# Time course of matrix metalloproteases and tissue inhibitors in bleomycin-induced pulmonary fibrosis

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To investigate simultaneously localization and relative activity of MMPs during extracellular matrix (ECM) remodeling in bleomycin-induced pulmonary fibrosis in rat, we analyzed the time course of the expression, activity and/or concentration of gelatinases MMP-2 and MMP-9, collagenase MMP-1, matrylisin MMP-7, TIMP-1 and TIMP-2, both in alveolar space (cellular and extracellular compartments) and in lung tissue. MMP and TIMP expression was detected (immunohistochemistry) in lung tissue. MMP activity (zymography) and TIMP concentration (ELISA) were evaluated in lung tissue homogenate (LTH), BAL supernatant (BALs) and BAL cell pellet (BALp) 3, 7, 14, and 28 days after bleomycin intratracheal instillation. Immunohistochemistry showed an extensive MMP and TIMP expression from day 7 in a wide range of structural and inflammatory cells in treated rats. MMP-2 was present mainly in epithelia, MMP-9 in inflammatory cells. MMP-2 and MMP-9 activity was increased respectively in BAL fluid and BAL cells, with a peak at day 7. TIMP-1 and TIMP-2 concentration (ELISA) enhancement was delayed at day 14. In conclusion gelatinases and their inhibitors are significantly activated during bleomycin-induced pulmonary fibrosis. Marked changes in gelatinases activity are observed early in the alveolar compartment, with a prevailing extracellular activity of MMP-2 and a predominant intracellular distribution of MMP-9, while enzyme activity changes in lung parenchyma were less evident. In the repairing phase the reduction of gelatinases activity is synchronous with a peak of alveolar concentration of their inhibitors.

Key words: metalloprotease, MMP; tissue specific inhibitor, TIMP; bleomycin; pulmonary fibrosis.

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Abnormal extracellular matrix (ECM) deposition and remodelling due to a disregulated balance of degradative enzymes and of fibrogenic stimuli is a recognized key event in the development of pulmonary fibrosis. Remodelling of ECM in both physiological and pathological situations is controlled primarily by the matrix metalloprotease (MMP) family of enzymes. MMPs are extracellular enzymes secreted as inactive zymogens (Corbel *et al.*, 2002) and activated after secretion. MMPs in the lung are produced by structural (bronchial, bronchiolar and alveolar epithelia, endothelia, fibroblasts) and inflammatory cells (macrophages, neutrophils) (Matsumoto *et al.*, 1996; Nagase, 1997). Expression and activity of MMPs and of their tissue specific inhibitors (TIMPs) have been studied in several human lung diseases characterized by basal membrane destruction and fibrosis (Hayashi *et al.*, 1996; Lanchou *et al.*, 2003), but most of the interest is associated with the potential targeting of MMPs in the therapy of pulmonary fibrosis, in particular idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP) (Selman *et al.*, 2000). Unfortunately, human studies do not allow following the expression and activation of MMPs and TIMPs during disease evolution. For this reason, a number of experimental models have been proposed to investigate the development of pulmonary fibrosis, among which lung injury induced via the endotracheal instillation of bleomycin is the most common (Aso *et al.*, 1976). Although the use of bleomycin-induced lung fibrosis as a model for UIP has recently been challenged for its predominantly inflammatory mechanism and acute evolution with eventual repair (Borzone *et al.*, 2001), it still represents an acceptable model to study cell-matrix interaction in ECM remodeling and fibrosis (Gabazza *et al.*, 2002).

Previous studies provided cumulative data on enzymes present both in alveolar spaces and in lung interstitium. However, it is possible that MMPs play

a different role in lung alveolar and interstitial compartments, as is suggested by their different cellular source and substrate specificity.

To better understand how the expression and activity of MMPs and their inhibitors are induced and evolve in the tissue and alveolar compartments of rat lungs during ECM remodeling in bleomycin-induced pulmonary injury and fibrosis, in the present study we analyzed the time course of the activity of gelatinases (MMP-2 and MMP-9), collagenase (MMP-1), matrylisin (MMP-7), and the concentration of TIMP-1 and TIMP-2, both in alveolar space (cellular and extracellular compartments) and in lung tissue, and compared the results with the immunohistochemical expression of the enzymes in the lungs.

## Materials and Methods

### Animals

Adult male Sprague-Dawley rats weighting 150 g were purchased from Charles River Italy s.p.a. (Milan, Italy). For the duration of the experiment, the animals were maintained in specific pathogen-free conditions, were fed with commercial rat food and water *ad libitum* with a 12-h dark/light cycle.

### Induction of bleomycin-induced pulmonary fibrosis

After general anesthesia administered with intra-muscular injection of tiletamine chlorohidrate (3,33 mg/100g body weight) and zolazepam chlorohidrate (3,33 mg/100g body weight), 20 rats received bleomycin sulfate (Nippon Kayaku Co. Ltd, Chiyoda Ku, Tokyo, Japan) 1.7 U in 0.4 mL of sterile solution (1.13U/100g body weight). Eight control rats received only 0.4 mL of saline solution. Bleomycin and sterile solutions were prepared immediately before administration and were administered as a single dose with a 25-gauge needle directly into the trachea, exposed via a small cervical skin incision and separation of the strap muscles. Five treated and two control animals were killed at day 3, 7, 14, and 28 after treatment by carotid exsanguination under deep anesthesia. The experimental protocol was approved by the Ethical Committee of the University of Pavia.

### Bronchoalveolar Lavage (BAL)

The lungs were exposed by a mid thoracotomy incision and the trachea was cut to place a polyethylene cannula (Angiocut, Becton Dickinson, Utah, USA). The lungs were lavaged *in situ* tree times with 3 aliquots of 5 mL of saline. The BAL fluid were pooled, and centrifuged for 10 min at 1500 rpm at 4 °C. Supernatant of BAL (BALs) was gently aspirated, concentrated by ultrafiltration at 4°C at 3000 rpm for 45 min (Millipore Ultrafree Biomax, 5000 NMWL, Bedford, Minn), aliquoted and frozen at –80°C for gel zymography analysis. Cell pellet of BAL (BALp) was resuspended in 250 μL of RPMI (Invitrogen Corporation) and stored at –80°C for gel zymography analysis. An aliquot (about $5 \times 10^5$ cells) of this cell suspension was used to prepare duplicate cytospin preparations, which were stained with May-Grünwald-Giemsa for light microscopy. A cell differential count was obtained by randomly reading at least 200 cells per slide, and expressed according to the percentage of macrophages, neutrophils, lymphocytes and eosinophils.

### Lung tissue processation

After lavage the lungs were removed *en bloc*, quickly weighted and placed in cold (4°C) buffer (30 mM Histidine, 250 mM sucrose, 2 mM EDTA, pH 7.2). Subsequently they were cut, and a part of each lobe (4-5 mm³) was frozen in liquid nitrogen and stored at -80°C, until use for gel zymography analysis. The remaining parenchyma was fixed with neutral buffered 10% formalin, embedded in paraffin according to routine laboratory protocols, and sectioned at a thickness of 4 μm for light microscopy and immunohistochemistry. Sections were stained with hematoxylin and eosin and Movat pentachrome; unstained sections were used for immunohistochemistry. Hematoxylin and eosin and Movat stained slides were examined to quantitatively assess the type and grade of tissue damage, inflammatory cell infiltration and tissue repair and fibrosis. Semiquantitative scoring was performed in accordance with the method described by Madtes *et al.* (1999).

### Immunohistochemistry

Immunohistochemical analysis of MMP and TIMP expression was performed with antibodies and antisera against MMP-1, MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 as detailed in Table 1. Tissue sections collected on silanate glass slides were deparaffinized and rehydrated. After inhibition of endogenous peroxidase and appropriate pretreatment tissue sections were incubated overnight at 4

**Table 1. MMPs and TIMPs concentrations for immunohistochemical assay. MMPs: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinase; MWO: microwave oven.**

| Name | Provider | Clone | Working dilution | Pretreatment |
|---|---|---|---|---|
| MMP-1 | LabVision Corporation (Fremont, Ca) | Rabbit polyclonal | 1:300 | None |
| MMP-2 | LabVision Corporation | Rabbit polyclonal | 1:1000 | Pepsin 30' 37°C |
| MMP-7 | Santa Cruz Biotechnologies (Santa Cruz, Ca) | Goat polyclonal (C-17) | 1:250 | Pepsin 20' 37°C |
| MMP-9 | LabVision Corporation | Mouse monoclonal (IIA5) | 1:50 | MWO 500W 10' |
| TIMP-1 | LabVision Corporation | Rabbit polyclonal | 1:800 | MWO 500W 10' |
| TIMP-2 | LabVision Corporation | Rabbit polyclonal | 1:1000 | MWO 500W 10' |

°C with primary antibodies, diluted as appropriate (Table 1) (Dako antibody diluent, DakoCytomation, Carpinteria, CA). The reactions were revealed with the avidin-biotin-peroxidase complex method (DakoCytomation LSAB+ System), using diaminobenzidine tetrahydrochloride as chromogen substrate (DakoCytomation, Liquid DAB). Each reaction set included a negative control obtained with substitution of the primary antibody with dilution buffer, and positive controls as suggested by the manufacturer. MMP and TIMP expression in lung inflammatory cells was also considered as internal positive control. Immunostained slides were examined to identify the cell types expressing each MMP and TIMP, and to semiquantitatively score the amount of protein present in the lung.

### Protein extraction

After washing in ice-cold saline, frozen lung samples were homogenized in an ice-cold extraction buffer (1:10 wt/vol) containing cacodylic acid (10 mM), NaCl (150 mM), $ZnCl_2$ (1 mM), $CaCl_2$ (20 mM), $NaN_3$ (1.5 mM), Triton -X 100 0.01% v/v (pH5.0). The maintenance of a low pH and temperature prevented proteolytic activation during the extraction process. The homogenate was then centrifuged (5 min. 10.000 rpm) and the supernatant harvested. Cells (BALp) obtained from centrifugation of BAL were lysed with 50 nM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100. The protein concentration of lungs, BAL supernatant and pellet was measured with the colorimetric Lowry method (1951). All samples were aliquoted and stored at -20°C before use.

### MMPs zymography

Lung extracts were normalized by a final protein concentration of 400 µg/mL using sample loading buffer (0.25 M Tris-HCl, 4% sucrose w/v, 10% SDS w/v and 0.1% bromophenol blue w/v, pH 6.8), while BALs and BALp were normalized to a final protein concentration of 300 µg/mL.

Diluted samples were loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin or collagen, under nonreducing conditions. The gels were run at 15 mA/gel trough the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), at the temperature of 4°C in a running buffer containing 25 mM Tris-HCl, 190 mM Glycine, 0.1% SDS w/v. After the SDS-PAGE the gels were washed twice in 2.5 % Triton X-100 for 30 min each to allow protein renaturation, rinsed in water, and incubated for 18 h in an incubation buffer at 37°C (50 mM Tris-HCl, $CaCl_2$ 5 mM, $NaN_3$ 0.02% w/v, pH 8). After incubation, the gels were stained with 0.125 % Coomassie blue R-250 in 30% methanol, 10% acetic acid, 60% water for 30 min., destained for 1h in 30% methanol, 10% acetic acid, 60% water, with the change of the solution after 30 min., to reveal zones of lysis; then the gels were dried and analyzed. Trypsin activation was required for MMP-1 assessment. Activation was carried out in Tris-Hcl 25 mM at 37°C for 15 min. containing 1:0.05 w/mL. The reaction was stopped by placing the mixture on ice. Trypsin concentration and incubation time were selected in preliminary experiments demonstrating that these conditions provided optimal condition of activation.

Extracts were normalized by protein content and then loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of type III denatured collagen, under non-reducing conditions. The gels were run, stained and destained as described above.

The determination of MMP-7 was obtained using commercial gels containing 1 mg/mL Casein (Bio-Rad Laboratories, Italy). Run conditions were the same as above.

Positive controls for SDS-PAGE zymography were included in all zymograms. The zymograms were analyzed with a densitometer (GS 710 Densitomer, Bio-Rad) and data were expressed as optical density (OD) normalized to protein content.

**Table 2.** Differential cell count of BAL fluid in control animals and in response to bleomycin (1.13U/100g body weight). Cell count is expressed as $10^6$ cells/mL of macrophages, neutrophils, lymphocytes and eosinophils. The data are expressed as mean ± standard deviation (SD).

|  | Macrophages | Neutrophils | Lymphocytes | Eosinophils |
|---|---|---|---|---|
| C | 0,19±0,05 | 0,01±0,01 | 0,04±0,02 | 0,00±0,00 |
| 3 d | 0,17±0,03 | 0,01±0,00 | 0,04±0,01 | 0,00±0,00 |
| 7 d | 0,08±0,03 | 0,08±0,03 | 0,06±0,01 | 0,05±0,04 |
| 14 d | 0,57±0,53 | 0,02±0,01 | 0,11±0,13 | 0,00±0,00 |
| 28 d | 0,32±0,13 | 0,07±0,06 | 0,12±0,07 | 0,00±0,00 |

### TIMPs analysis

Lung homogenates and BALs were assayed for TIMP-1 and TIMP-2 contents with commercially available ELISA kits (Biotrak; Amersham Pharmacia Biotech), according to manufacturer instructions. The kits detect total TIMP-1 or TIMP-2, free and complexed with metalloproteinases. Total TIMP concentration was expressed in ng/mL reported to mg/mL of proteins of each sample.

### Statistical analysis

Control animals at different time point were considered together.

Non- parametric tests were employed in the statistical analysis of the data because of the small sample size and because the data were not normally distributed (Kolmogorov-Smirnov test). We used the Kruskal-Wallis ANOVA test and for post hoc analysis the Mann-Whitney U test.

All tests were two-sided. Statistica 6.0 (Statsoft, Inc. 2003, Tulsa, OK, US.) was used for statistical computations.

## Results

### Differential cellular analysis of BAL

Cell counts from control and treated animals are depicted in Table 2. In brief, BALp obtained from control animals consisted of more than 80% macrophages. On day 7 the differential cellular count of BAL showed an increased cell number with increased percentage of neutrophils (28%), eosinophils (18%) and lymphocytes (23%), and a decrease of macrophages (31%). Total cell count was markedly increased at day 14 after bleomycin administration, with lymphocyte and macrophage recruitment. At day 28, cell count was reduced but still 2fold higher than normal; percentage cell distribution showed an increase of polymorphonuclear granulocytes and macrophage reduction.

### Histological evaluation

Rat lung damage following bleomycin instillation did not differ from what has been described in previous studies (Borzone *et al.,* 2001). Three days after administration, lungs began to show increased alveolar and septal cellularity, with alveolar bronchiolization, hyperplasia of type 2 pneumocytes and focal endoalveolar hyaline membranes. The damage was more extensive at day 7, with higher inflammation scores and minimal increase of fibrosis. Starting from day 14, deposition of interstitial collagen appeared, with retraction of surrounding parenchyma, and the number of macrophages both in alveolar spaces and interstitium peaked; fibrosis scores were higher than those of inflammation. At day 28, fibrosis scores were still high, although partial regression was observed in some cases, while inflammation scores were reduced. Macrophages were numerous in the alveolar spaces. Control lungs did not show significant inflammation or fibrosis at any time point.

### Immunohistochemical expression of MMPs and TIMPs

Antibodies used for immunohistochemical reactions recognize both inactive proenzimes and activated MMPs. For this reason, the immunohistochemical study of bleomycin treated lungs showed expression of most proteins in a wide range of structural and inflammatory cells, which obviously did not reflect the actual enzyme activity in lung. However this overexpression was related with the treatment, since in control lungs only MMP-1 and TIMP-1 were occasionally expressed in alveolar macrophages, and a MMP-2 focal reactivity was present in endothelia and bronchial epithelia. MMP-7 was only focally expressed in bronchiolar epithelial cells.

Briefly (Figure 1), starting from day 7, intense MMP-2 expression appeared in reactive bronchiolar and alveolar epithelia, endothelia and macrophages. A high number of MMP-2 positive cells, mostly alveolar and interstitial macrophages, were also present at day 14 and 28. Immunoreactivity for MMP-9 was recognized from day 7 in infiltrating neutrophils, macrophages and in hyperplastic epithelia, also progressing with the degree of inflammation and with macrophage infiltration. MMP-7 was expressed in inflammatory cells and, to a lesser extent, in bronchiolized and type 2 alveolar epithelia. Its appearance was delayed, at day
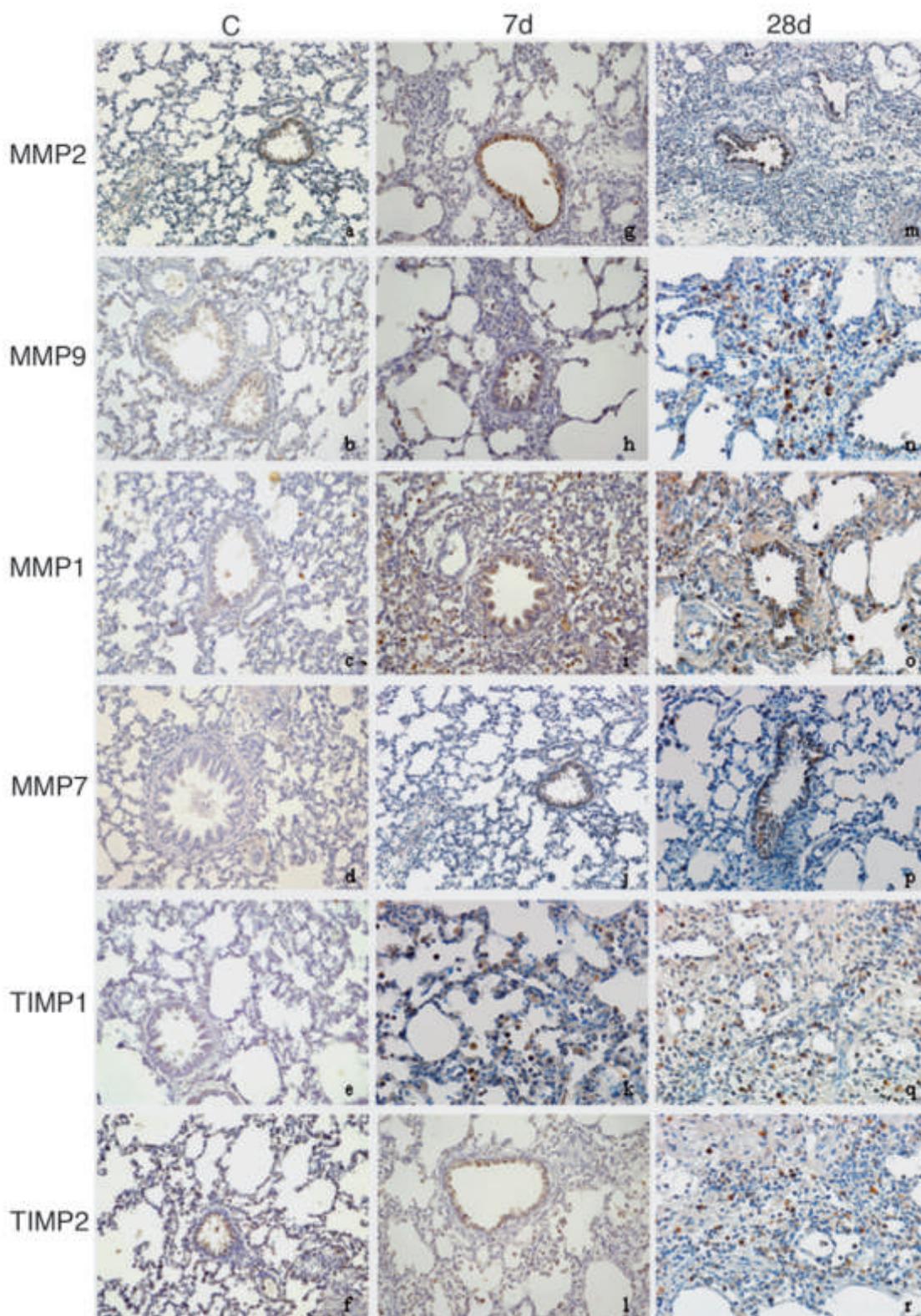
**Figure 1. Photomicrographs showing the expression of MMPs and TIMPs in bleomycin-treated rat lungs. Gelatinases MMP-2 and MMP-9 expression was increased in airways and inflammatory cells shortly after treatment (7 days); MMP-2 was expressed preferentially in epithelial cells, while MMP-9 was present mainly in inflammatory cells. At 28 days, MMP2 expression was reduced, while interstitial inflammatory cells were highly reactive for MMP9. MMP-1 and MMP-7 were present respectively in alveolar macrophages and in epithelial and inflammatory cells at highest intensity in late phase after bleomycin treatment (28 days). TIMP-1 and TIMP-2 expression appeared at 7 days and was maximal in interstitial cells in fibrotic areas in the reparative phase (28 days). MMP and TIMP expression was negligible in controls. Original magnification X 20.**

**Table 3. Gelatinolitic activity values of MMP-2 and MMP-9 in LTH, BALs and BALp. The data were expressed as median and interquartile range.**

| | MMP-2 | | | MMP-9 | | |
|---|---|---|---|---|---|---|
| | LTH Median (25%-75%) | LTH Median (25%-75%) | BALs Median (25%-75%) | BALp Median (25%-75%) | BALs Median (25%-75%) | BALp Median (25%-75%) |
| C | 0,91 (0,90-1,10) | 0,17 (0,14-0,19) | 0,00 (0,00-0,14) | 0,07 (0,00-0,11) | 0,69 (0,49-2,18) | 0,00 (0,00-0,00) |
| 3 d | 1,28 (1,18-1,34) | 0,13 (0,12-0,15) | 0,00 (0,00-0,00) | 0,28 (0,19-0,32) | 0,91 (0,58-1,64) | 0,00 (0,00-0,00) |
| 7 d | 1,52 (1,38-1,83) | 0,22 (0,13-0,38) | 0,40 (0,00-0,61) | 3,25 (1,68-6,11) | 4,33 (0,63-6,99) | 1,32 (0,00-1,88) |
| 14 d | 1,28 (1,20-1,48) | 0,09 (0,06-0,16) | 0,00 (0,00-0,00) | 0,49 (0,26-0,63) | 0,56 (0,49-0,76) | 0,00 (0,00-0,00) |
| 28 d | 1,11 (1,06-1,19) | 0,18 (0,15-0,19) | 0,37 (0,27-0,41) | 0,00 (0,00-0,63) | 1,81 (1,30-2,50) | 0,29 (0,00-0,58) |

**Table 4. TIMP-1 and TIMP-2 concentration values in LTH and BALs detected by ELISA; collagenolitic activity values of MMP-1 and matrilysin MMP-7 in LTH. The data were expressed as median and interquartile range.**

| | TIMP-1 | | TIMP-2 | | MMP-1 | MMP-7 |
|---|---|---|---|---|---|---|
| | BALs Median (25%-75%) | LTH Median (25%-75%) | BALs Median (25%-75%) | LTH Median (25%-75%) | LTH Median (25%-75%) | LTH Median (25%-75%) |
| C | 0,22 (0,09-0,25) | 0,53 (0,47-0,91) | 4,29 (1,06-7,32) | 0,37 (0,15-0,77) | 0,27 (0,24-0,77) | 0,39 (0,00-0,41) |
| 3 d | 0,16 (0,13-0,87) | 0,79 (0,35-1,18 ) | 6,85 (6,38-9,00 ) | 0,35 (0,11-4,02) | 0,32 (0,30-0,34) | 0,11 (0,00-1,28) |
| 7 d | 0,00 (0,00-0,00) | 0,25 (0,13-0,33) | 11,51 (1,33-18,03) | 0,00 (0,00-3,13) | 0,29 (0,24-1,62) | 0,00 (0,00-0,11) |
| 14 d | 0,00 (0,00-0,00) | 0,95 (0,59-1,18 ) | 20,55 (16,21-22,54) | 5,79 (4,63-7,12) | 0,26 (0,23-0,33) | 0,13 (0,07-0,22) |
| 28 d | 0,91 (0,07-2,80) | 0,14 (0,13-0,16 ) | 2,56 (0,22-6,55 ) | 0,00 (0,00-0,00) | 1,37 (1,21-1,50) | 0,00 (0,00-0,00) |

14, and remained elevated in inflammatory and reparative-fibrotic foci at day 28. MMP-1 was expressed in alveolar and interstitial macrophages, and its expression increased in accordance with their number. TIMP-1 was detected in macrophages and fibroblasts and TIMP-2 in macrophages, fibroblasts and alveolar epithelia. Their expression appeared at day 7 and progressively increased at day 14 and 28.

### Gel Zymography results

As illustrated in Figure 2a, after bleomycin instillation the overall activity of MMP-2 showed a trend toward an increase at day 7. In LTH MMP-2 significantly (Kruskal-Wallis test: $p=0.004$) increased at day 7 and subsequently decreased. In BALs MMP-2 ($p=0.0973$) at day 7 was sixfold compared with controls, fourfold and sevenfold compared with results at day 3 and 14, respectively. In BALp MMP-2 activity ($p=0.053$) was detected only at day 7 and 28 in treated rats; at day 7 it was fivefold greater as compared to day 28. Median values and interquartile ranges of densitometric evaluation of MMP2 gel zymography are reported in Table 3.

MMP-9 activity (Figure 2b) was extremely low in all compartments of control lungs. In treated rats ($p=0.0018$) it significantly increased at day 7 only in the cellular compartment of the BAL, when it was elevenfold, sixfold and fortysixfold greater compared to day 3, day 14 and controls respective-

ly. A lower activity was detected in BALs, peaking at day 7. In LTH a low level of MMP-9 activity was constant along the time. Median values and interquartile ranges of densitometric evaluation of MMP9 gel zymography are reported in Table 3. An example of zymogram of pro-MMP2 and pro-MMP-9 in lung tissue is shown in Figure 4.

MMP-1 ($p=0.03$), as shown in Figure 3a, peaked at day 28 in LTH. In LTH MMP-7 activation was higher in controls than in treated rats ($p=0.095$) (Figure 3b). In BALp no MMP-7 activity was present in controls; only at day 7 zymography showed an MMP-7 activity in treated rats. Median values and interquartile ranges of densitometric evaluation of MMP1 and MMP7 gel zymography are reported in Table 4.

### TIMPs determination

The peak of TIMP-2 (Figure 2c) was delayed with respect to the increase in MMP activity. In both BALs and LTH TIMP-2 at day 14 was fivefold increased respect to controls (Kruskal-Wallis test: in BALs $p=0.012$ and in LTH $p=0.0077$). After bleomycin instillation, TIMP-1 (Figure 2d) was not detectable in BALs ($p=0.0043$) at 7 and 14 days, and peaked at 28 day. In LTH TIMP-1 ($p=0.0037$) showed a bimodal trend, with two peaks, at 3 and 14 days. No TIMPs activity was observed in BAL cellular compartment. Median values and interquartile ranges of TIMP activity are reported in Table 4.
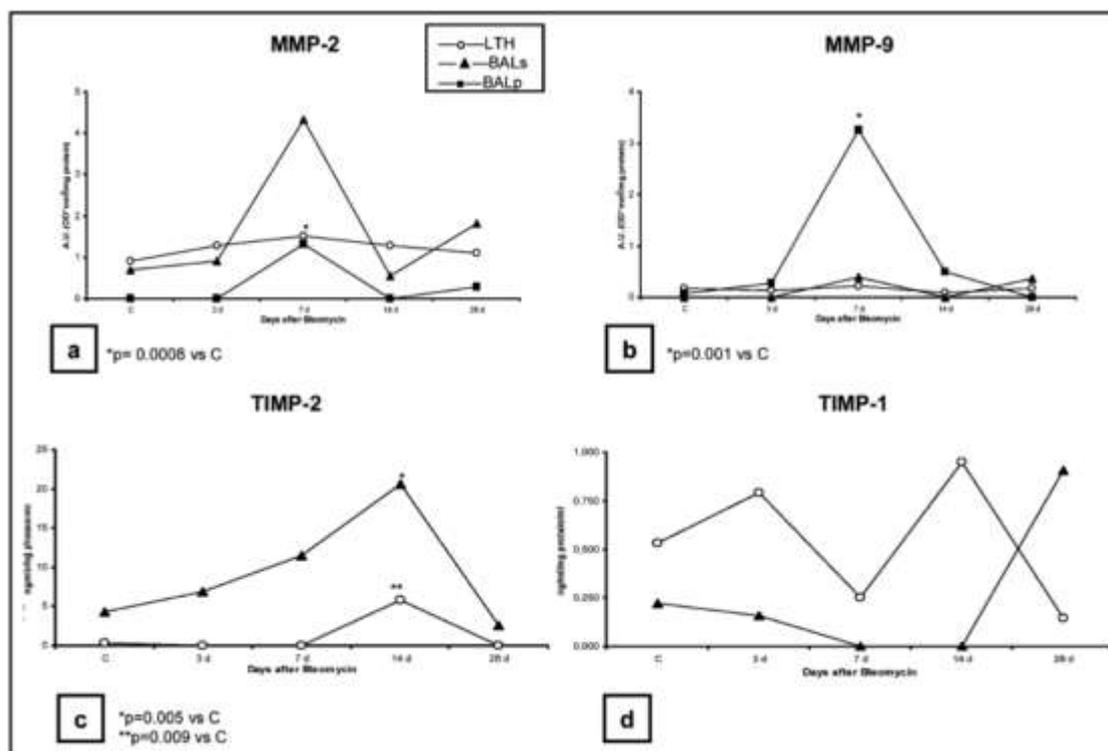
**Figure 2.** Gelatinolitic activity of MMP-2 (a) and MMP-9 (b) in control and bleomycin treated rats in BALs, BALp and LTH by gelatin zymographic analysis expressed in A.U. (OD*mm²/mg protein). TIMP-1 and TIMP-2 concentration in LTH and BALs detected by ELISA (ng/ml/mg protein/mL) is shown in (c) and (d). The data are expressed as median. p: Mann-Whitney U test (treated rats at each time point versus controls).
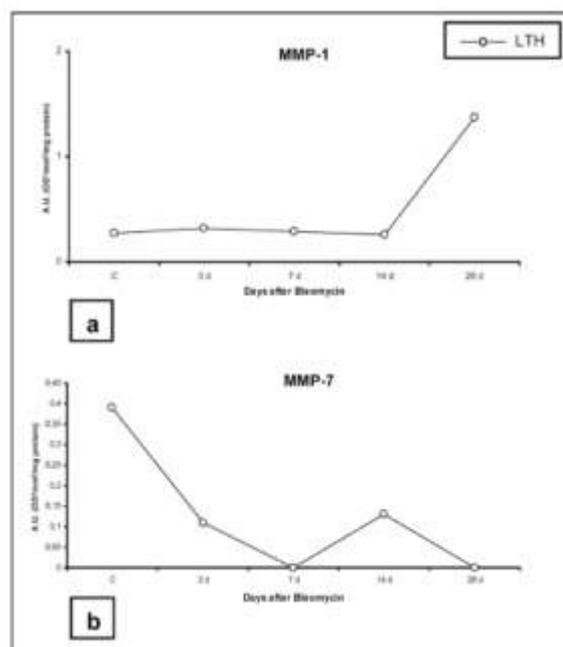


**Figure 3.** Collagenolitic activity of MMP-1 (a) and activity of matrilysin MMP-7 (b) in control and bleomycin treated rats in LTH by collagen and casein zymography analysis expressed in A.U.(OD*mm²/mg protein). The data are expressed as median.
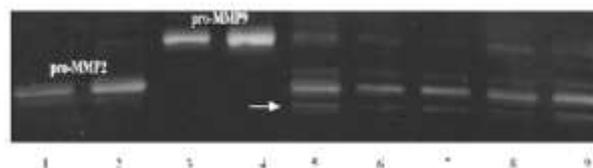


**Figure 4.** Example of pro-MMP2 and pro-MMP-9 zymogram in lung tissue. Standard latent pro-MMP2 and pro-MMP9 (purified from mammalian cells) at 25-50 ng/mL concentration (lane 1,2 and 3,4). Extract from rat lung tissue (lane 5-9).

## Discussion

In the present study, we investigated the time course of MMPs and TIMPs expression and activity in rat lungs after bleomycin injury, separately in alveolar space cellular and extracellular compartments, and in lung tissue. Our data showed that gelatinases and their inhibitors are significantly activated in the lung after bleomycin-induced injury and development of fibrosis, while MMP-1 does not seem to play a relevant role. In particular, marked changes in gelatinase activity were observed in the alveolar compartment, with a prevailing extracellular activity of gelatinase A and a

predominant intracellular distribution of gelatinase B, while enzyme activity changes in lung parenchyma were less evident. It was also observed that the rapid reduction of gelatinase activity at the beginning of the repairing phase (14-28 days) was synchronous with a peak of alveolar concentration of their specific inhibitors TIMP-1 and TIMP-2. Immunohistochemical analyses documented ex novo expression of MMPs and TIMPs after bleomycin administration both in lung structural and inflammatory cells.

Several experimental studies, developed in bleomycin-induced lung fibrosis model, showed a variable increase of gelatinase B both at the mRNA and protein level (Yaguchi *et al.,* 1998; Betsuyaku *et al.,* 2000; Pardo *et al.,* 2003) in whole lung homogenates and BAL fluid. In our experiments, in which the massive alveolar lavage probably removed a large part of endoalveolar inflammatory cells, MMP-9 activity was constant in lung tissue, while we observed a synchronous increase of activity in BAL, reaching the maximal level 7 days after treatment. This corresponds to the peak of recruitment of polymorphonuclear granulocytes in alveolar spaces as shown in BAL cell count. The amount of extracellular activity of MMP-9 was negligible with respect to intracellular compartment, suggesting that only a small amount of MMP-9 is delivered in the alveolar fluid by activated alveolar inflammatory cells, in accordance with previous studies in animal models and also in human pulmonary fibrosis (Bakowska and Adamson, 1998; Lemjabbar *et al.,* 1999).

In our study, gelatinase-A activity increased after treatment and peaked at day 7 in both alveolar compartment (cells and extracellular fluid) and, to a lesser but significant extent, in lung tissue as already demonstrated in other studies (Yaguchi *et al.,* 1998; Kunugi *et al.,* 2001; Bakowska and Adamson, 1998). Both interstitial and alveolar macrophages and epithelial cells could be responsible for enzyme production and delivery in interstitium and alveolar fluid, since they were strongly immunoreactive for MMP-2. The absence or weak activity of MMP-2 in BAL cells may suggest that the enzyme is mainly delivered by lung structural cells. In this contest, MMP-2 has been indicated as critical not only for gelatinolytic basement membrane damage, but also for alveolar epithelial cell migration during the repair process after lung injury (Kunugi *et al.,* 2001).

In the alveolar fluid TIMP-1 concentration decreased, with respect to controls, during the first two weeks after treatment, followed by a peak at 28 days, synchronous with the presence of large number of activated macrophages in the alveolar spaces which can be considered responsible for the increased TIMP-1 concentration in the last two weeks. The high baseline level of TIMP-1 in lung alveolar fluid could explain the virtual absence of MMP-9 activity during the first days after treatment, while the MMP-9 peak at 7 days is synchronous with the disappearance of TIMP-1 in BAL extracellular fluid, possibly due to progressive depletion of cellular reserves of the inhibitor. As it occurred with MMP-9/TIMP-1, the increase of TIMP-2 concentration in BAL fluid at 14 days corresponded to the infiltration of macrophages in the alveolar spaces and to the observed reduction of MMP-2 in the same compartment. This observation further supports the hypothesis of a prevalence of nondegradative enzymes in the fibrotic phase of bleomycin lung injury (Selman 2000). TIMP-2 concentration in lung tissue was relevant only at 14 days, in contrast with the marked expression observed with immunohistochemistry, both in cells and interstitial space at any time after treatment raising the hypothesis of antibody cross-reaction with other inhibitors.

Immunohistochemistry showed MMP-1 expression in alveolar and interstitial macrophages, but not in lung structural cells; this is not in contrast with previous studies of MMP-1 gene expression (Balbin *et al.,* 2001). The enhancement at day 28 in treated rats of lung tissue MMP-1 supports a role of the late activation of macrophage MMP-1 in the regression of bleomycin-induced fibrosis in the present model.

The role of MMP-7 was recently hypothesized in human and experimental lung fibrosis (Zuo *et al.,* 2002; Cosgrove *et al.,* 2002). Matrilysin has been shown to play a role in epithelial wound repair (Dunsmore *et al.,* 1998) as confirmed by our immunohistochemical data showing highest expression of MMP-7 in interstitial macrophages and in alveolar and bronchiolar epithelia in the repairing phase (14-28 days). However, at biochemical assays, the highest activity in lung tissue was observed in controls, while after treatment it was variably reduced. The highest activity observed in normal lung tissue suggests a role of MMP-7 in tissue homeostasis rather than in fibrogenesis. In sum-

mary, our data showed that in rat lung after bleomycin injury a phase of enhanced activity of lytic enzymes, with distinct extracellular or intracellular localization, is followed by marked inhibitor activation synchronous with the development of fibrosis. Although it is clearly recognized that extracellular matrix remodeling in UIP differs from bleomycin model, some similarities appear between our results and gelatinase and TIMP activities observed in UIP patients (Ramos 2001). Although analyses in man are limited by sampling issues, more biochemical data on lung tissue, and on cellular and extracellular alveolar compartments are needed in order to better understand the progression of extracellular matrix remodeling during the development of idiopathic pulmonary fibrosis and to prospect possible therapeutic approaches with specific inhibitors.

## References

Aso Y, Yoneda K, Kikkawa Y. Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice. Lab Invest 1976; 35:558-68.

Bakowska J, Adamson IYR. Collagenase and gelatinase activities in bronchoalveolar lavage fluids during bleomycin-induced lung injury. J Pathol 1998; 185:319-23.

Balbin M, Fueyo A, Lopez JM, Alvarez J, Sanchez LM, Quesada V, Bordallo J, Murphy G, Lopez-Otin C. Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. J Biol Chem 2001; 276:10253-62.

Betsuyaku T, Fukuda Y, Parks WC, Shipley JM, Senior RM. Gelatinase B is required for alveolar bronchiolization after intratracheal bleomycin. Am J Pathol 2000; 157:525-35.

Borzone G, Moreno R, Urrea R, Meneses M, Oyarzun M, Lisboa C. Bleomycin-induced chronic lung damage does not resemble human idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2001; 163:1648-53.

Corbel M, Belleguic C, Boichot E, Lagente V. Involvement of gelatinases (MMP-2 and MMP-9) in the development of airway inflammation and pulmonary fibrosis. Cell Biol Toxicol 2002; 18:51-61.

Cosgrove GP, Schwarz MI, Geraci MW, Brown KK, Worthen GS. Overexpression of matrix metalloproteinase-7 in pulmonary fibrosis. Chest 2002; Suppl 121:25S-26S.

Dunsmore SE, Saarialho-Kere UK, Roby JD, Wilson CL, Matrisian LM, Welgus HG, Parks WC. Matrilysin function and expression in airway epithelium. J Clin Invest 1998; 102:1321-31.

Gabazza EC, Taguchi O, Adachi Y. Bleomycin-induced lung fibrosis: the authors should have used another method to induce pulmonary lesions resembling human idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2002; 165:845-46.

Hayashi T, Stetler-Stevenson WG, Fleming MV, Fishback N, Koss MN, Liotta LA, et al. Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lung of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. Am J Pathol 1996; 149:1241-56.

Kunugi S, Fukuda Y, Ishizaki M, Yamanaka N. Role of MMP-2 in alveolar epithelial cell repair after bleomycin administration in rabbits. Lab Invest 2001; 81:1309-18.

Lanchou J, Corbel M, Tanguy M, Germain N, Boichot E, Theret N, et al. Imbalance between matrix metalloproteinases (MMP-9 and MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in acute respiratory distress syndrome patients. Crit Care Med 2003; 31:536-42.

Lemjabbar H, Grosset P, Lechapt-Zalcman E, Franco-Montoya ML, Wallaert B, Harf A, Lafuma C. Overexpression of alveolar macrophage gelatinase B (MMP-9) in patients with idiopathic pulmonary fibrosis. Effects of steroid and immunosuppressive treatment. Am J Respir Cell Mol Biol 1999; 20:903-13.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem 1951; 193:265-75.

Madtes CD, Elston AL, Hackman RC, Dunn AR, Clark JG. Trasforming growth factor-alpha deficiency reduces pulmonary fibrosis in transgenic mice. Am J Respir Cell Mol Biol 1999; 20:924-34.

Matsumoto S, Katoh M, Watanabe T, Masuho Y. Molecular cloning of rabbit matrix metalloproteinase-2 and its broad expression at several tissue. Biochim Biophys Acta 1996; 1307:137-39.

Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem 1997; 378:151-60.

Pardo A, Ruiz V, Arreola JL, Ramirez R, Cisneros-Lira J, Gaxiola M, et al. Bleomycin-induced pulmonary fibrosis is attenuated in Á-glutamyl transpeptidase-deficient mice. Am J Respir Crit Care Med 2003; 167:925-32.

Ramos C, Montano M, Garcia-Alvarez J, Riuz V, Uhal BD, Selman M, et al. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol 2001; 24:591-98.

Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R, Barrios R and et al. TIMP-1, -2, -3, and –4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? Am J Physiol Lung Cell Mol Physiol 2000; 279:L562-74.

Yaguchi T, Fukuda Y, Ishizaki M, Yamanaka N. Immunohistochemical and gelatin zymography studies for matrix metalloproteinases in bleomycin-induced pulmonary fibrosis. Pathol Int 1998; 48:954-63.

Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, et al. Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci USA 2002; 99:6292-7.

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