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Differential Expression of Extracellular Matrix Remodeling Genes in a Murine Model of Bleomycin-Induced Pulmonary Fibrosis

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Exposure to the chemotherapeutic drug bleomycin leads to pulmonary fibrosis in humans and has been widely used in animal models of the disease. Using C57BL/6 bleomycin-sensitive mice, pulmonary fibrosis was induced by multiple intraperitoneal injections of the drug. An increase in the relative amounts of steady-state α1(I) procollagen, α1(III) procollagen, and fibronectin mRNA as well as histopathological evidence of fibrosis was observed. The effect of bleomycin on the expression of the enzymes responsible for extracellular matrix degradation, the matrix metalloproteinases (MMPs), and their inhibitors (TIMPs), was selective and showed temporal differences during the development of fibrosis. Of the MMPs tested, bleomycin treatment resulted in the up-regulation of gelatinase A and macrophage metalloelastase gene expression in whole-lung homogenates, whereas gelatinase B, stromelysin-1, and interstitial collagenase gene expression was not significantly changed. *Timp2* and *Timp3*, the murine homologues of the respective TIMP genes, were constitutively expressed, whereas *Timp1* was markedly up-regulated during fibrosis. The strong correlation between enhanced extracellular matrix gene expression, differential MMP and TIMP gene expression, and histopathological evidence of fibrosis suggest that dysregulated matrix remodeling is likely to contribute to the pathology of bleomycin-induced pulmonary fibrosis. *(Am J Pathol 1998, 152:821–828)*

Fibrotic lung diseases are characterized by excessive synthesis and deposition of connective tissue in the distal airspace.[1] Although there are multiple etiologies of pulmonary fibrosis, one cause in humans and in widely used animal models of the disease is exposure to the chemotherapeutic drug, bleomycin. Bleomycin treatment results in acute lung injury, followed by inflammation, fibroblast proliferation, and dysregulated matrix remodeling, which culminates in thickened alveolar walls, derangement of the airspace, patchy alveolar collapse, and focal scarring.[2,3] Although various aspects of matrix production have been studied extensively, the contribution of matrix degradation to the pathology of the disease has not been as well evaluated.

Degradation of the extracellular matrix is controlled primarily by the matrix metalloproteinases (MMPs), a family of zinc-dependent, secreted enzymes that, collectively, are capable of degrading the major components of the matrix by virtue of their individual substrate specificities. The MMPs include the collagenases, gelatinases, stromelysins, macrophage metalloelastase, matrilysin, and membrane-type MMPs. The MMPs are regulated at the level of gene transcription, by latent proenzyme activation, and are inhibited by a family of secreted proteins known as tissue inhibitors of matrix metalloproteinases (TIMPs), which bind to the MMP active site and also to latent forms of the specific MMPs.[4–6] The TIMP family consists of TIMP-1, TIMP-2, TIMP-3, and the recently discovered TIMP-4.[7,8] Although each member of the TIMP family shares a common inhibitory function, the individual members exhibit structural and functional differences and are thought to play distinctly different physiological roles in normal and pathological states based on their differential patterns of cell- and tissue-specific expression.[9,10]

A highly regulated balance of active MMPs and TIMPs is maintained during normal tissue metabolism. An imbalance in these ratios has been implicated in a number of pathological disorders, including tumor invasion and metastasis, arthritis, fibrotic diseases, atherosclerosis, and emphysema.[11] To date, there have been no *in vivo* studies of the contribution of MMPs and TIMPs to the development of bleomycin-induced pulmonary fibrosis. Using a murine model of the disorder, we compared the expression of MMP and TIMP genes with expression of extra-

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cellular matrix genes and histological evidence of pulmonary fibrosis. The results of our study revealed a strong correlation between enhanced extracellular matrix gene expression, differential MMP and TIMP gene expression, and histopathological evidence of fibrosis, suggesting that dysregulated matrix remodeling may contribute to the pathology of the disease.

Materials and Methods

Animals and Treatment

Pathogen-free female C57BL/6 mice (20 to 23 g) were purchased from Harlan-Sprague Dawley (Indianapolis, IN) and were maintained in a viral- and pathogen-free facility to minimize pulmonary infection. In triplicate, mice were injected intraperitoneally (i.p.) with 1 U of bleomycin (Blenoxane, Bristol-Meyers Oncology, Princeton, NJ) dissolved in sterile saline, every other day, for a total of 4 U. Control mice, in triplicate, were injected i.p. with sterile saline every other day, for a total of four injections. At various time points during bleomycin treatment, mice were sacrificed by terminal anesthesia with pentobarbital (approximately 250 mg/kg).

Histology

Lungs were perfused through the right ventricle of the heart with 2% paraformaldehyde in phosphate buffer to maintain pulmonary architecture. Lungs were fixed and embedded in paraffin. Right lobes were sectioned and stained with either hematoxylin and eosin (H&E) or with Masson's trichrome blue stain.

RNA Isolation and Blot Analysis

Freshly dissected, nonperfused lungs were rapidly placed in guanidinium isothiocyanate buffer (GITC), frozen in liquid nitrogen, and stored at −70°C until use. Total lung RNA was prepared using the GITC extraction and cesium chloride centrifugation method of Chirgwin et al[12] as modified by Maniatis et al.[13] Five to ten micrograms of RNA were electrophoresed through 0.8% agarose gels containing formaldehyde. RNA length standards (0.4 to 9.5 kb) were obtained from Gibco BRL (Gaithersburg, MD). Gels were stained with ethidium bromide and destained overnight in 0.1 mol/L ammonium acetate to assess RNA integrity and equivalent loading. RNA was transferred to Gene Screen Plus (New England Nuclear, Boston, MA) following the manufacturer's specifications.

Hybridization probes were gel-purified cDNA or genomic DNA inserts of the following plasmids generously provided as noted: *Timp1* (the murine homologue of the gene encoding TIMP-1), provided by Dr. David Denhardt[14]; *Timp2* (the murine homologue of the gene encoding TIMP-2) and *Timp3* (the murine homologue of the gene encoding TIMP-3), provided by Dr. Dylan Edwards[15,16]; murine interstitial collagenase (MMP-13), provided by Dr. Yves Eeckhout and Dr. Hideaki Nagase[17]; murine gelatinase A (MMP-2), provided by Dr. Karl Tryggvason[18]; murine gelatinase B (MMP-9), provided by Dr. Nancy Berliner[19]; rat stromelysin-1 (MMP-3), obtained from American Type Culture Collection[20] (Rockville, MD); murine macrophage metalloelastase (MME) (MMP-12[21]); murine α1(I) procollagen and murine α1(III) procollagen, provided by Dr. Benoit deCrombrugghe[22]; and rat fibronectin, provided by Dr. Richard Hynes.[23]

cDNA probes were labeled with [^{32}P]dCTP by random priming (Gibco BRL) or by Ready-To-Go DNA Labeling Beads (Pharmacia Biotech, Piscataway, NJ). Hybridization was for 16 hours at 42°C in 50% formamide, 5X SSC (SSC is standard saline citrate: 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 1X Denhardt's solution, 20 mmol/L phosphate buffer (pH 7.6), 1% SDS, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate. After hybridization, filters were washed twice at room temperature in 1X SSC, followed by two rinses at 65°C in 1X SSC/1% sodium dodecyl sulfate (SDS), and a final room temperature wash in 0.1X SSC. Kodak XAR-5 film was exposed at −70°C with Dupont Cronex Lightning Plus intensifying screens (Dupont, Wilmington, DE). To determine relative RNA levels, autoradiographic film was digitized via Imapro QCS 3200 (Imapro Corp., Ottowa, Ontario, Canada) flatbed scanner using Adobe Photoshop plug-in running on Macintosh 7100/80 AV (Apple, Cupertino, CA). Resulting digital images were analyzed with VTrace (Image Analysis Facility, University of Iowa) running on SGI Indigo^2 (Silicon Graphics, Mtn. View, CA).

Immunohistochemistry

Lungs were perfused with 2% paraformaldehyde in phosphate buffer, fixed, and embedded in paraffin as described. Sections were pretreated with 0.05 mol/L glycine in phosphate-buffered saline and blocked with 5% bovine serum albumin. Immunostaining was carried out using a 1:1000 dilution of an affinity-purified rabbit polyclonal antibody directed against MME.[21] After overnight incubation at 4°C, sections were incubated for 1 hour with a 1:500 dilution of secondary antibody (Texas-Red-conjugated goat anti-rabbit IgG; Molecular Probes, Eugene, OR). Negative controls were prepared by omitting the primary antibody from the staining procedure. Sections were photographed using a BioRad MRC 1024 laser scanning confocal microscope.

Zymography

Gelatinases A and B were detected in tissue homogenates prepared from saline-injected control and bleomycin-treated lungs. Frozen tissue (kept at −80°C until assay) was thawed and then solubilized and incubated (3:10, w/v) in 0.5% Triton X-100/20 mmol/L phosphate buffer (pH 7.0) for 24 hours at 4°C to release MMPs. After solubilization, samples were centrifuged and supernatants were collected for zymography. Before loading onto gels, supernatants were mixed with nonreducing loading buffer as previously described.[24] Protein content of supernatants was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). For electrophoresis,

10% SDS-polyacrylamide gel electrophoresis gelatin-substrate gels were prepared as previously described.[24] Unstained broad-range protein standards (New England BioLabs, Beverly, MA) and conditioned media from human HT1080 cells, which contain gelatinases A and B, were run in parallel to determine the molecular weights of gelatinases present in lung tissue samples. Gels were stained, dried, and scanned (HP ScanJet IIc, Hewlett-Packard Corp., Hopkins, MN), using computer image analysis software (NIH Image Program). Images were analyzed using the electrophoretic gel lane calculation options in the program. Standardization was done with an optical density step program as previously described.[24]

Results

Development of Pulmonary Fibrosis in Bleomycin-Treated Mice

The onset and duration of bleomycin-induced pulmonary fibrosis in mice is well documented and depends upon the mouse strain, the dose of bleomycin, and the mode of instillation.[25] Among the murine strains that have been studied, C57BL/6 mice are bleomycin-sensitive and develop pulmonary fibrosis.[26,27] Although the majority of animal models of the disease are based on a single intratracheal instillation of bleomycin, we elicited the fibrotic response by i.p. injection of the drug. Although this is not a novel approach, we chose this method of instillation to more closely parallel the systemic approach used in chemotherapy.

To establish the most effective dose of bleomycin using this regimen, mice were injected i.p. with either saline, a total of 2 U of bleomycin (administered as injections of 0.5 U of bleomycin every other day over a 7-day period), or 4 U of bleomycin (administered as injections of 1 U of the drug every other day over a 7-day period). Multiple injections of bleomycin reduced morbidity compared with a single i.p. injection of the cumulative dose of the drug. The relative amounts of extracellular matrix mRNAs present in whole-lung homogenates from C57BL/6 mice sacrificed 21 days after the final bleomycin injection were determined by RNA blotting. By assessing the steady-state mRNA levels of fibronectin and the interstitial collagens type I and type III, which have been shown previously to be up-regulated by bleomycin in other animal models of the disease,[28–30] no elevation of extracellular matrix gene expression or histopathological evidence of fibrosis was evident in mice that received a total of 2 U of bleomycin, whereas a sevenfold increase in α1(I) procollagen mRNA as well as less marked increases in α1(III) procollagen mRNA and fibronectin mRNA were observed in mice that received 4 U of the drug (Figure 1A). H&E staining of lung tissue taken from mice treated with 4 U of bleomycin provided histopathological evidence of pulmonary fibrosis at 2 months after treatment as determined by patchy alveolar derangement and subpleural thickening that appeared more advanced by 4 months after treatment (Figure 1B). Based on these results, as well as Masson's trichrome blue staining of C57BL/6 lung

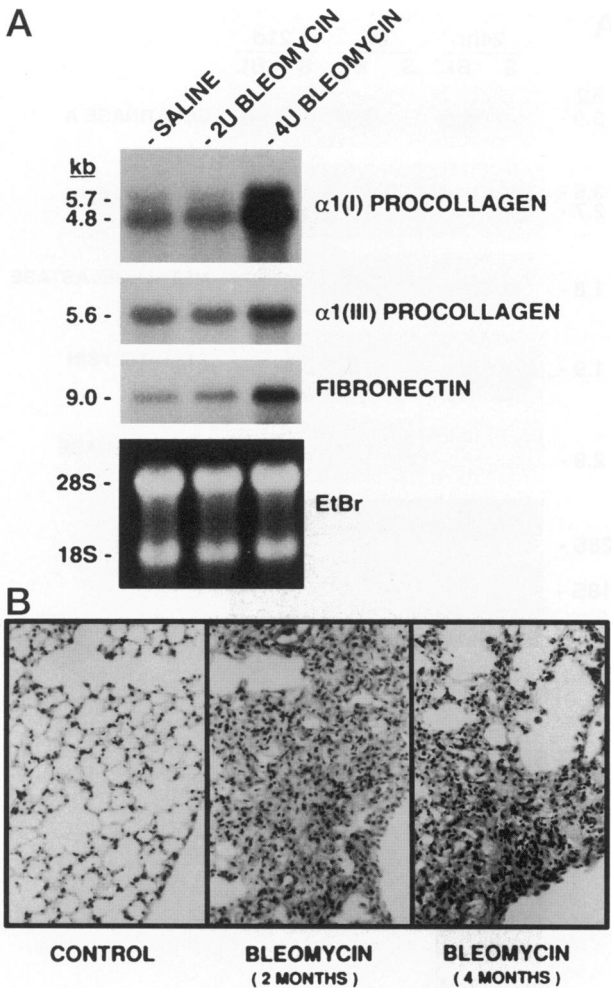

Figure 1. The effect of bleomycin treatment on the development of pulmonary fibrosis. **A:** RNA blot analysis of steady-state, total cellular lung RNA (10 μg) isolated 21 days after the final i.p. injection of either saline (**lane 1**), a total of 2 U of bleomycin (**lane 2**), or a total of 4 U of bleomycin (**lane 3**) in C57BL/6 mice. Blots were hybridized with ^{32}P-labeled cDNA probes for α1(I) procollagen, α1(III) procollagen, and fibronectin. **B:** Patchy subpleural fibrosis was observed in H&E-stained lung tissue. Note the alveolar collapse and subpleural thickening at 2 months after treatment and more advanced fibrosis seen at 4 months after treatment. Original magnification, ×250.

sections at 2 and 4 months after treatment to confirm collagen deposition in the fibrotic loci, hydroxyproline measurements of lung tissue, and the failure to induce pulmonary fibrosis in the bleomycin-insensitive BALB/c mouse strain using this drug regimen (data not shown), we elicited the fibrotic response in C57BL/6 mice using multiple injections of sublethal doses of bleomycin (1 U of bleomycin i.p. every other day for a total of 4 U of the drug) in subsequent experiments.

Differential MMP Gene Expression during Bleomycin Treatment

Steady-state levels of MMP transcripts in whole-lung homogenates from mice that received either saline or 4 U of bleomycin were assessed by RNA blot analysis using a series of MMP cDNA hybridization probes (Figure 2). The effect of bleomycin treatment on MMP gene expression

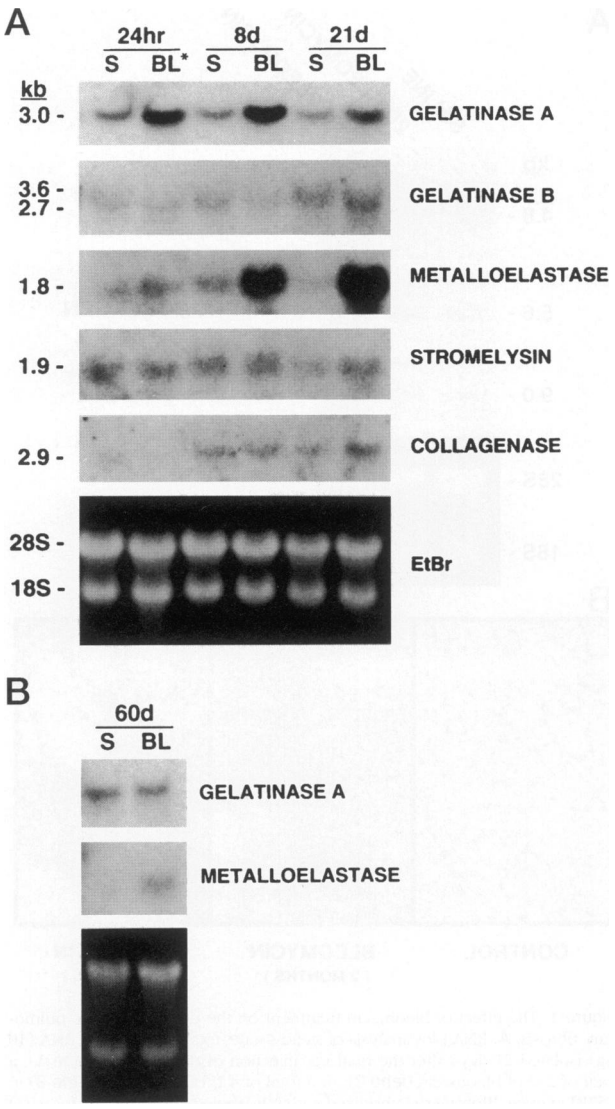

Figure 2. Differential MMP gene expression during bleomycin-induced pulmonary fibrosis. **A:** RNA blot analysis of steady-state, total cellular RNA (10 μg) from saline-injected (S) or bleomycin-injected (BL) mice. Blots were sequentially hybridized, stripped of radioactivity, and rehybridized with ^{32}P-labeled cDNA probes for gelatinase A and gelatinase B, MME, stromelysin, and interstitial collagenase as indicated: **lanes 1, 3,** and **5,** saline-injected control mice; **lane 2,** 24 hours after the second bleomycin injection (total, 2 U; *mid-point of drug course); **lane 4,** 8 days after the fourth and final bleomycin injection (total, 4 U); **lane 6,** 21 days after the fourth and final bleomycin injection (total, 4 U). **B:** RNA blot analysis of steady-state, total cellular RNA (10 μg) from saline-injected (S) or bleomycin-injected (BL) mice 60 days after treatment.

was selective and showed temporal differences. The most dramatic induction of MMP gene activity by bleomycin treatment was that of murine MME. MME transcripts, present in low abundance in control and treated lungs until 8 days after the completion of bleomycin instillation, increased 10-fold in 21-day post-bleomycin-treated lungs, and temporally paralleled the marked increase in α1(I) procollagen steady-state mRNA observed at this stage in the development of the disease (see top panel in Figure 5B). By 60 days after treatment, when patchy subpleural fibrosis was histopathologically evident, both MME and α1(I) procollagen mRNAs returned to near-baseline levels. Using an affinity-purified rabbit polyclonal antibody, MME was localized in the fibrotic lesions (Figure 3). Although fluorescence was detected in alveolar and interstitial macrophages, it was also detected throughout the lung matrix. This staining pattern has been observed previously for both MME and the human orthologue, human macrophage metalloelastase (HME) (S. Shapiro, unpublished observation) and is thought to be due to high-affinity binding of MME to elastin and perhaps other matrix components.

Unlike MME, stromelysin-1 steady-state mRNA was abundant in control and treated lungs but showed no significant changes throughout bleomycin treatment (Figure 2). Transcripts of interstitial collagenase were consistently difficult to detect in control and treated lungs by RNA blotting and did not change significantly throughout bleomycin treatment. Of the MMPs tested, only gelatinase A gene expression increased during the early stages of bleomycin treatment. Gelatinase A transcripts, present in normal lungs as previously reported,[18] increased in abundance two- to threefold above basal values as early as 24 hours after the second bleomycin insult and remained at this level throughout bleomycin treatment and the progression of fibrosis, returning to near-basal values at 60 days after treatment. The relative amount of gelatinase B steady-state mRNA, also present in normal lung tissue, did not change significantly during treatment. To assess the presence of gelatinases A and B in control and fibrosing lungs (21 days after treatment), whole-lung homogenates from nonperfused tissue were subjected to substrate gel zymography using gelatin as a substrate and conditioned media from HT1080 cells as molecular weight standards for gelatinases A and B (Figure 4). As predicted from their difference in molecular weight under nonreducing conditions, mouse gelatinase B proenzyme migrated more slowly than the human proenzyme.[31] As the lung tissue used in our study included both parenchymal and nonparenchymal cells, the overall gelatinolytic activity reflects the contribution of numerous cell types. Healthy and fibrosing lungs synthesized predominantly gelatinase A and B proenzymes as well as the less abundant active forms. Gelatinolytic activity was slightly elevated in bleomycin-treated lungs. The magnitude of increase in gelatinase B proenzyme was similar to that previously noted in isolated rat alveolar macrophages treated with bleomycin *in vitro*.[32] These results confirm the gelatinase Northern data and suggest no major changes in post-translational activation.

Temporal Appearance of Timp *Transcripts during the Development of Bleomycin-Induced Pulmonary Fibrosis*

To address whether the increases in MMP mRNAs seen during the early stages of bleomycin-induced injury and the development of fibrosis were associated with changes in *Timp* gene expression, we determined steady-state transcript levels in control and bleomycin-treated lungs by RNA blotting using a series of *Timp* cDNA hybridization probes (Figure 5). Constitutive ex-

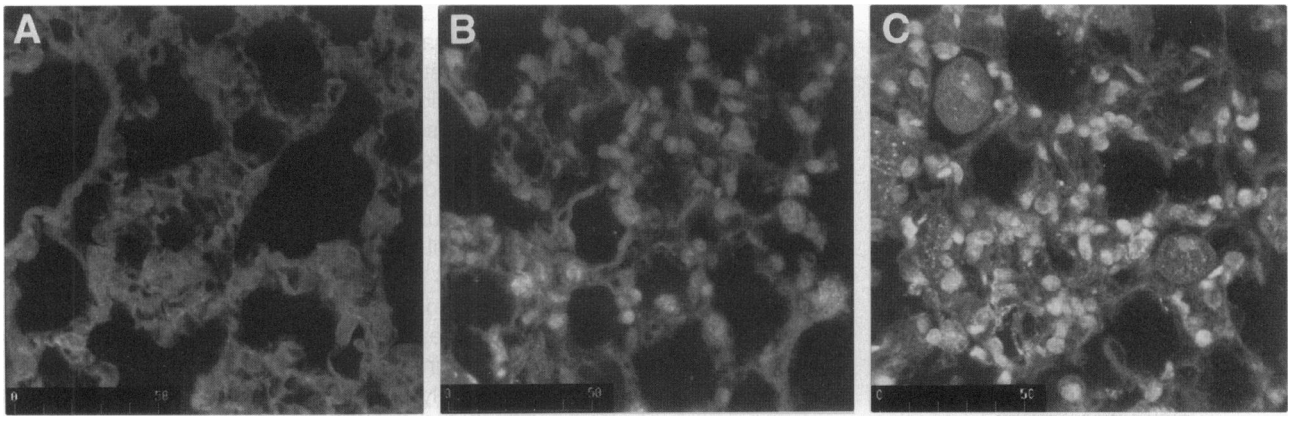

Figure 3. Immunohistochemical localization of macrophage metalloelastase in fibrotic lesions. Saline-injected (**A**) and 60-day post-bleomycin-treated (**B** and **C**) lungs were immunostained for MME as described. MME was localized in macrophages of fibrotic lesions. Bar, 50 μm.

pression of *Timp1*, *Timp2*, and *Timp3* was observed in saline-injected control animals. The relative abundance of the 3.5- and 1.0-kb *Timp2* transcripts and the 4.5-kb *Timp3* transcript was greater than that of the 0.9-kb *Timp1* transcript, in agreement with previous reports of *Timp* gene expression in adult mouse lung.[15,33,34] The effect of bleomycin treatment on increased *Timp* expression in C57BL/6 mice was selective. *Timp2* and *Timp3* transcript levels remained at basal values throughout the course of bleomycin treatment, whereas *Timp1* mRNA increased sixfold as early as 24 hours after the second drug insult (Figure 5A) and coincided with the early up-regulation of gelatinase A gene expression (Figure 2, lanes 1 and 2). A striking increase in the level of *Timp1* steady-state mRNA was observed during the development of fibrosis 21 days after the final bleomycin instillation (Figure 5B). The increase in *Timp1* mRNA seen at this stage was paralleled by marked increases in α(I) procollagen mRNA and in MME mRNA (Figure 2A). Sixty days after treatment, when patchy subpleural fibrosis was histologically evident, *Timp1* as well as MME and α1(I) procollagen mRNA levels decreased to near-basal values. The return of mRNA levels to constitutively expressed levels in whole-lung homogenates suggests that the fibrotic response has subsided and that the residual gene activity seen by RNA blotting represents normal matrix metabolism in unaffected regions of the lung. This notion is supported by previous studies citing an inability to detect signal for α1(I) and α2(I) procollagen and α1(III) procollagen transcripts over end-stage fibrotic lesions in bleomycin-treated mice using *in situ* hybridization analysis.[35,36]

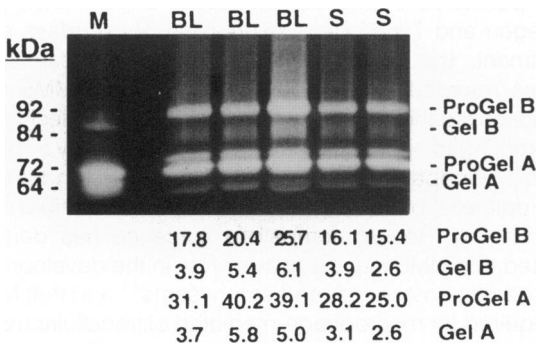

Figure 4. Presence of gelatinases A and B in lung homogenates. Gelatin substrate zymogram of lung homogenates from saline-injected control mice (S) and from bleomycin-injected mice 21 days after treatment (BL). Molecular weights (M) were determined from human HT1080 cells that constitutively express gelatinase A and B. Gelatinolytic values were determined by densitometry and are expressed as relative absorbance of lysis zones divided by sample protein content and appear below the zymogram.

Discussion

The regulation of pulmonary extracellular matrix remodeling during normal tissue metabolism and its dysregulation in numerous lung diseases results from a complex interplay of MMP transcriptional regulation, proenzyme activation, and inhibition by TIMPs in nearly all of the resident cells of the lung (reviewed in Refs. 37 and 38). In the case of bleomycin-induced pulmonary fibrosis, acute endothelial and epithelial damage initiates a sequence of events starting with the influx of a variety of inflammatory cells, including macrophages, neutrophils, and lymphocytes, into the alveolar space. These cells elaborate various cytokines, growth factors, and other mediators that influence the synthesis and activity of MMPs, which presumably function to repair the damaged alveolar extracellular matrix.[39,40] Certain of these MMPs, including stromelysin-1, gelatinase A, gelatinase B, and MME, are potentially capable of amplifying the disruption of alveolar architecture by virtue of their specificity for various components of the basement membrane, thus enabling further infiltration of inflammatory cells and fibroblasts into the alveolar space and thereby setting into motion the dysregulated matrix remodeling that follows. The study presented here is the first report of differential expression of several MMP and TIMP genes in an *in vivo* murine model of bleomycin-induced pulmonary fibrosis. Our results suggest that differential MMP expression may underlie the pathogenesis of the disease.

The earliest elevation of MMP gene expression after bleomycin treatment was that of gelatinase A, the capacity of which to degrade the major component of the basement membrane, type IV collagen, makes this MMP a candidate for repair of alveolar basement membrane

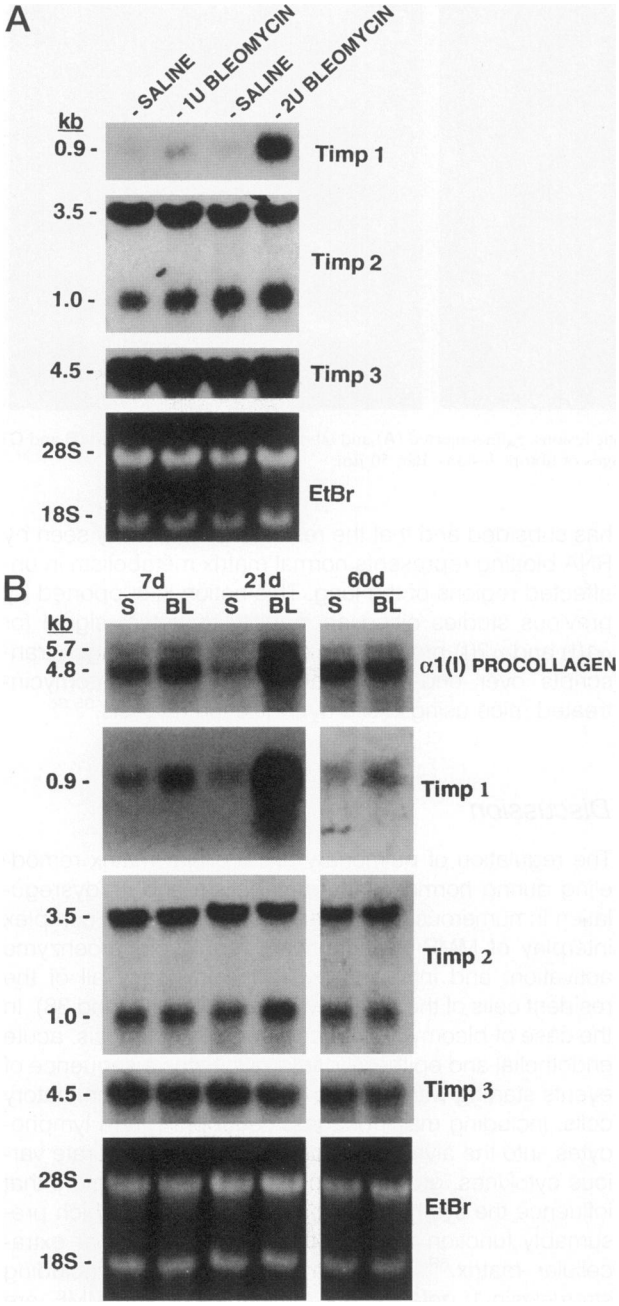

Figure 5. Timp *gene expression during the development of bleomycin-induced pulmonary fibrosis.* **A:** RNA blot analysis of steady-state, total cellular RNA (5 μg). Blots were sequentially hybridized, stripped of radioactivity, and rehybridized with ^{32}P-labeled cDNA probes for *Timp1, Timp2, and Timp3 as follows:* **lanes 1**, **3**, and **5**, saline-injected control mice; **lane 2**, 24 hours after the first bleomycin injection (total, 1 U); **lane 4**, 24 hours after the second bleomycin injection (total, 2 U). **B:** RNA blot analysis of steady-state, total cellular lung RNA (10 μg) isolated from mice that received saline (S) or 4 U of bleomycin (BL) and were analyzed at the prefibrotic stage (7 days after bleomycin treatment, **lanes 1** and **2**), during the development of fibrosis (21 days after treatment, **lanes 3** and **4**), and 60 days after treatment (**lanes 5** and **6**). Blots were sequentially hybridized, stripped of radioactivity, and rehybridized with ^{32}P-labeled cDNA probes for α1(I) procollagen, *Timp1, Timp2, and Timp3.*

damage as well as a potential contributor to more extensive endothelial and epithelial damage. In contrast, gelatinase B and stromelysin-1 steady-state mRNA levels did not change significantly after bleomycin treatment. As we did not measure the enzymatic activity of stromelysin-1 with its broad range of substrate specificity for almost all components of the basement membrane, its potential contribution to the pathology of the disorder will require additional examination. However, detection of gelatinase A and B proenzymes, as well as minor amounts of the active forms of the enzymes in healthy and fibrosing lungs, suggests that these MMPs may contribute to alveolar basement membrane remodeling. It is noteworthy that the early increase in gelatinase A mRNA (24 hours after the second bleomycin insult) was paralleled by a sixfold increase in *Timp1* mRNA. If these increases are reflected in elevations of active gelatinase A and TIMP-1 polypeptides, this, as well as the potential contributions of TIMP-2 and TIMP-3, may regulate gelatinase A activity as well as overall MMP activity that may be modulated at susceptible sites in the lung. Results from an immunohistological study indicate that gelatinase A colocalizes with type IV collagen in intact alveolar basement membranes of healthy human lungs as well as in endothelial and disrupted epithelial basement membranes in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis.[38] That gelatinase A activity may be modulated at these sites is suggested by the co-localization of TIMP-2. As fibrosis in these patients progressed, immunoreactivity of both gelatinases A and B and TIMP-1 and -2 increased and was present in myofibroblasts and epithelial cells lining the proliferative lesions. Comparable examination of bleomycin-induced fibrotic lesions will be useful in identifying the cell types expressing MMPs and TIMPs during the initiation and progression of fibrosis. Although *Timp3* transcripts are abundant in mouse lungs, and are homogeneously distributed throughout the parenchyma,[34] the contribution of TIMP-3 to pulmonary matrix remodeling is not yet known.

The most striking increase of bleomycin-induced MMP gene expression was that of MME, which was up-regulated 8 days after the final bleomycin insult, followed by a more marked induction 21 days after treatment, which was paralleled by marked elevations of both α1(I) procollagen and *Timp1* gene expression. By 60 days after treatment, the steady-state transcripts of these three genes returned to near-baseline levels and MME was immunohistochemically localized in the fibrotic lesions. It is tempting to speculate that MME may also play a role in attempted repair or continued damage of the endothelial and epithelial basement membranes at susceptible sites in the lung. Recent compelling evidence has demonstrated that MME plays a primary role in the development of cigarette-smoke-induced emphysema[41] and that MME is required for macrophage-mediated extracellular matrix and tissue invasion both *in vivo* and *in vitro*.[42] Both MME and the human orthologue, HME, have potent elastolytic activity as well as the capacity to degrade components of the basement membrane, including fibronectin, laminin, entactin, chondroitin sulfate, and heparan sulfate.[43–45] Interestingly, MME does not degrade interstitial collagen,

which may explain the presence of fibrosis despite extensive MME expression. Moreover, MME may influence inflammatory cell recruitment and enhance macrophage accumulation and subsequent inappropriate matrix remodeling. MME expression is restricted primarily to tissue macrophages, including alveolar macrophages, and with the exception of its abundant expression in the actively remodeling term placenta, normal human tissues do not express this MMP.[44,46] As noted previously for the elevation of gelatinase A gene expression during the early stages of bleomycin injury, the marked elevation of MME gene expression at 21 days after treatment was accompanied by a striking increase in *Timp1* mRNA. As *Timp1* is transcriptionally regulated by a number of cytokines, growth factors, and other mediators present in the pulmonary milieu,[47–50] the up-regulation of *Timp1* at this stage may reflect a dynamic change in remodeling pressure in the fibrosing lung that may modulate MMP biosynthesis and activity. Closer examination of developing fibrotic lesions using *in situ* hybridization and immunolocalization analyses, together with measurements of MME catalytic activity and its potential regulation by TIMP binding at the site of injury, will address this possibility.

Finally, bleomycin-induced pulmonary fibrosis culminates in the overproduction of interstitial collagen in the distal airspace. That this may be the consequence of an imbalance in the ratio of interstitial collagenase, one of the major metalloproteinases responsible for the degradation of interstitial collagen, and TIMPs has been suggested in studies of fibrotic diseases of the kidney, lung, and liver.[38,51–55] In these tissues, a decrease in the biosynthesis or activity of interstitial collagenase and an up-regulation of TIMP-1 and, in some cases, TIMP-2, together with localization of TIMPs in fibrotic lesions, is thought to contribute to the pathology of fibrosis. Transcripts of interstitial collagenase were consistently difficult to detect in control and bleomycin-treated lung tissue by RNA blotting and did not change significantly throughout the course of treatment. Although rodents possess collagenase-3 (MMP-13), this enzyme appears to be expressed in place of collagenase-1 in rodent macrophages and other cell types. Although it is possible that collagenase-3 expression may be up-regulated as fibrosis progresses, it appeared that elevated α1(I) procollagen gene expression was not accompanied by a parallel increase in collagenase-3 biosynthesis during the time course of our study. Examination of collagenase-3 biosynthesis and enzymatic activity throughout a more extended time course will address the question of whether bleomycin-induced pulmonary fibrosis follows a similar pattern as noted in other fibrotic diseases.

In summary, an imbalance in the relative ratios of active MMPs and TIMPs has been shown to contribute to the pathology of a number of diseases. Experimental models of acute lung injury using bleomycin and other agents, together with studies of patients with diverse lung diseases (reviewed in Refs. 37, 38, and 56), have highlighted the contributions of cytokines, growth factors, inflammation, altered alveolar structure, and dysregulated matrix remodeling to the pathogenesis of these disorders. The results of our study have shown that bleomycin-induced pulmonary fibrosis results in the differential expression of MMP and TIMP genes. If the relative ratio of active MMPs and TIMPs is modulated at susceptible sites in the lung, such an imbalance could play a critical role in the pathogenesis of the disease. Additional examination of developing fibrotic lesions using the murine model of bleomycin-induced pulmonary fibrosis presented in this study will lead to a better understanding of the nature of the cell types involved, and the complex mechanisms by which fibrosis is initiated and progresses.

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