TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment?

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IDIOPATHIC PULMONARY FIBROSIS (IPF) is a progressive and usually lethal interstitial lung disease of unknown etiology characterized by varying degrees of inflammation and fibrosis in the lung parenchyma (14).

The fibrotic response is generally considered an irreversible process and is characterized by a striking increase in fibroblast population and a profound and complex change in extracellular matrix (ECM) turnover. That brings about a progressive accumulation of connective tissue proteins (5) and, in addition, basement membrane disruption that is observed mainly in the early stages of the disease (25). In this context, an imbalance between the synthesis and degradation of ECM molecules in the local lung microenvironment appears to be of central importance in the pathogenesis of the fibrotic component of IPF.

Matrix metalloproteinases (MMPs), the mediators of matrix degradation, are a family of zinc endoproteinases that share structural domains and are collectively capable of degrading essentially all ECM components (2). At present, the human MMP gene family contains 17 members that can be divided by structure and substrate specificity into several subgroups including collagenases, gelatinases, stromelysins, and membrane-type (MT) MMPs; other MMPs do not appear to fall into any of these subgroups (36, 37). The regulation of these enzymes reveals similarities and differences. They differ in cellular sources and inducibility, but they share the property of being synthesized as inactive zymogens in which activation can occur through intracellular, extracellular, or cell surface-mediated proteolytic mechanisms (2, 26, 37).

A pivotal extracellular control of MMP catalytic activity is accomplished by members of a specific family of inhibitors named tissue inhibitors of metalloproteinases (TIMPs). There are currently four members of the TIMP family (TIMP-1 to -4) that, besides their common MMP inhibitory action, differ in expression patterns and other properties such as association with latent MMPs, cell growth-promoting activity, cell survival-promoting activity, and apoptosis (1, 7, 9).

The possible role of both MMPs and TIMPs in IPF is at present unclear. It has been considered that TIMPs are good candidates for tissue fibrosis, but studies in lung fibrosis are scanty. There are two recent studies (6, 11) exploring the localization of TIMP-2, and in one of them (11), TIMP-1 was also evaluated. However, the expression and localization of TIMP-3 and TIMP-4 have not been examined in any fibrotic lung disorder.

The aim of this study was to determine the localization of TIMP-1, TIMP-2, TIMP-3, and TIMP-4 in lung tissue obtained from patients with IPF. Additionally,
we colocalized TIMP-2 and TIMP-3, which have been related with cell growth-promoting activity and apoptosis, respectively (1, 10), with the nuclear proteins Ki67 (associated with proliferation) and p27 (inhibitor of the cell cycle). To have a more integrated picture of matrix remodeling, we also examined a variety of MMPs including the collagenase subfamily (MMP-1, MMP-8, and MMP-13), the gelatinase subfamily (MMP-2 and MMP-9), and MT1-MMP.

MATERIALS AND METHODS

Study population. Twelve nonsmoking patients with IPF were included in this study (8 men and 4 women; mean age 58.1 ± 13.2 yr). The protocol was approved by the Ethical Committee of the Instituto Nacional de Enfermedades Respiratorias (Mexico DF, Mexico). Diagnosis of IPF was supported by clinical, radiologic, computed tomography scan, and functional findings of interstitial lung disease and was corroborated by open lung biopsy (22, 27). The morphologic diagnosis of IPF was based on typical microscopic findings and included patchy, nonuniform alveolar septal fibrosis and interstitial inflammation consisting mostly of mononuclear cells but also of neutrophils and eosinophils; a variable macrophage accumulation was observed in the alveolar spaces as was the cuboidalization of the alveolar epithelium. Biopsies lacked granulomas, vasculitis, microorganisms, and inorganic material as seen by polarized light microscopy. None of the patients had been treated with steroids or other immunosuppressive drugs at the time of biopsy. As controls, histologically normal lung tissues obtained at necropsy from six nonsmoking adult individuals (4 men and 2 women; mean age 43.2 ± 11.1 yr) who died of causes unrelated to lung diseases were utilized.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was performed with a standard technique (32). Briefly, the fiber-optic bronchoscope was wedged in two separate segments of the right middle lobe or lingula, and 300 ml of normal saline were instilled in 50-ml aliquots, with an average return of 70%. The recovered BAL fluid was filtered through sterile gauze, measured, and then centrifuged at 250 g for 10 min at 4°C. The supernatants were kept frozen at −70°C until used. The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS), and an aliquot was used to evaluate the total number of cells. Other aliquots were fixed in carbowax, and three slides per sample were stained with hematoxylin and eosin, Giemsa, and toluidine blue and used for differential cell counts. Six nonsmoking healthy volunteers were lavaged as control subjects (3 women and 3 men; 33.5 ± 6 yr old).

Immunohistochemistry. Tissue sections were deparaffinized, rehydrated, and then blocked with 0.45% H2O2 in methanol for 30 min followed by normal serum (Vector Laboratories, Burlingame, CA) diluted 1:20 in PBS for 20 min. Before the immune reaction, antigen retrieval with 0.1 M citrate buffer, pH 6.0, was performed. The sources of the primary monoclonal antibodies were Fuji Chemical Industries (Toyama, Japan) for MMP-1, MMP-8, MMP-9, MMP-13, TIMP-1, TIMP-2, and TIMP-3 and Calbiochem (San Diego, CA) for MMP-2. TIMP-4 polyclonal antibody was kindly donated by S. S. Apte (Cleveland Clinic Foundation, Cleveland, OH). The monoclonal antibody for mitotic inhibitor/pressor protein p27 was from NeoMarkers (Fremont, CA), and Ki67 rabbit polyclonal antibody was from Novocastra Laboratory (Newcastle, UK). Antibodies were applied and incubated at 4°C overnight. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) was used according to the manufacturer’s instructions. 3-Amino-9-ethylcarbazole (BioGenex) in acetate buffer containing 0.05% H2O2 was used as the substrate. The sections were counterstained with hematoxylin (19). The primary antibody was replaced by nonimmune serum for negative control slides.

To identify epithelial cells, myofibroblasts, and macrophages, parallel sections were immunostained for cytokeratin 7, α-smooth muscle actin, and HAM56, respectively (DAKO, Carpinteria, CA).

In the case of double-label immunostaining, either Ki67 or p27 antibody was used first followed by a secondary biotinylated antibody and an avidin-biotin-peroxidase complex (Vectastain Elite ABC peroxidase kit, Vector Laboratories). Color was revealed with 3’, 3’-diaminobenzidine in PBS containing 0.05% H2O2. Afterward, either TIMP-2 or TIMP-3 antibody was used, followed by a secondary biotinylated antibody with avidin-biotin and alkaline phosphatase complexes (Vectastain ABC alkaline phosphatase kit, Vector Laboratories). The color reaction was accomplished by incubation with Fast Red chromogen (Biomed, Foster City, CA).

Evaluation of immunohistochemical results. The intensity of the staining in different lung tissue components was evaluated in the IPF patients as described previously (11). A scale from 0 to 3 was used as follows: 0, negative; 1, mild staining; 2, moderate staining; and 3, strong staining.

In situ hybridization. Riboprobes for in situ hybridization were generated from human cDNA TIMP-4 cloned in a pGem-T vector provided by S. S. Apte (Cleveland Clinic Foundation). The plasmid was linearized before translation with Kpn I. An antisense 487-bp fragment was transcribed with T7 and a sense 508-bp fragment with SP6 RNA polymerase, respectively. The transcription of sense and anti-sense transcripts was performed with a labeling mixture containing digoxigenin-UTP (Boehringer Mannheim, Mannheim, Germany).

In situ hybridization was performed on 4-μm sections as previously described (19). Briefly, the sections mounted on silanized slides were incubated in 0.001% proteinase K (Sigma, St. Louis, MO) for 20 min at 37°C. After acetylation with acetic anhydride, the sections were prehybridized for 1 h at 45°C in a hybridization buffer. The sections were incubated with the digoxigenin-labeled probes at 45°C overnight. Some sections were hybridized with a digoxigenin-labeled sense RNA probe. The tissues were incubated with a polyclonal sheep anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for 1 h at room temperature. The color reaction was performed by incubation with Fast Red chromogen (Biomed). The sections were lightly counterstained with hematoxylin.

Fibroblast isolation and culture. Human fibroblast-like cells were obtained from normal and IPF lungs as previously described (20). Cells were isolated by trypsin dispersion and grown in Ham’s F-12 medium (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum. The cells were cultured at 37°C in 5% CO2-95% air in 25-cm2 Falcon flasks with F-12K medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. When the fibroblasts reached early confluence, the medium was replaced with serum-free F-12K medium containing phorbol 12-myristate 13-acetate (PMA). Fibroblasts were collected for RNA extraction.

Flow cytometry. In parallel experiments, fibroblasts were fixed with ice-cold 70% ethanol and stored at −20°C until assayed. The cells were washed and incubated for 1 h at 37°C
with FITC-conjugated monoclonal anti-human α-smooth muscle actin antibody diluted 1:400 in 1% bovine serum albumin in PBS, pH 7.3. The fibroblasts were washed and resuspended in PBS for fluorescence-activated cell sorter analysis as described elsewhere (35).

**Zymography.** SDS-polyacrylamide gels containing gelatin (1 mg/ml) were used to identify proteins with gelatinolytic activity from BAL supernatants (21). After electrophoresis, the gels were washed in a solution of 2.5% Triton X-100 (15 × 2 min) to remove SDS, washed extensively with water, and incubated overnight at 37°C in 100 mM glycine, pH 7.6, containing 10 mM CaCl₂ and 50 mM ZnCl₂. Identical gels were incubated in the presence of 20 mM EDTA as controls. The gels were stained with Coomassie

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**Fig. 1.** Tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 immunolocalization in idiopathic pulmonary fibrosis (IPF) and normal lungs. **A** and **B**: TIMP-1-immunoreactive interstitial cells in IPF lungs. Original magnification: ×10 in A; ×40 in B. **B**, inset: positive interstitial macrophages. Original magnification, ×100. **C** and **D**: IPF lungs exhibiting immunolabeled subepithelial myofibroblasts stained for TIMP-2 (arrow). Original magnification, ×10. **D**, inset: positive fibroblasts. Original magnification, ×100. **E** and **F**: normal lungs were usually negative for TIMP-1 and TIMP-2, respectively. Original magnification, ×40. **G**: negative control omitting the primary antibody. Original magnification, ×40.
brilliant blue R250 and destained in a solution of 7.5% acetic acid and 5% methanol.

**RT-PCR.** Total RNA extracted from human fibroblasts as previously described (20) was used for RT-PCR to explore gelatinase B expression. Total RNA extracted from IPF and control lungs was used to analyze MMP-13 and TIMP-4 expression. RNA was reverse transcribed to synthesize cDNA according to the manufacturer’s protocol (GIBCO BRL).

PCR amplification (Perkin-Elmer 9600, Branchburg, NJ) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP-9, MMP-13, and TIMP-4 was performed with a cDNA working mixture in a 25-μl reaction volume containing 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 200 μM deoxynucleotide triphosphates, 1 μM specific 5’ and 3’ primers, and 1 U of Taq DNA polymerase (Perkin-Elmer).

The set of primers used for the GAPDH PCR was 5’-CATC-CATCCCGTGACCTTAT-3’ and 5’-GCATGACTCTCACAATGCGA-3’, with an amplified product of 395 bp. Nucleotides 5’-TCATGACCTCATCTTC-3’ and 5’-GAACAGCTGCACTTAT-3’ were used as primers to amplify a 134-bp segment for collagenase-3 cDNA. For TIMP-4 amplification, the primers used were 5’-CCAGAGGTCAGGTGGTAA-3’ (antisense) and 5’-ACACCCAGACAGGTATC-3’ (sense) for a fragment size of 446 bp. Gelatinase B primers were 5’-GTGGGGCTCAAGGTGTGGAAT-3’ (antisense) and 5’-GTGCTGGCTGCTGCTTGGCTG-3’ (sense), with an amplified product of 303 bp.

To quantify the housekeeping gene GAPDH, a competitor was constructed by cutting an internal fragment of 155 bp of a GAPDH cDNA (originally 1,233 bp) cloned in a PBR 322 plasmid with Nco I. The modified GAPDH cDNA was subsequently religated. The competitor (cGAPDH) sequence was obtained by PCR amplification of the modified plasmid with the primers for GAPDH. The competitor size was 240 bp.

Fourfold serial dilutions of the standard competitor (5, 10, 15, and 20 pg) were coamplified with a constant amount of cellular cDNA (1 μl). Cycling conditions were 95°C for 10 min for 1 cycle; 95°C for 30 s, 58°C for 30 s, and 72°C for 120 s for 40 cycles; and 72°C for 7 min for the final incubation. Aliquots (5 μl) of the PCR product were resolved in 1.5% agarose gel containing ethidium bromide. Band intensities were quantitated by scanning densitometry with a Kodak digital science electrophoresis documentation and analysis system 120 (Kodak, Rochester, NY). The logarithm of the GAPDH-to-cGAPDH ratio was plotted as a function of the logarithm of

![Fig. 2. TIMP-3 immunolocalization in IPF and normal lungs. A and B: immunoreactive TIMP-3 bound mainly to elastic lamina of vessels and to extracellular matrix in IPF lungs. Original magnifications: ×2.5 in A; ×40 in B. No TIMP-3 signal was noticed in normal lungs (C). Original magnification, ×40. D: interstitial macrophages with immunoreactive TIMP-3 staining in IPF lung section. Original magnification, ×40. E: negative control omitting the primary antibody. Original magnification, ×40.](http://ajplung.physiology.org/2017/05/08.1663.png)
the known cGAPDH amount. The point of equivalence represents the concentration of GAPDH in the cDNA sample. Dilutions were performed to reach 5 pg/μl of GAPDH. For each amplification, 10 pg of GAPDH were used.

For MMP-13 and gelatinase B amplification, cycling conditions were 95°C for 10 min for 1 cycle; 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s for 35 cycles; and 72°C for 7 min for the final incubation. For TIMP-4 amplification, the conditions were 95°C for 10 min for 1 cycle; 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s for 38 cycles; and 72°C for 7 min for the final incubation. Aliquots (5 μl) of the PCR product were resolved on a 1.5% agarose gel containing ethidium bromide.

RESULTS

TIMP-1, TIMP-2, TIMP-3, and TIMP-4 localization. TIMP-1 was detected in 8 of 12 IPF lungs, usually localized in isolated interstitial cells, mainly macrophages and fibroblast-like cells (Fig. 1, A and B). This inhibitor was observed in areas of dense scar tissue.

Regarding TIMP-2, it was found in almost all IPF lungs, primarily associated with subepithelial fibroblast/myofibroblast foci (Fig. 1C), some of them partially occupying the alveolar spaces (Fig. 1D). Myofibroblasts were identified by immunoreactive α-smooth

Fig. 3. TIMP-4 mRNA and immunoprotein localization in IPF and control lungs. A and B: in situ hybridization of antisense TIMP-4 riboprobe showing positive epithelial cells (A) and interstitial macrophages and plasma cells (B, arrow) in IPF lung. Original magnification, ×40. C: sense TIMP-4 riboprobe. Original magnification, ×40. D: immunohistochemistry of TIMP-4 showing several positive plasma cells (arrows). Original magnifications: ×40; ×60 in inset. E: negative control discarding the primary antibody. Original magnification, ×40. F: normal lung. Original magnification, ×10.
TIMP-3 showed the most intense extracellular staining and was mainly found coupled to the elastic lamina of most vessels, revealing the characteristic duplication of this structure in pulmonary fibrosis (Fig. 2, A and B). This observation was corroborated in 75% of examined IPF lungs. Less intense staining was noticed in thickened alveolar septa. In contrast, in normal lungs, no positive staining was observed even in the elastic lamina of vessels (Fig. 2C). In some areas of IPF lungs, numerous interstitial cells, mainly macrophages (identified by HAM56; data not shown), were strongly stained (Fig. 2D). Additionally, in two IPF samples, some alveolar epithelial cells were also positive (see Fig. 5F). Control IPF samples incubated without the primary specific antibody were negative (Fig. 2E).

By in situ hybridization, TIMP-4 transcript was localized in some alveolar epithelial cells (Fig. 3A), although a strong positive signal was revealed in interstitial areas, mainly in macrophages and, interestingly, plasma cells. (Fig. 3B). The immunoreactive protein paralleled the mRNA observations, confirming the finding of TIMP-4 in plasma cells in almost all IPF lungs (Fig. 3D). Immunohistochemistry for TIMP-4 protein was negative in normal lungs (Fig. 3P). Tissues hybridized with the sense probe or without the primary antibody showed no reactivity (Fig. 3, C and E).

RT-PCR for TIMP-4 mRNA. Expression of TIMP-4 in two IPF lungs and two control lungs was analyzed by RT-PCR. As illustrated in Fig. 4, the expected 446-bp fragment was revealed after 40 PCR cycles only in IPF samples (lanes 3 and 4). Control lungs did not reveal a positive result, although an equivalent cDNA concentration as analyzed by GADPH amplification was used (Fig. 4, lanes 1 and 2).

Ki67 and p27 immunostaining and colocalization with TIMP-2 and TIMP-3. Ki67 and p27 were analyzed in IPF lungs. In general, nuclear p27 staining was more abundant than Ki67 staining, although there were regional variations in immunoreactivity. Most proliferating cells as revealed by the presence of the Ki67 antigen were alveolar epithelial cells and fibroblast-like cells mainly located in areas of fibroblast foci protruding to the alveolar spaces (Fig. 5A). On the other hand, the inhibitory cell cycle protein p27 was usually present in most inflammatory interstitial and intraluminal cells as well as in endothelial cells (Fig. 5B).

Colocalization of Ki67 and TIMP-2 occurred in areas of fibroblasts, mostly in the periphery of fibroblast foci (Fig. 5C), whereas in the same areas where TIMP-2 was positive, p27 was negative (Fig. 5D).

Nuclear p27 detection colocalized with TIMP-3 in some interstitial inflammatory cells, in endothelial cells where elastic lamina showed extracellular TIMP-3 accumulation, and in some epithelial cells (Fig. 5, E and F). In general terms, no colocalization of TIMP-3 and Ki67 was noticed (Fig. 5F), whereas TIMP-3 was observed in alveolar epithelial cells and in some interstitial cells showing a negative staining for Ki67.

Collagenase immunolocalization. Immunoreactive MMP-1 (collagenase-1) was noticed in reactive alveolar epithelial cells and/or in clusters of alveolar macrophages in all IPF lungs (Fig. 6A). Besides, hyperplastic type 2 pneumocytes lining honeycomb cysts in areas of fibroconnective tissue deposition showed strong staining for MMP-1 (Fig. 6B). Cytokeratin staining corroborated the epithelial nature of these cells (data not shown). Intriguingly, practically no interstitial cells producing MMP-1 were noticed.

Immunoreactive MMP-8 (collagenase-2) was localized in 75% of the IPF lungs, usually in a few neutrophils inside vessels, and in two patients, the label was also observed in the interstitium (Fig. 6C). MMP-13 (collagenase-3) was not detected either by immunohistochemistry or by RT-PCR of IPF lungs even when up to 60 cycles were assayed. In three control lungs, scattered alveolar macrophages stained for MMP-1 were noticed as well as some neutrophils displaying MMP-8 (Fig. 6, D and E). Control samples incubated with nonimmune sera were negative (Fig. 6F).

Gelatinases A and B and MT-1 MMP immunolocalization. Regarding type IV collagenases, MMP-2 (gelatinase A) was found in some foci of subepithelial myofibroblasts (Fig. 7A) located close to areas of basement membrane disruption. In 8 of 12 of the IPF lungs, MMP-2 was also observed associated with the ECM surrounding fibroblast foci (Fig. 7B). Normal lungs showed no positive staining (Fig. 7C).

MT1-MMP that has been associated with the activation of pro-MMP-2 was noticed in widely spaced interstitial cells, mainly macrophages (Fig. 7D), and in three cases in reactive alveolar epithelial cells (data not shown). MT1-MMP was not observed in normal lungs (Fig. 7E). IPF tissues incubated without the primary specific antibody were negative (Fig. 7F).
Fig. 5. Immunohistochemical localization of p27 and Ki67 and colocalization with TIMP-2 and TIMP-3. p27 and Ki67 were revealed with diaminobenzidine, and TIMP-2 and TIMP-3 were revealed with Fast Red. A: Ki67 in IPF lung section. Arrow, positive nucleus in epithelial cells; arrowhead, signaling subepithelial positive nuclei. Original magnification, ×10. B: IPF lung showing numerous p27-positive cells. Original magnification, ×10. Lung sections in A and B were counterstained with eosin. C and D: fibroblast foci in IPF lung colocalizing cytoplasmic TIMP-2 with nuclear Ki67 and TIMP-2 with p27, respectively. Original magnification, ×10. E: colocalization of TIMP-3 with p27. Original magnification, ×40. Inset: positive cytoplasmic TIMP-3 and nuclear p27 in interstitial cells. Original magnification, ×40. F: double localization of Ki67 and TIMP-3 showing that most epithelial cells were positive for TIMP-3 and negative for Ki67. Original magnification, ×40. Inset: epithelial cells colocalizing TIMP-3 with p27. Original magnification, ×100.
MMP-9 (gelatinase B) was found in almost all IPF lungs, primarily in intravascular and interstitial neutrophils (Fig. 8A). In four patients, a weaker staining was also noticed in clusters of alveolar macrophages. Interestingly, positive staining was appreciated in some subepithelial myofibroblasts and reactive alveolar epithelial cells (Fig. 8B). Similar to MMP-2, extracellular localization of MMP-9 in areas of dense scars was also seen (Fig. 8C). The majority of the control lungs showed scattered neutrophils positive for MMP-9, whereas macrophages were usually negative (Fig. 8D). Control samples incubated with nonimmune serum were negative (Fig. 8E).

A summary of the immunolocalization results of TIMPs and MMPs in IPF lungs is depicted in Table 1.

**RT-PCR for fibroblast gelatinase B mRNA.** To corroborate the expression of MMP-9 by lung fibroblasts, we examined the expression of MMP-9 in cells obtained from normal lungs (~8% myofibroblasts by α-actin staining and fluorescence-activated cell sorter counting) and in those derived from IPF lungs (40–80% myofibroblasts). Total RNA from nonstimulated and

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**Fig. 6.** Collagenase-1 and collagenase-2 immunolocalization in IPF and normal lungs. Immunoreactive collag- enases were revealed by 3,3′-diaminobenzidine. A: IPF lung showing several epithelial cells (arrows) and alveolar macrophages (arrowhead) with intracytoplasmic signal for matrix metalloproteinase (MMP)-1. Original magnification, ×40. B: IPF lung with MMP-1-positive cuboidal epithelial cells (arrows). Original magnification, ×40. C: immunoreactive MMP-8 in several intravascular neutrophils (double arrowhead). Original magnification, ×40. D: positive MMP-1 alveolar macrophage (arrowhead) in a control lung. Original magnification, ×40. E: MMP-8-stained neutrophils were sporadically found in normal lung (double arrowhead). Original magnification, ×40. F: negative control section without the primary antibody. Original magnification, ×40.
PMA-stimulated fibroblasts was reverse transcribed, and cDNA was amplified as described in MATERIALS AND METHODS. Only fibroblasts derived from IPF lungs showed a 303-bp fragment amplification that was increased approximately threefold after PMA treatment (Fig. 9, lanes 3–6). An equivalent cDNA concentration as measured by GADPH amplification with an internal competitor showed no amplification even after PMA stimulation (Fig. 9, lanes 1 and 2).

BAL. Significantly less fluid was recovered from IPF patients (55.4 ± 9.7 vs. 71.3 ± 12.1% in control subjects; P < 0.01). BAL fluid obtained from IPF patients revealed a twofold increase in total cell number and exhibited a significant increase in the percentage of neutrophils and eosinophils compared with those in the control samples (Table 2).

**SDS-PAGE zymography.** To identify BAL fluid gelatinolytic activities, aliquots adjusted to 20 μl of lavage fluid were analyzed by gelatin substrate gel zymography. A representative gelatin zymogram comparing IPF BAL fluid with that from normal subjects is shown in Fig. 10. BAL normal samples showed faint bands of 72- and 92-kDa activities corresponding to pro-MMP-2 and pro-MMP-9, respectively (Fig. 10, lanes N). IPF patients revealed a marked increase of BAL fluid progelatinase B activity (Fig. 10, lanes 1–4), and in most samples, the activated form of 86 kDa was also evident. In addi-

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**Fig. 7.** MMP-2 and membrane type (MT) 1 MMP localization in IPF and normal lungs. Immunoreactive proteins were revealed by 3,3′-diaminobenzidine, and the sections were lightly counterstained with hematoxylin. A: cluster of fibroblasts showing immunoreactive MMP-2 in an IPF lung (arrow). Original magnification, ×10. B: higher magnification of the same field. Original magnification, ×100. C: normal lung showing no positive staining for MMP-2. Original magnification, ×10. D: immunohistochemical localization of MT1-MMP in several interstitial cells in an IPF lung (arrowhead). Original magnification, ×100. E: normal lungs showed no MT1-MMP signal. Original magnification, ×10. F: IPF sample with primary antibody omitted. Original magnification, ×40.
tion, gelatinolytic bands of higher molecular mass, likely representing lipocalin-associated progelatina-
se B specific in neutrophils, were also noticed. Densitometric analysis showed a 2.5- to 7-fold in-
crease in progelatinase B activity compared with that in healthy individuals (Fig. 10). Likewise, IPF
BAL fluids displayed a 5- to 15-fold increase in progelatinase A activity compared with that in con-
trol samples. In the majority of IPF fluids, the 68-
kDa active form of pro-MMP-2 was also observed. All

Table 1. MMP and TIMP immunoreactivity in IPF lungs

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Values are staining intensity graded as 0, no staining; 1, weak or mild staining; 2, moderate staining; and 3, strong staining. MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 MMP; TIMP, tissue inhibitor of metalloproteinase.
gelatinolytic bands were fully inhibited by EDTA (data not shown).

**DISCUSSION**

A dynamic balance between synthesis and degradation of ECM components is required for lung structure and functional maintenance. The loss of regulated turnover may result in a number of different lung diseases including lung emphysema and pulmonary fibrosis.

IPF is characterized by a chronic inflammatory process, with the presence of widely distributed small aggregates of actively proliferating fibroblasts and myofibroblasts, which, in turn, are responsible for the excessive ECM deposition, mainly fibrillar collagens, and alveolar structural remodeling (14). The disease is of a patchy nature, and the progression of the fibrotic lesion until a mature scar at any given time and place reflects an imbalance in ECM synthesis and degradation.

The MMPs, also called matrixins, play a central role in all processes involving ECM remodeling, and an inappropriate regulation of their activities may have pathological implications. Regulation is controlled at several levels including gene transcription and activation of a latent enzyme. Locally, in the extracellular space, MMPs are tightly regulated by TIMPs.

In IPF, the remodeling processes may involve various MMPs and their specific inhibitors, but little is known about their possible participation during lung fibrogenesis.

Our results showed that there are important differences in both localization and abundance of collagenases, gelatinases, and TIMPs in fibrotic tissues. Thus the collagenases MMP-1 and MMP-8, which are responsible for the degradation of interstitial fibrillar collagens, showed a more localized expression compared with TIMPs, and MMP-13, another collagenase, was not found. MMP-1 was usually noticed in alveolar epithelial cells and alveolar macrophages, whereas in the thickened alveolar septa where increased bundles of collagen are accumulating, no positive signal was noticed.

Considering that the main function of collagenases is the removal of fibrillar collagens, the absence in the expression of this enzyme in the interstitium, at least as detected by the methods used in this work, might explain, in part, the presence of scars that do not undergo resorption. Additionally, it has been proposed that MMP-1 expression is induced in primary keratinocytes by contact with native type I collagen and that its degradation initiates keratinocyte migration during reepithelialization (24). In this context, it can be speculated that degradation of basement membrane by upregulated gelatinases in IPF may induce upregulation of collagenase-1 in alveolar epithelial cells, facilitating type 2 cell migration and proliferation (15, 16).

In contrast, TIMP expression was more abundant and usually noticed associated with the ECM or in interstitium-located cells. Particularly TIMP-3, which is studied here for the first time in IPF, was copiously observed throughout the lung parenchyma. This inhibitor was found exquisitely decorating the elastic lamina in vessels and within the thickened alveolar septa. Actually, a striking feature of TIMP-3 is that it is ECM associated, whereas TIMP-1 and TIMP-2 are freely diffusible extracellular proteins (7). TIMP-3 is not only important in matrix turnover but also has been shown to induce apoptosis by the stabilization of tumor necrosis factor-α receptors by an effect dependent on TIMP-3 inhibitory function (31). Moreover, TIMP-3 overexpression in vascular smooth muscle cells promoted death by apoptosis by an effect independent of MMP inhibition (1). Interestingly, it has been recently demonstrated in mesangial cells that p27, a protein inhibitor of cell proliferation, may also be involved in apoptosis (13). Thus it seems that under certain conditions, a p27-mediated increase in cyclin-dependent kinase-2 activity leads to apoptosis. In our study, TIMP-3 colocalized with p27 in alveolar epithelial cells and interstitial inflammatory cells. Apoptosis of type 2 pneumocytes has been demonstrated in vivo in IPF lungs, and it has been suggested that chronic epithelial cell death may avoid normal reepithelialization, enhancing the fibrotic response (33). Several mechanisms may be in-

![Fig. 9. Analysis of fibroblast gelatinase B expression by RT-PCR. Total RNA extracted from human lung fibroblasts was reverse transcribed, and cDNA was amplified with use of the primers in MATERIALS AND METHODS. Lanes 1 and 2, normal fibroblasts (8% myofibroblasts) without and with, respectively, phorbol 12-myristate 13-acetate (PMA); lanes 3 and 4, IPF fibroblasts (78% myofibroblasts) without and with, respectively, PMA; lanes 5 and 6, IPF fibroblasts (57% myofibroblasts) without and with, respectively, PMA; lane 7, U-2 OS cells treated with PMA as a positive control.](image-url)
fibroblasts obtained from IPF lungs. The fibroblasts in these lungs expressed MMP-9 in vitro, as we also showed by RT-PCR in cells obtained from human normal lungs, the findings that fibroblasts obtained from IPF lungs expressed the transcript and that expression of gelatinase B was closely related to the percentage of myofibroblasts were of particular importance. Additionally, BAL fluid gelatin zymography showed increased gelatinase activities attributable to MMP-2 and MMP-9 in IPF patients.

TIMP-4 was also explored for first time in IPF lungs. This inhibitor can effectively inhibit human MMPs, and it has been suggested that as TIMP-2, it is more specific for MMP-2. Moreover, it binds to the COOH-terminal domain of MMP-2 in a manner similar to TIMP-2 (12). In normal tissues, TIMP-4 mRNA is confined primarily to the adult heart, and no transcript has been detected in normal lungs [8]. In the present study, we demonstrated by RT-PCR, in situ hybridization, and immunohistochemistry that TIMP-4 is highly expressed in IPF lungs, primarily by interstitial macrophages, clusters of plasma cells, and alveolar epithelial cells. Although its role in IPF is largely unknown, the widespread expression found in this study strongly suggests that it may contribute to a profibrotic microenvironment in the IPF lungs.

MT1-MMP has been mainly implicated in the activation of gelatinase A through a trimolecular complex comprising MT1-MMP, MMP-2, and TIMP-2 (3). Intriguingly, by immunohistochemistry, we found that although gelatinase A and TIMP-2 were expressed by the same type of cells, primarily myofibroblasts, MT1-MMP was mainly observed in interstitial cells. Studies are ongoing in our laboratory to analyze the distribution of other MT-MMPs in this disease.

In summary, our findings indicate that during lung fibrogenesis, 1) there is a wider distribution of TIMPs compared with the collagenases MMP-1 and MMP-8 in the lung parenchyma. These findings, together with previous results from our laboratory (18, 23, 28–30) in which we have consistently found a decrease in collagenolytic activity associated with the development of fibrosis, suggest that in IPF a nondegrading fibrillar collagen microenvironment is prevailing; and 2) excessive MMP-2 and MMP-9 production might play a role in basement membrane disruption, enhancing fibroblast invasion to the alveolar spaces.

As previously reported (6, 11), subepithelial myofibroblasts in IPF lungs exhibited immunoreactive MMP-9. Considering that lung fibroblasts do not express MMP-9 in vitro, as we also showed by RT-PCR in cells obtained from human normal lungs, the findings that fibroblasts obtained from IPF lungs expressed the transcript and that expression of gelatinase B was closely related to the percentage of myofibroblasts were of particular importance. Additionally, BAL fluid gelatin zymography showed increased gelatinase activities attributable to MMP-2 and MMP-9 in IPF patients.

Fig. 10. Identification of gelatinolytic enzymes in bronchoalveolar lavage (BAL) fluid by SDS-PAGE gelatin zymography. BAL samples were mixed with an equal volume of Laemmli sample buffer containing 3% SDS. Right: lanes 1–4, IPF samples; lanes N, control samples. A, gelatinase (GEL) A; B, gelatinase B. Left: quantitative image analysis of the surface and intensity of the gelatinolytic bands. F1–F4, IPF samples; N1 and N2, control samples. Results are expressed as activity in arbitrary units (see MATERIALS AND METHODS).

Volved in alveolar epithelial cell apoptosis, including the Fas-Fas ligand pathway [17] and fibroblast secretion of apoptotic factors [34], and the results of the present study suggest the possible participation of TIMP-3.

TIMP-2 was found almost exclusively associated with fibroblast foci. A fibroblast focus is characterized by a distinct cluster of fibroblasts and/or myofibroblasts within the alveolar wall. The fibroblast foci are distributed throughout the lung parenchyma, representing a characteristic morphological feature in IPF and indicating that fibrosis is actively ongoing (14). These foci may occur in the interstitium as well as in the alveolar spaces (14–16). In the latter, fibroblasts migrate through gaps in the alveolar epithelial basement membranes and proliferate within the alveolar spaces, producing intraluminal fibrosis. This process might be at least partially associated with the secretion of the gelatinases MMP-2 and MMP-9. In this context, both MMP-2 and MMP-9 were observed in subepithelial myofibroblasts and occasionally in areas of denuded alveolar basement membranes, suggesting that these MMPs may play a role in the migration of these cells to the alveolar spaces. As mentioned, subepithelial myofibroblasts were also positive for TIMP-2. Although MMP inhibition is the main function of TIMPs, paradoxically, TIMP-2 might be influencing an activation of progelatinase A through a trimolecular complex comprising MT1-MMP, MMP-2, and TIMP-2 (3).

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