrophoretically in a 1% formaldehyde gel and transferred to a nylon membrane (Zetabind, Cuno, Meridian, CT). Specific primers and the respective cDNA templates for TIMP-2 and SLPI were used to generate 32P-labeled probes by PCR. Hybridization was performed at 50°C for 16 h. Autoradiography was performed at ~70°C on Kodak X-OMAT-AR film (Rochester, NY). A Fotodyne densitometer (New Berlin, WI) with AMBIS software (San Diego, CA) was used for densitometry analysis. Equal RNA loading was confirmed by methylene blue staining of 18S and 28S bands.

**Biotinylation of rabbit polyclonal anti-rat TIMP-2 and anti-rat SLPI IgG**

Anti-rat TIMP-2 or anti-rat SLPI (2.0 mg) was dialyzed in PBS overnight at 4°C in 50 mM sodium bicarbonate buffer, pH 8.5. NHS-LC-Biotin (74 mg/ml) was added to the BAL fluids following dextran sedimentation and hypotonic RBC lysis, the cells were treated with 2.7-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxyethyl ester (Sigma). A 96-well minichamber was used for chemotaxis assays as previously described (39). Neutrophils (2.25 × 106/well) were loaded into upper compartments of polycarbonate filters containing a porosity of 3 μm. Cells were incubated for 30 min at 37°C (5% CO2, humidified). The filter was removed, and nonmigrating cells wiped off. Fluorescence was read on a Cytofluor 2300 plate reader (Perceptive Bio-systems, Framingham, MA) at 630 nm. MIP-2 (10-10–10-9 μM) was used as a positive control for all chemotaxis studies. The BAL content of rat CXC chemokines, MIP-2 and cytokine-induced neutrophil chemoattractant (CINC), was determined by ELISA. The 2 h time was selected because this is when BAL levels of MIP-2 and CINC peak (39). C5a functional activity in BAL fluids was defined by the use of rabbit anti-C5a, which blocks neutrophil chemotactic activity of rat C5a (40). For this study normal rabbit IgG or anti-rat C5a IgG (each at 5 μg/ml) was added to the BAL fluids before the chemotaxis assay. For calculation of change inhibition in chemotactic activity, values for negative controls were subtracted from all positive control groups, and then the present reduction was calculated for comparisons between positive control groups.

**Statistical analysis**

All values were expressed as the mean ± SEM. Datasets were examined with one- and two-way analysis of variance, and individual group means were then compared with Student’s t test. For calculations of the percent change, mean negative control values were first subtracted from values in positive control groups and in each treatment group.

**Results**

**Cloning of rat TIMP-2**

Rat TIMP-2 has been cloned by other groups (13, 14). Our rat TIMP-2 DNA demonstrated >99% identity with the published rat nucleotide and amino acid sequences, with a 663-nucleotide open reading frame that was 96 and 92% homologous to the murine (12) and human (7, 11) TIMP-2 sequences, respectively (data not shown). The open reading frame encoded a 220-amino acid peptide with a 26-amino acid signal peptide and a mature protein consisting of 194 amino acids. The deduced amino acid sequence shared 99 and 97% homology with the published murine and human sequences for TIMP-2, respectively (data not shown). Analysis of the protein structure revealed an estimated molecular mass of 20 kDa.

**TIMP-2 mRNA time course in IgG immune complex-induced lung injury**

Expression of mRNA for TIMP-2 in lung extracts from animals undergoing lung injury due to intrapulmonary deposition of IgG immune complexes was determined as a function of time (0–6 h) after initiation of injury. RNA was analyzed by Northern blots probed with a full-length radiolabeled cDNA for rat TIMP-2. Results are shown in Fig. 1, in which relative OD as a function of time was determined in lung extracts. Equal loading was demonstrated by methylene blue staining of 18S and 28S bands (upper frame inset). While constitutive expression was found in lung RNA, maximal up-regulation was found 0.5 h after initiation of the lung inflammatory reaction, with up-regulation continuing to 4 h, followed by a decrease at 6 h. Northern blot analysis showed distinct TIMP-2 mRNA transcripts of 1.0 and 3.5 kb (Fig. 1, bottom frame), as has been previously reported for human (7, 11) and murine (12) TIMP-2. Recent data demonstrate that the difference in size of the mRNAs of the human TIMP-2 gene is the result of
the use of different polyadenylation signals within the 3’ end of the gene (41).

**TIMP-2 protein expression in BAL fluids from IgG immune complex-injured rats**

In gels in which electrophoresis was performed under reducing conditions, Western blot analysis was conducted with anti-rat TIMP-2. TIMP-2 was not detected in BAL fluids obtained at time zero (Fig. 2), but was detected in BAL fluids in a position near the 18.5-kDa marker at 0.5, 1, 2, 4, 6, and 8 h after initiation of lung inflammatory responses. The m.w. standards are shown in the extreme left lane. Anti-rat TIMP-2 demonstrated reactivity for both the recombinant rat and human TIMP-2 (Fig. 2, extreme right two lanes). Under nonreducing conditions anti-TIMP-2 Ab detected at the 4 h point a band of approximately 110 kDa (data not shown). This finding suggests that TIMP-2 exists as a complex with an enzyme(s) in the inflamed lung.

**Cloning of rat SLPI**

The deduced nucleotide and amino acid sequences derived from three independently cloned rat SLPI cDNAs are shown in Fig. 3. The first 21 bases of the sequence represent the primer that was used to clone the cDNA. The predicted open reading frame encodes a mature protein of 106 amino acids with an estimated molecular mass of 14 kDa. The first 25 amino acids form the putative signal peptide. Further analysis of the deduced amino acid sequence revealed 67% homology with human SLPI and 91% homology with murine SLPI amino acid sequences, with all 16 cysteine residues conserved (Fig. 4).

**SLPI mRNA time course in IgG immune complex lung injury**

Expression of mRNA for SLPI in lung extracts from animals undergoing lung injury was determined as a function of time (0 – 8 h) after initiation of the reaction (Fig. 5). The pattern was different from that found for rat TIMP-2 (Fig. 1). mRNA for rat SLPI was analyzed by Northern blot probed with a full-length open reading frame cDNA for rat SLPI. The results are shown in Fig. 5, A and B, in which the time course of expression (after normalization of signals as explained in Materials and Methods) was determined in the IgG immune complex model of lung injury (Fig. 5B). Normal lung (at 0 h) contained no detectable mRNA for SLPI; very little mRNA could be detected at 1 and 2 h, but there was a gradual increase in expression of lung SLPI mRNA, especially at 4 h and continuing to at least 8 h (Fig. 5A). Northern blot analysis showed only one mRNA transcript of 0.7 kb for rat SLPI.

**SLPI protein expression in BAL fluids from IgG immune complex-injured rats**

Immunoreactive SLPI material was detected by Western blot analysis of BAL fluids at 0.5, 1, 2, 4, 6, and 8 h after initiation of the lung inflammatory reactions (Fig. 6). No constitutive SLPI was found in BAL fluids (obtained at time zero). The bands in BAL samples closely aligned with recombinant human SLPI. In data not shown, recombinant human and rat SLPI aligned in the same position. Under nonreducing conditions anti-SLPI Ab detected a band of approximately 115 kDa (data not shown) at the 4 h point.
suggesting that SLPI exists as a complex with its substrate in the inflamed lung.

**Effects of in vivo blockade of TIMP-2 and SLPI in lung injury**

Rabbit polyclonal blocking Abs to rat TIMP-2 and SLPI were used to assess the extent to which their intratracheal instillation would alter the severity of inflammatory lung injury. Negative control animals received anti-BSA intratracheally in the absence of i.v. injected BSA. In positive controls, unless otherwise indicated, 250–300 mg preimmune IgG was mixed with the anti-BSA. In all studies anti-TIMP-2 or anti-SLPI in the indicated amounts was instilled intratracheally with the anti-BSA. The intensity of the lung injury was assessed by the permeability index and by neutrophil counts in BAL fluids. The results from five separate experiments are summarized in Table I. The presence of anti-TIMP-2 Ab (Expt. A–C) caused a significant increase (29–52%) in the intensity of the lung injury, as reflected by extravascular leak of albumin, compared with that in positive control animals treated with preimmune IgG. In experiments not shown, negative controls that received anti-BSA intratracheally, but with omission of the i.v. infusion of 10 mg of BSA, and that also received 300 μg of anti-TIMP-2 or anti-SLPI intratracheally had permeability indexes that were the same as those found in otherwise untreated negative control animals (data not shown). The effect of anti-TIMP2 on neutrophil content of BAL fluids was also assessed. As shown in Table I, compared with animals receiving preimmune IgG there was a significant increase in BAL neutrophils in animals receiving anti-TIMP-2, rising from 85–115%, depending on the particular experiment.

Similar protocols were employed in the case of the anti-SLPI Ab (Table I, Expt. D and E). The instillation of SLPI Ab in two separate experiments markedly increased the permeability index, rising from 56–85% compared with that in positive control animals receiving preimmune IgG. Treatment with anti-SLPI induced an increase in the permeability index that appeared to be dose dependent, with 250 and 500 μg of the Ab causing 54 and 85% increases, respectively, in the permeability index. In terms of BAL neutrophil counts, treatment with anti-SLPI Ab caused significant increases in the number of BAL neutrophils (53–81%) compared with that in the positive control groups treated with preimmune IgG.

**Chemotactic activity in BAL fluids**

To assess the possible mechanism(s) by which treatment of animals with anti-TIMP-2 or anti-SLPI caused increased neutrophil accumulation, BAL fluids were examined 2 and 4 h after the initiation of lung inflammatory reactions for chemotactic activity for neutrophils. Total chemotactic activity, chemokine content (MIP-2 and CINC), and C5a-related chemotactic activity were determined.
increase (tactic activity, while treatment with anti-SLPI caused a 1.64-fold increased levels of neutrophils in BAL fluids of positive controls. Thus, increased lung injury and statistically significant compared with levels in positive controls animals treated with anti-TIMP-2 or anti-SLPI, these were not statistically significant. In the presence of anti-TIMP-2 or anti-SLPI levels increased by nearly fivefold in the positive controls pretreated with preimmune IgG. In the presence of anti-TIMP-2 and CINC levels peaked in the lung inflammatory model employed (39). As shown by the data in Table III, chemokine content in BAL fluids under these experimental conditions correlates with increased chemotactic activity in BAL fluids at 4 h.

Chemokine content in BAL fluids

Chemokine content in BAL fluids was assessed at 2 h, as this is the time at which MIP-2 and CINC levels peak in the lung inflammatory model employed (39). As shown by the data in Table III, MIP-2 levels increased by nearly fivefold in the positive controls pretreated with preimmune IgG compared with those in negative controls. Likewise, CINC levels increased threefold in the positive controls pretreated with preimmune IgG. In the presence of anti-TIMP-2 or anti-SLPI, no further increase in chemokine content appeared. While there were slight increases in CINC levels in animals treated with anti-TIMP-2 or anti-SLPI, these were not statistically significant compared with levels in positive controls treated with preimmune IgG. Thus, increased lung injury and increased levels of neutrophils in BAL fluids of positive controls treated with anti-TIMP-2 or anti-SLPI were not reflected by increases in BAL CXC chemokines.

C5a-related chemotactic activity in BAL fluids

To assess to what extent chemotactic activity in BAL fluids of positive control animals at 2 and 4 h could be related to C5a content, the experiments described in Table IV were conducted. Two and four hour BAL fluids from negative controls (in which i.v. infusion of BSA was omitted) had chemotactic values, as revealed by relative fluorescence units of $<10$ (data not shown). Similar to the findings described in Table II, at 2 h there were $>20$-fold increases in chemotactic activity in each of the three positive control groups. The addition of anti-C5a IgG (5 $\mu$g/ml) failed to reduce the chemotactic activity in the 2 h BAL samples compared with companion samples to which were added preimmune IgG (5 $\mu$g/ml; Table IV). In the BAL fluids obtained at 4 h, chemotactic activity was significantly increased (nearly doubled) in all three positive control groups compared with that in BAL fluids obtained at 2 h, and these increases were statistically significant. The total amount of chemotactic activity in the 4 h samples from positive control rats treated with preimmune IgG was significantly higher than that at 2 h (368 $\pm$ 46 vs 212 $\pm$ 14). The 4 h BAL samples from animals pretreated with anti-TIMP-2 or anti-SLPI contained significantly ($p < 0.05$) elevated levels (by 52–57%) of chemotactic activity compared with the 4 h BAL samples from rats treated with preimmune IgG. In all three positive control groups, the addition of anti-C5a to 4 h BAL fluids substantially reduced the level of measurable neutrophil chemotactic activity, from 76 to

As shown in Table II, there was a $>20$-fold increase in overall chemotactic activity in BAL fluids at both 2 and 4 h following initiation of the inflammatory reaction in the positive control group treated with 300 $\mu$g of preimmune IgG. In the presence of either anti-TIMP-2 or anti-SLPI, no significant increase ($<5\%$) in chemotactic activity occurred at 2 h. However, at 4 h, treatment with anti-TIMP-2 caused a 1.70-fold increase ($p = 0.001$) in chemotactic activity, while treatment with anti-SLPI caused a 1.64-fold increase ($p = 0.02$) in chemotactic activity compared with the values in the positive controls treated with preimmune IgG. Thus, the increase in neutrophil accumulation in BAL fluids under these experimental conditions correlates with increased chemotactic activity in BAL fluids at 4 h.

### Table I. In vivo effect of blockade of TIMP-2 or SLPI on lung inflammatory injury

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Intervention</th>
<th>Permeability Index</th>
<th>Percent Increase</th>
<th>BAL Neutrophils ($\times 10^6$)</th>
<th>Percent Increase</th>
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</thead>
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<tr>
<td>A</td>
<td>Preimmune IgG (300 $\mu$g)</td>
<td>0.45 $\pm$ 0.04</td>
<td>47 ($p &lt; 0.05$)</td>
<td>2.0 $\pm$ 0.45</td>
<td>95 ($p = 0.02$)</td>
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<td></td>
<td>Anti-TIMP-2 (300 $\mu$g)</td>
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<td>39.6 $\pm$ 0.94</td>
<td>85 ($p = 0.03$)</td>
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<tr>
<td>B</td>
<td>Preimmune IgG (300 $\mu$g)</td>
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<td>2.6 $\pm$ 0.09</td>
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<td>Anti-TIMP-2 (300 $\mu$g)</td>
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<td>85 ($p = 0.03$)</td>
<td>1.9 $\pm$ 0.17</td>
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<tr>
<td>C</td>
<td>Preimmune IgG (250 $\mu$g)</td>
<td>0.46 $\pm$ 0.03</td>
<td>0.04</td>
<td>4.1 $\pm$ 0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-TIMP-2 (250 $\mu$g)</td>
<td>0.70 $\pm$ 0.04</td>
<td>52 ($p = 0.004$)</td>
<td>4.1 $\pm$ 0.34</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Preimmune IgG (300 $\mu$g)</td>
<td>0.28 $\pm$ 0.02</td>
<td>3.4 $\pm$ 0.38</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-SLPI (300 $\mu$g)</td>
<td>0.44 $\pm$ 0.05</td>
<td>57 ($p = 0.01$)</td>
<td>5.2 $\pm$ 0.58</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Preimmune IgG (250 $\mu$g)</td>
<td>0.13 $\pm$ 0.02</td>
<td>2.1 $\pm$ 0.11</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-SLPI (250 $\mu$g)</td>
<td>0.20 $\pm$ 0.02</td>
<td>54 ($p = 0.01$)</td>
<td>3.8 $\pm$ 0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-SLPI (500 $\mu$g)</td>
<td>0.24 $\pm$ 0.03</td>
<td>85 ($p &lt; 0.01$)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* For each experiment, $n = 10$/group. All interventions consisted of addition of the indicated material to the anti-BSA, which was instilled intratracheally at 300 $\mu$g/ml; Table IV). In the presence of anti-TIMP-2 or anti-SLPI (250 $\mu$g each), the BAL neutrophil counts ($\times 10^6$) were 0.49 $\pm$ 0.16, 0.266 $\pm$ 0.184, and 0.292 $\pm$ 0.173, respectively. The mean of neutrophil levels ($\times 10^6$), which have also been corrected for negative control values, in negative control lungs ranged from 0.29 $\pm$ 0.07 to 0.74 $\pm$ 0.001. ND, not done. In negative controls receiving PBS, anti-TIMP-2, or anti-SLPI (250 $\mu$g), the BAL neutrophil counts ($\times 10^6$) were 0.49 $\pm$ 0.16, 0.266 $\pm$ 0.184, and 0.292 $\pm$ 0.173, respectively.

### Table II. Chemotactic activity in BAL fluids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 h Relative Fluorescence</th>
<th>4 h Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls, preimmune IgG</td>
<td>$&lt;10$</td>
<td>$&lt;10$</td>
</tr>
<tr>
<td>Positive controls, preimmune IgG</td>
<td>241 $\pm$ 64, p, N.S.</td>
<td>295 $\pm$ 25, p = 0.001</td>
</tr>
<tr>
<td>Positive controls, anti-TIMP-2</td>
<td>251 $\pm$ 106, p, N.S.</td>
<td>501 $\pm$ 114, p = 0.01</td>
</tr>
<tr>
<td>Positive controls, anti-SLPI</td>
<td>250 $\pm$ 65, p, N.S.</td>
<td>485 $\pm$ 30, p = 0.02</td>
</tr>
</tbody>
</table>

* For all chemotaxis assays, 50 $\mu$l of BAL fluid (from a total of 5.0 ml) was added to lower compartments of the chambers. The reference neutrophil chemotactic response in vitro to $10^{-8}$ M FMLP was 930 $\pm$ 22. Positive control groups received 300 $\mu$g preimmune IgG or 300 $\mu$g anti-TIMP-2 or anti-SLPI intratracheally. For each of the treatment protocols, $n = 6$.

* See section in Materials and Methods.
90% (p = < 0.05). Thus, the increased chemotactic activity in 4 h BAL fluids of animals treated with anti-TIMP-2 or anti-SLPI appeared to be associated predominately with increased C5a-dependent chemotactic activity.

The ability of anti-C5a to suppress most of the neutrophil chemotactic activity in the 4 h BAL specimens may be due to the fact that in any chemokines that peak at 2 h (39) and are rapidly declining by 4 h, long residual chemokines may have been oxidatively or proteolytically functionally degraded and not detected in the chemotaxis assay.

**TNF-α activity in BAL fluids**

BAL levels of TNF-α were evaluated at 2 and 4 h using treatment protocols similar to those described in Tables I–IV. TNF-α levels rose from <30 pg/ml in the negative control group to 2300 ± 1200 and 6427 ± 1518 pg/ml at 2 and 4 h, respectively, in the positive control groups pretreated with 300 μg of preimmune IgG (data not displayed). In positive control groups pretreated with anti-TIMP-2, BAL levels at 2 and 4 h were 1900 ± 240 and 9335 ± 629 pg/ml, respectively, while 2 and 4 h BAL fluids from anti-SLPI-treated animals had values of 2100 ± 1500 and 7632 ± 768, respectively. When TNF-α levels at 2 h and 4 h in the positive control groups (which also received 300 μg of preimmune IgG with the anti-BSA) were compared with the values at the same time points in animals receiving either 300 μg of anti-TIMP-2 or 300 μg of anti-SLPI, there were no statistically significant differences. Thus, treatment with anti-TIMP-2 or anti-SLPI did not significantly affect BAL levels of TNF-α.

### Expression of TIMP-2 and SLPI in rat lungs

Immunostaining for TIMP-2 and SLPI was conducted using frozen sections of rat lungs at 0 and 4 h. The results are shown in Fig. 7. In the case of TIMP-2, no staining was found in lungs obtained at time zero either in vessels (Fig. 7A, upper area) or in bronchioles (Fig. 7A, lower area). In striking contrast, at 4 h (Fig. 7B) staining for TIMP-2 was found in alveolar epithelial cells (arrowheads) and in endothelial cells (arrow). In the case of SLPI, no staining was found in lung tissue at 0 h (Fig. 7C), whereas at 4 h staining for SLPI was found on surfaces of alveolar epithelial cells (Fig. 7D, solid arrowheads), in vascular endothelial cells (solid arrows), and in cells that appeared to be macrophages (open arrow). As indicated above, the appearance of SLPI in BAL fluids (Fig. 2) seems somewhat discordant with the appearance of mRNA for SLPI (Fig. 5). It is possible that SLPI is generated in two waves, the first being early (1–2 h) and involving alveolar macrophages, and the second involving other cell types, such as endothelial and epithelial cells. This could explain the differences in protein presence (in BAL fluids) and mRNA appearance (in whole lung homogenates).

### Metalloproteinase activity in BALs

To ascertain whether there was any metalloproteinase activity associated with TIMP-2, BAL fluids were obtained 0 and 4 h after initiation of lung injury. Zymography was performed after incubating samples with anti-TIMP-2 Ab or nonspecific IgG and Gammaind Sepharose beads. No MMP-2 or MMP-9 activity was found to be associated with anti-TIMP-2 Ab at the 0 h point (Fig.

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**Table III.** Chemokines in BAL fluids at 2 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIP-2 (ng/ml)</th>
<th>CINC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls</td>
<td>63.9 ± 27.4</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Positive controls + preimmune IgG</td>
<td>342.6 ± 13.91</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Positive controls + anti-TIMP-2</td>
<td>291.2 ± 23.6</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>Positive controls + anti-SLPI</td>
<td>312.8 ± 15.7</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

*The animals received 300 μg intratracheally of preimmune rabbit IgG, anti-SLPI, or anti-TIMP-2. Chemokine levels were measured by ELISA in BAL fluids at 2 h after onset of reactions.

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**Table IV.** C5a-related chemotactic activity in BAL fluids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive controls, preimmune IgG (300 μg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive controls, anti-TIMP-2 (300 μg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive controls, anti-SLPI (300 μg)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Chemotactic activity in BAL fluids of negative controls at 2 and 4 h was <10 units of relative fluorescence. For each of the values shown in this table, n = 5. The various treatment protocols were similar to those described in Table I. BAL fluids were examined for neutrophil chemotactic activity in the presence of normal rabbit IgG or rabbit anti-rat C5a IgG (each at 5 μg/ml).

*The negative control and FMLP values are described in footnotes of Table II.

*When these values were compared to values in the closest column (normal IgG, 4 h), all p values were <0.05.
However, at 4 h both MMP-2 and MMP-9 activities were associated with the anti-TIMP-2 Ab (Fig. 8), but not with the control nonspecific IgG (data not shown), thus confirming that complex formation does take place between TIMP-2 and MMPs in BALs from injured lungs.

Discussion

Neutrophils and lung macrophages play a critical role in the development of inflammatory lung reactions by releasing tissue-destructive mediators such as oxidants, cytokines, and serine and metalloproteases (1–3). Since endogenous plasma protease inhibitors do not have the ability to fully prevent stimulated neutrophils from degrading substrates (42, 43), interest has focused on endogenous protease inhibitors that exist within tissues. TIMP-2 is released from a variety of lung cells and is one of the resident protease inhibitors assumed to be important in the regulation of tissue MMPs (1, 4). TIMP levels have been shown to be inducible by a variety of cytokines (44–46). SLPI is also produced by cells lining the tracheobronchial tree and is one of the resident inhibitors thought to be important in the regulation of serine protease activities (22, 24, 47, 48). SLPI has been shown to be up-regulated in epithelial cells by cytokines such as IL-1β and TNF-α (48). The in vivo finding of complexes of SLPI with human neutrophil elastase in bronchial secretions suggests that SLPI functions as an elastase inhibitor (24, 47, 48). Collectively, it seems likely that the balance of endogenous proteases and anti-proteases (TIMPs and SLPI) in lung at least in part determines the outcome of inflammatory-induced lung injury, but there is little direct evidence to support this conclusion.

Recent studies suggest that membrane-type MMPs are also regulated by TIMP-2 (49, 50). Therefore, the activity of a given MMP in vivo may depend directly on the tissue level and availability of TIMP-2. Since MMP expression has been correlated with invasiveness of tumor cells, it has been suggested that the ability of tumor cells to cross basement membranes may partly be due to the expression of MMPs (51, 52).

As demonstrated in the current studies, the rat SLPI gene encodes a protein of 106 amino acids, with a 25-amino acid containing signal peptide. Comparison of the deduced amino acid sequence of rat SLPI with the established sequences of human (15, 16) and murine SLPI (17, 18) has revealed 67 and 91% homologies, respectively. In our studies, Northern blot analysis has shown SLPI mRNA in inflamed rat lung with an approximate size of 0.7 kb,
similar to that reported for human SLPI (20, 53). There are few studies describing in vivo expression of SLPI or TIMP-2 in lung inflammatory responses, although several reports describe protective effects of exogenously administered SLPI in lung models of injury (31, 54–57). SLPI has also been reported to function as an anti-inflammatory agent by interfering with the signal transduction pathway leading to monocyte metalloprotease production (58). SLPI also inhibits TNF-α in vitro responses of macrophages to LPS (17).

Recent work has demonstrated that intrapulmonary administration of recombinant human TIMP-2 or SLPI results in suppression of IgG immune complex-induced alveolitis (31). This attenuation was unexpectedly associated with a reduction in lung myeloperoxidase content, suggesting that MMPs and serine proteases somehow affect neutrophil recruitment and that this cell recruitment process may be regulated by TIMP-2 and SLPI, as defined by exogenous administration of TIMP-2 or SLPI. The data in the current studies indicate that both mRNA and protein for TIMP-2 and SLPI are up-regulated during the course of the lung inflammatory response, and that synthesis of these proteins is not confined to a single cell type. The data show that Ab-induced blockade of endogenous TIMP-2 or SLPI leads to intensified lung damage following deposition of IgG immune complexes. Endogenous TIMP-2 and SLPI seem to regulate the inflammatory response by attenuating the development of tissue injury and by regulating the recruitment of neutrophils. The enhanced recruitment of neutrophils in the presence of anti-TIMP-2 or anti-SLPI was not related to increased levels in BAL fluids of CXC chemokines (MIP-2 and CINC) or TNF-α, each of which is known to be required for neutrophil recruitment in the lung injury model (34, 39). However, enhanced neutrophil recruitment in the presence of anti-TIMP-2 or anti-SLPI appears to be due to the increased C5a-related neutrophil chemo-tactic activity present in BAL fluids at 4 h. Since it is well established that serine proteases from neutrophils can generate a neutrophil chemotactic fragment from C5 (59), these data suggest that endogenous MMPs and serine proteases may also have the ability to cleave C5 in a similar manner, producing C5a.

In the current studies, the Northern blot data have indicated induction of mRNA for TIMP-2 (data not shown) and SLPI following intrapulmonary deposition of IgG immune complexes, although the time course for up-regulation was not the same for the two endogenous anti-true proteases. Western blot analysis of BAL fluids has revealed up-regulation of both TIMP-2 and SLPI during the inflammatory response. Immunostaining has shown localization of these inhibitors in alveolar epithelial and vascular endothelial cells and in what appear to be lung macrophages. The ability of blocking Abs to rat TIMP-2 or SLPI to significantly increase neutrophil recruitment and to increase the intensity of injury (as measured by leakage of serum albumin) suggests that these inflammatory responses are indeed regulated by endogenous enzyme inhibitors of neutrophil recruitment and that this cell recruitment process may be regulated by TIMP-2 and SLPI during the inflammatory response by attenuating the development of tissue injury and by regulating the recruitment of neutrophils. The enhanced recruitment of neutrophils in the presence of anti-TIMP-2 or anti-SLPI was not related to increased levels in BAL fluids of CXC chemokines (MIP-2 and CINC) or TNF-α, each of which is known to be required for neutrophil recruitment in the lung injury model (34, 39). However, enhanced neutrophil recruitment in the presence of anti-TIMP-2 or anti-SLPI appears to be due to the increased C5a-related neutrophil chemotactic activity present in BAL fluids at 4 h. Since it is well established that serine proteases from neutrophils can generate a neutrophil chemotactic fragment from C5 (59), these data suggest that endogenous MMPs and serine proteases may also have the ability to cleave C5 in a similar manner, producing C5a.

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