American Journal of Pathology, Vol. 149, No. 4, October 1996

Immunohistochemical Study of Metalloproteinases and Their Tissue Inhibitors in the Lungs of Patients with Diffuse Alveolar Damage and Idiopathic Pulmonary Fibrosis

Tomayoshi Hayashi,*† William G. Stetler-Stevenson,‡ Marian V. Fleming,† Nancy Fishback,† Michael N. Koss,† Lance A. Liotta,‡ Victor J. Ferrans,* and William D. Travis†

From the Pathology Section, National Heart, Lung, and Blood Institute, and the Laboratory of Pathology,‡ National Cancer Institute, National Institutes of Health, Bethesda, Maryland, and the Department of Pulmonary and Mediastinal Pathology,† Armed Forces Institute of Pathology, Washington, DC*

Immunohistochemical and confocal microscopic studies of the localization of matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs), and type IV collagen were made in lung tissues from patients with normal pulmonary histology (n = 3), diffuse alveolar damage (n = 14), and idiopathic pulmonary fibrosis (n = 12). Pretreatment with pepsin revealed otherwise undetectable MMP- and TIMP-immunoreactive sites. In normal lung, MMP-2, MMP-9, TIMP-1, and TIMP-2 were localized in ciliated cells, endothelial cells, pneumocytes, macrophages, and smooth muscle cells; fibroblasts showed a strong reaction only for MMP-2. Only TIMP-2 showed co-localization with type IV collagen. Myofibroblasts and epithelial cells expressed increased reactivity for MMPs and TIMPs in both disorders. The reactivities for MMPs and TIMPs were stronger in diffuse alveolar damage. MMP-2 showed focal co-localization in capillary endothelial and disrupted epithelial basement membranes, suggesting activation of collagenolysis. A protective effect against this lysis was suggested by the extensive co-localization of TIMP-2 with type IV collagen and fibrillar collagens. Alveolar buds showed increased reactivity for MMPs and TIMPs in their lining epithelial cells, myofibroblasts, and their basement membranes; however, their matrices were mostly unreactive. These findings emphasize the complexity of the roles of MMPs and TIMPs in collagen turnover in diffuse alveolar damage and idiopathic pulmonary fibrosis. (Am J Pathol 1996, 149:1241–1256)

The development of fibrotic changes can be conceptualized as resulting from an imbalance in the equilibrium of the normal processes of synthesis and degradation of extracellular matrix (ECM) components. Morphological and biochemical studies have demonstrated that acute tissue injury initiates a series of changes, including infiltration by acute inflammatory cells and release of various cytokines and other mediators, that induce proliferation of fibroblasts and myofibroblasts, followed by the synthesis of increased amounts of connective tissue components by these cells. The extent to which alterations in matrix proteolysis play a role in the development and regression of fibrosis is poorly understood. The lysis of the various types of collagens is regulated by the balance between the activities of the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs).

The family of MMPs has at least 10 soluble, secreted members, which have several characteristics in common including 1) requirement of Zn atoms for protease activity, 2) secretion in proenzyme form, 3) activation by proteolytic removal of the amino terminus, and 4) inhibition by specific TIMPs.[1,2] MMP-2 and MMP-9 (also referred to as gelatinase A and gelatinase B) rapidly degrade denatured collagens (gelatin) as well as a number of native collagen types

Accepted for publication June 11, 1996.

Address reprint requests to Dr. Victor J. Ferrans, Chief, Pathology Section, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 2N240, 10 Center Drive, MSC-1518, Bethesda, MD 20892-1518.

that contain regions in which the helical structure of the protein is disrupted.[1,2] High levels of the 72-kd MMP-2 are associated with lysis of basement membranes and metastatic invasion of tumor cells. MMP-2 and MMP-9 are distinct from other members of the MMP family in that they possess a unique region that is immediately adjacent to the putative metal-binding domain, is homologous to the gelatin-binding domain of fibronectin, and may function in substrate binding. MMP-2 and MMP-9 also differ from other members of the family by their ability to interact, as latent proenzymes, with their endogenous inhibitors, the TIMPs.[3–5] These proenzyme-inhibitor complexes are specific in that pro-MMP-2 binds TIMP-2 and pro-MMP-9 binds TIMP-1.[1,2] TIMP-1 is a 28.5-kd glycoprotein that forms a 1:1 complex with all activated MMPs as well as with the proenzyme form of MMP-9.[6–8] TIMP-2 is a 21-kd nonglycosylated protein that shares 37% amino acid identity and 65.6% overall homology with TIMP-1; however, these two proteins are immunologically distinct.[9]

Accumulating evidence indicates the importance of the MMPs and TIMPs in lung development[10–12] as well as in a variety of pulmonary disorders, including idiopathic pulmonary fibrosis (IPF),[13,14] emphysema,[15] bronchiectasis,[16] acute lung injury,[17] pleural effusions,[18] and lung carcinomas.[19–21] However, only a few studies have reported the immunohistochemical localization of MMPs and TIMPs in the lung. Bronchial epithelial cells and smooth muscle cells in normal lung were reported to show a faint positive reaction for type IV collagenase.[22] Fibroblasts and endothelial cells in the stroma of lung carcinomas showed a strong reaction for MMP-2.[23] Biochemical studies have demonstrated that various collagenases are produced by lung fibroblasts,[13] macrophages,[24] and neutrophils.[25] TIMPs also are known to be present in lung fibroblasts[13] and alveolar macrophages[26] as well as in various cell types in fetal lung.[27] Messenger RNAs for MMPs and TIMPs also have been identified in a variety of cell types in the lungs.[9,12,19,23,28]

Immunohistochemical techniques for the detection of MMPs and TIMPs have not been applied systematically to the study of non-neoplastic pulmonary diseases. In the present study, we have used conventional immunohistochemical methods as well as multiple antibody labeling techniques in conjunction with laser scanning confocal fluorescence microscopy to investigate changes in the activity and distribution of MMPs and TIMPs in open lung biopsy specimens from patients with diffuse alveolar damage (DAD) and IPF. We have paid special attention to the co-localization of type IV collagen with the MMPs and TIMPs, because this type of collagen is a preferred substrate of both MMP-2 and MMP-9 and is of critical importance in maintaining the structural integrity of the alveolar wall. Furthermore, disruption of the epithelial basement membranes is known to be associated with the migration of myofibroblasts into the alveolar spaces, the formation of alveolar buds, and the structural remodeling that characterizes the fibrotic lung disorders.[29]

Materials and Methods

Patients

The study materials consisted of 26 open-lung biopsy specimens from 14 patients with DAD (10 men and 4 women; age range, 18 to 73 years; mean age, 51.1 ± 16.4 years) and 12 patients with IPF (5 men and 7 women; age range, 30 to 71 years; mean age, 51.8 ± 13.5 years). For control studies, lung tissues were obtained at necropsy from 3 adult subjects who died of causes unrelated to pulmonary disease, were nonsmokers, and showed normal pulmonary histology. The diagnosis in each of these patients was established on the basis of standard clinical criteria and of histopathological studies of the biopsy specimens.[30] The patients with IPF had the clinicopathological features of usual interstitial pneumonia.

Preparation of Tissues

For histological study, the tissues were fixed with neutral buffered 10% formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin (H&E), Masson trichrome, and Movat pentachrome methods.

Immunohistochemical Studies

Immunohistochemical studies were made using primary antibodies against MMP-2, MMP-9, TIMP-1, and TIMP-2. Immunohistochemical staining procedures were also used for the detection of type IV collagen to compare the patterns of distribution of this component with those of the MMPs and TIMPs. Both peroxidase- and fluorochrome-conjugated secondary antibodies were used in conjunction with these primary antibodies.

Antibodies

The primary antibodies against MMP-2 (Ab-45), MMP-9 (Ab-110), TIMP-1, and TIMP-2 were rabbit

polyclonal antibodies. Details of the preparation, characteristics, and specificity of these antibodies have been presented previously.[9,31,32] The anti-peptide antibodies against MMP-2 and MMP-9 react with both the activated and latent forms of these enzymes.[31,32] The antibody against type IV collagen (M785, Dako Corp., Emeryville, CA) was a mouse monoclonal antibody.

Peroxidase Staining Procedures

For the demonstration of MMP-2, MMP-9, TIMP-1, and TIMP-2, sections were stained with and without pretreatment with pepsin as described below. For the demonstration of type IV collagen, all sections were treated with pepsin. The sections were deparaffinized, hydrated, and treated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature (RT) to eliminate endogenous peroxidase activity. After washing, some of the sections were treated with 0.4% pepsin (P-7125, Sigma Chemical Co., St. Louis, MO) in 0.01 mol/L HCl for 30 minutes at 37°C. The sections were then washed and incubated with 1.5% normal goat or horse serum for 20 minutes at RT to suppress nonspecific binding of the antibodies. The sections were then incubated overnight at 4°C with the primary antibodies against MMP-2, MMP-9, TIMP-1, and TIMP-2 and for 30 minutes at RT with the antibody against type IV collagen. For sections not treated with pepsin, the antibodies were diluted 1:1000 for MMP-2, 1:750 for MMP-9, 1:4000 for TIMP-1, and 1:1000 for TIMP-2. For sections treated with pepsin, the antibodies were diluted 1:2000 for MMP-2, 1:1000 for MMP-9, 1:4000 for TIMP-1, 1:1000 for TIMP-2, and 1:50 for type IV collagen. The sections were then processed by the avidin-biotin-peroxidase complex method using the Vector Elite kit (Vector Laboratories, Burlingame, CA) and the Vector VIP kit to produce a violet color at the sites of reactivity. This method was preferred because in many tissue sections brownish deposits of hemosiderin interfered with the specific detection of the color of the reaction product resulting from incubation with diaminobenzidine. The sections were then counterstained with hematoxylin and mounted.

Double-Staining Procedures for Immunofluorescence

For immunofluorescent staining, sections were deparaffinized and treated with pepsin as described above, washed and incubated with the mixture of 10% normal goat serum and 10% normal horse serum for 20 minutes at RT, and then incubated overnight at 4°C with the mixture of rabbit polyclonal (MMP-2, MMP-9, TIMP-1, or TIMP-2) and mouse monoclonal antibody against type IV collagen. In the mixture, the antibodies were diluted 1:50 for type IV collagen, 1:400 for MMP-2, 1:200 for MMP-9, 1:800 for TIMP-1, and 1:200 for TIMP-2. The sections were then incubated with the mixture of the two secondary antibodies (fluorescein-isothiocyanate-conjugated horse anti-mouse IgG, diluted 1:100 (F1-2000 from Vector Laboratories), and Texas-red-conjugated goat anti-rabbit IgG, diluted 1:100 (T1-1000 from Vector Laboratories) for 1 hour at RT. After washing, the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (D-9542 from Sigma), diluted 1:10,000, and the sections were examined with a confocal microscope (Leica model TCS4D/DMIRBE) equipped with argon and argon-krypton lasers for UV (351 to 364 nm), blue (488 nm), and green (568 nm) excitation.

Immunohistochemical Control Procedures

Negative control immunohistochemical procedures included 1) omission of the primary antibody from the staining protocol and 2) substitution of the primary antibody by normal rabbit or mouse IgG in appropriate concentrations.

Evaluation of Immunohistochemical Results

The intensity of the staining observed in different tissue components was evaluated on a scale of 0 to 3 as follows: 0, negative; 1, mild; 2, moderate; and 3, strong staining.

Results

Histological Findings

In DAD, 3 of the patients showed changes that were compatible with an early stage of the disorder and consisted of hyaline membranes and fibrin masses in the alveolar spaces, edema of the alveolar septa, and infiltration by variable numbers of acute and chronic inflammatory cells. In the other 11 patients, the changes were more consistent with the organizing phase of the disorder and were characterized by formation of alveolar buds and thickening of the alveolar septa by edema, proliferation of myofibroblasts, and early fibrotic changes.

In contrast to this, the patients with IPF showed much less individual variation in their lung biopsy findings, which were characterized by patchy areas

Table 1. *Immunoreactivity of Components of Lesions of Diffuse Alveolar Damage and Idiopathic Pulmonary Fibrosis for MMPs and TIMPs*

Site	MMP-2			MMP-9			TIMP-1			TIMP-2		
	NOR	DAD	IPF	NOR	DAD	IPF	NOR	DAD	IPF	NOR	DAD	IPF
Bronchiolar epithelial cells	2	2	2	0–1	1	1	1	1	1–2	0–1	0–1	1
Type I pneumocytes	1–2	1	1	0–1	0–1	0–1	0–1	0–1	0–1	0–1	0–1	0–1
Type II pneumocytes	1	1	1	0	0–1	1	0–1	1	0–1	0–1	0–1	0–1
Bronchiolar cuboidal epithelial cells	2	2	2	1	1	2	0–1	0–1	0–1	1	0–1	0–1
Epithelial cells lining alveolar buds	–	2–3	2	–	2	2–3	–	2	2–3	–	2	2
Interstitial fibroblasts/myofibroblasts	2–3	2–3	2–3	0	0–2	0–1	0	2	0–1	0–1	2	0–1
Myofibroblasts in alveolar buds	–	2–3	2	–	2	1–2	–	2	1–2	–	2–3	2–3
Vascular smooth muscle cells	1	1	1	1	1	1	1	1	1–2	1	1	1–2
Airway smooth muscle cells	1	1	1	2	1–2	1–2	1	1	1–2	1	1	1–2
Endothelial cells	2–3	2–3	2–3	0–1	0–1	0–1	1	1	1	2	2	2
Tissue macrophages	1–2	1–2	1–2	0–1	0–1	0–1	0–1	0–1	0–1	0–1	0–1	0–1
Alveolar macrophages	1–2	1–2	1–2	0–1	0–1	1–2	0–1	1	1–2	0–1	0–1	0–1
Lymphocytes	0–1	0–1	0–1	0	0–1	0–1	0	0–1	0–1	0	0	0
Neutrophils	0	0	0	3	3	3	0	0	0	0	0	0
Interstitial matrix	0	0	0	0	0	0	1	1	1	1	0–1	1
Dense collagen	0	0	0	1–2	1	1	1	1	1	1	0–1	1
Matrix of alveolar buds	–	0	0	–	0	0	–	0	0	–	0	0–1
Hyaline membranes	–	0–1	–	–	0–1	–	–	1–2	–	–	2	–

The intensity of the immunohistochemical staining was graded as follows: 0, no reaction; 1, mild or weak staining; 2, moderate staining; 3, intense or strong staining. NOR, normal; DAD, diffuse alveolar damage; IPF, idiopathic pulmonary fibrosis; –, not applicable.

of dense fibrosis, structural remodeling, continuing formation of alveolar buds, and less pronounced proliferation of myofibroblasts than that observed in the patients with DAD.

Immunoperoxidase Studies

The results of the immunohistochemical staining methods are presented in Table 1.

Control Patients

MMP-2: In sections not treated with pepsin before immunohistochemical staining, the reaction for MMP-2 was moderate in ciliated epithelium and weak to negative in bronchiolar cuboidal cells, basal cells, and endothelial cells. Goblet cells and Clara cells were negative. Type I and type II pneumocytes showed a focal, weakly positive reaction. After pepsin treatment (Figure 1, A and B), an increase in reactivity was observed in bronchiolar cuboidal cells, ciliated cells, smooth muscle cells, endothelial cells, and pneumocytes. The latter two cell types showed a diffusely positive reaction. However, little or no change was observed in the reactivity of basal cells, goblet cells, and Clara cells. The stromal matrix was unreactive, with or without pepsin pretreatment. Without pepsin pretreatment, fibroblasts/myofibroblasts showed a negative reaction. After pepsin treatment, the staining of the cytoplasm of fibroblasts around bronchial tissue and arteries became moderately intense. Lymphocytes showed a negative to weak reaction. Neutrophils were unreactive. Tissue and alveolar macrophages showed a weak to moderate reaction. The staining of these cells was not altered by pepsin treatment.

MMP-9: Without pepsin pretreatment, the reaction for MMP-9 was negative in alveolar epithelial cells and endothelial cells, negative to weakly positive in bronchial ciliated cells, bronchiolar cuboidal cells, basal cells, and Clara cells, weak in vascular smooth muscle, and moderate in bronchiolar smooth muscle. After pepsin treatment (Figure 1C), the intensity of the reaction in bronchial ciliated cells, bronchiolar cuboidal cells, and smooth muscle cells showed a mild increase; endothelial cells showed a weak reaction and type I pneumocytes showed a weak but focal reaction, but type II pneumocytes remained unreactive. The reaction in perivascular and peribronchial collagen was negative but became weak to moderate after pepsin treatment. The connective tissue matrix of alveolar septa, the fibroblasts/myofibroblasts, and the lymphocytes were unreactive with or without pepsin treatment. Neutrophils showed a weak reaction, which was greatly increased by pepsin pretreatment. Tissue and alveolar macrophages were negative but became weakly reactive after treatment with pepsin.

TIMP-1: Without pepsin pretreatment, bronchial ciliated epithelial cells and endothelial cells were weakly reactive for TIMP-1; basal cells, Clara cells, and bronchiolar cuboidal cells were negative;

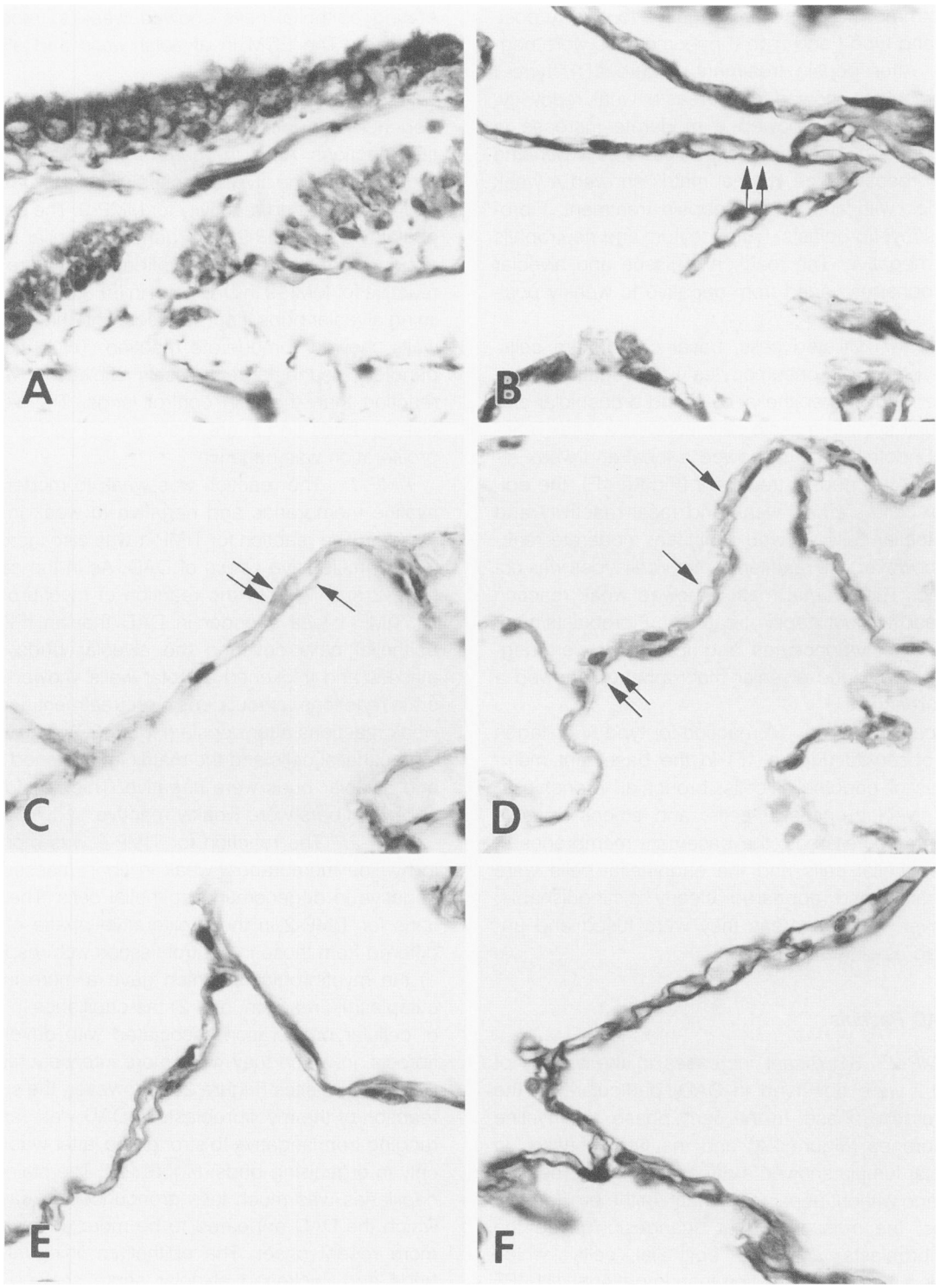


Figure 1. *Immunoperoxidase reactions in bronchiolar* (A) *and alveolar* (B *to* F) *structures in normal lung. All sections were pretreated with pepsin.* A: *MMP-2. The reaction is strong to moderate in bronchiolar ciliated cells and moderate in bronchiolar smooth muscle cells. The stromal matrix is unreactive. Magnification,* ×*1000.* B: *MMP-2. A thin, linear reaction is seen in the endothelial cells along the alveolar walls. The type I pneumocytes also show a positive reaction* (double arrow). *Magnification,* ×*820.* C: *MMP-9. The reaction is weak in endothelial cells* (arrow) *and weak and focal in type I pneumocytes* (double arrow). *Magnification,* ×*820.* D: *TIMP-1. Type I pneumocytes show a weak, focal reaction* (double arrow). *Endothelial cells show a focal but more intense reaction* (arrow). *Magnification,* ×*820.* E: *TIMP-2. Alveolar wall shows linear staining, with areas of single and double layers that probably represent basement membranes* (*as indicated by confocal microscopy observations; compare with Figure 4B*). *Magnification,* ×*820.* F: *Type IV collagen. The staining is limited to the basement membranes of alveolar epithelial cells and endothelial cells. In some areas, the basement membranes of the epithelial cells and the endothelial cells are separated and appear clearly distinguishable; however, in other areas they are fused and appear as single lines. Magnification,* ×*820.*

smooth muscle cells were negative to weakly positive, and type I and type II pneumocytes were negative. After pepsin treatment (Figure 1D), type I pneumocytes showed focal areas of weak reactivity, endothelial cells showed a moderate increase in staining, and vascular smooth muscle cells became mildly reactive. The stromal matrix showed a weak reaction with and without pepsin treatment. Fibroblasts/myofibroblasts, lymphocytes, and neutrophils were negative. The reaction in tissue and alveolar macrophages varied from negative to weakly positive.

TIMP-2: Ciliated cells, basal cells, Clara cells, and type I and II pneumocytes were negative; bronchiolar cuboidal epithelial cells and bronchiolar and vascular smooth muscle cells showed a weak reaction. Endothelial cells showed a focal and weak reaction. After pepsin treatment (Figure 1E), the epithelial cells showed weak and focal reactivity and endothelial cells showed a diffuse, moderate reaction; however, the reaction in other cell types was not altered. The stromal matrix showed weak reaction with and without pepsin treatment. Fibroblasts/myofibroblasts, lymphocytes, and neutrophils were negative. Tissue and alveolar macrophages showed a weak reaction.

Type IV Collagen: A reaction for type IV collagen was observed (Figure 1F) in the basement membranes of endothelial cells, bronchial, bronchiolar, and alveolar epithelial cells, and smooth muscle cells. In some areas, the basement membranes of the epithelial cells and the endothelial cells were separated and appeared clearly distinguishable; however, in other areas they were fused and appeared as single lines.

DAD Patients

MMP-2: Significant increases in the activity of MMP-2 were observed in DAD, particularly in the proliferative phase. In the early phase, the hyaline membranes (Figure 2A) and the fibrin masses in alveolar lumina showed a negative to weak reaction with and without pepsin treatment. In the proliferative phase, the most important changes involved the myofibroblasts and the epithelial cells, which showed a stronger reaction than in patients with IPF and in control patients. Myofibroblasts in some of the more organized and fibrosing alveolar buds showed surface reactions both in DAD and in IPF. Epithelial cells in nonfibrotic areas showed a normal (weak) reaction. The epithelial cells lining fibrin masses, alveolar buds, and thickened septa were moderately to strongly reactive (Figure 2B). Necrotic and degenerating epithelial cells showed weak to moderate reactivity. The ECM in alveolar walls and alveolar buds was unreactive.

MMP-9: Hyaline membranes, fibrin masses, and degenerating epithelial cells showed weak to negative reactions. As in the case of MMP-2, the myofibroblasts and the alveolar epithelial cells in DAD also showed increased reactivity for MMP-9. The patterns of staining for MMP-9 were generally similar in DAD and in IPF; however, the myofibroblasts were more reactive for MMP-9 in DAD than in IPF. Epithelial cells lining alveolar buds, fibrin masses, and thick alveolar walls showed a moderate reaction. Thick collagen bundles around larger vessels showed a weaker reaction than those in control lungs. The ECM in alveolar buds and in areas showing myofibroblastic proliferation was negative.

TIMP-1: The reaction was weak to moderate in hyaline membranes and negative to weak in fibrin masses. The reaction for TIMP-1 was also increased in the proliferative phase of DAD. As in the case of MMP-2 and MMP-9, the reaction of myofibroblasts for TIMP-1 was stronger in DAD than in IPF. The epithelial cells covering the alveolar buds, fibrin masses and thickened alveolar walls showed moderate reactions without pepsin pretreatment and very weak reactions after pepsin (Figure 2C). Degenerating epithelial cells and the matrix in thickened septa and alveolar buds were negative. Thick bundles of collagen fibers were weakly reactive.

TIMP-2: The reaction for TIMP-2 was moderate in hyaline membranes, weak in fibrin masses, and negative in degenerating epithelial cells. The reactions for TIMP-2 in the proliferative phase of DAD differed from those in control tissues with respect to 1) the myofibroblasts, which gave a more intense cytoplasmic reaction, and 2) the capillaries in areas of cellular proliferation associated with developing fibrosis, in which they were more intensely reactive than in other sites (Figure 2D). However, the surface reaction of the myofibroblasts in DAD was variable, ranging from negative to strong (the latter was found only in organizing buds; Figure 2E). The staining of capillaries was much less pronounced in cases in which the DAD appeared to be more acute and of more recent onset. The epithelium lining alveolar buds and thickened alveolar septa showed more intense reaction than did the epithelium in more normal areas of the biopsy specimens. This reaction was not modified by pretreatment with pepsin. The ECM in alveolar buds was negative and varied from negative to weakly reactive in other areas.

Type IV Collagen: The basement membranes of epithelial and endothelial cells in thickened alveolar

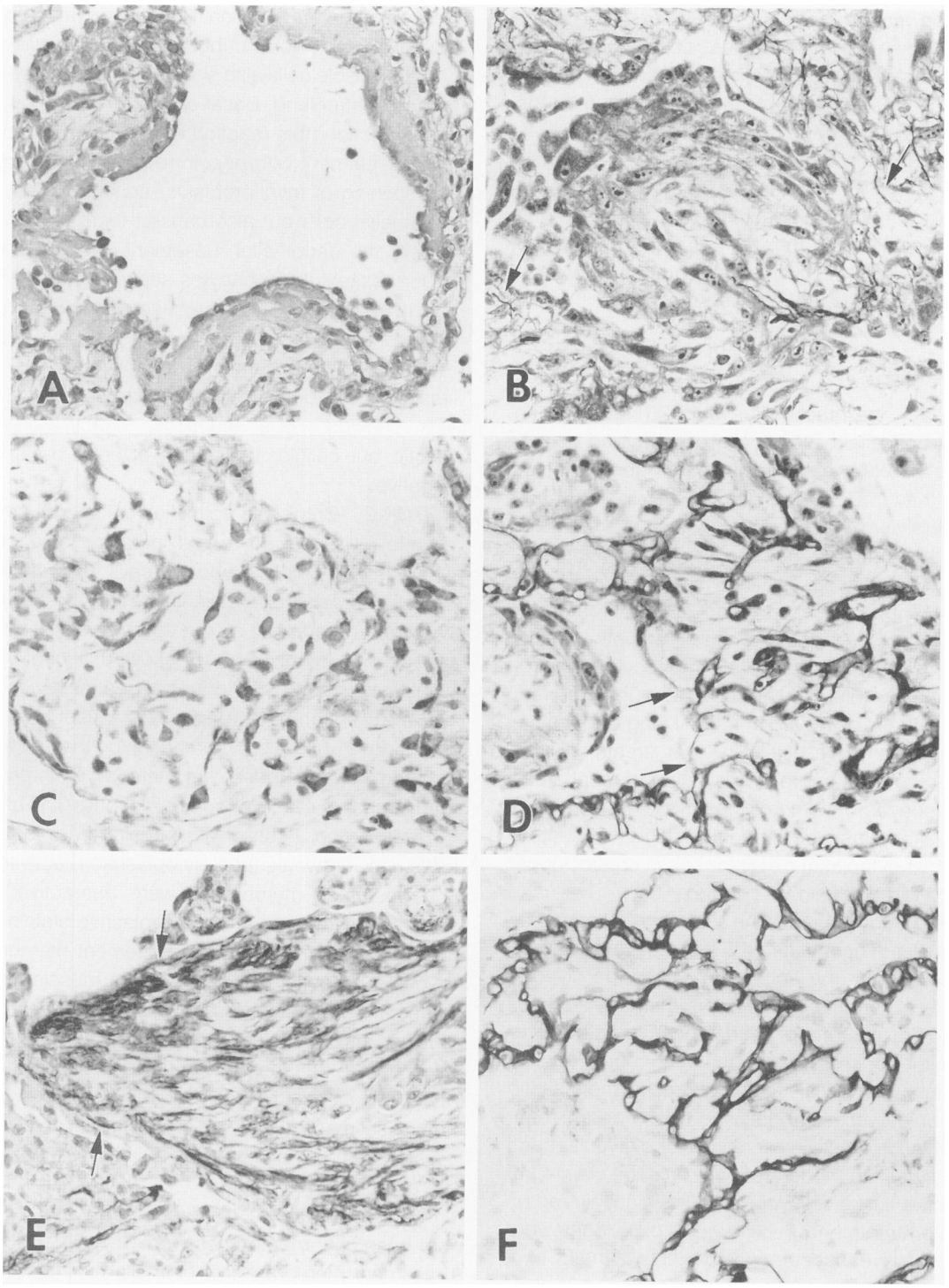


Figure 2. *Immunoperoxidase reactions in early* (A) *and proliferative* (B *to* F) *lesions in DAD. All sections were pretreated with pepsin.* A: *MMP-2. Hyaline membranes lining alveolar walls show a weak reaction, which is more pronounced in the luminal surfaces of the membranes. Magnification,* × *560.* B: *MMP-2. Epithelial cells covering alveolar bud and thickened alveolar septa show a moderate cytoplasmic reaction. Myofibroblasts in alveolar bud show a moderate cytoplasmic reaction, but their basement membranes are unreactive. Fragments of basement membranes present in the interstitium* (arrows) *show a strong reaction. The matrix in the thickened septa and the alveolar bud is unreactive. Magnification,* × *560.* C: *TIMP-1. A moderate cytoplasmic reaction is present in myofibroblasts in thickened alveolar septa. The matrix is negative. Magnification,* × *560.* D: *TIMP-2. A weak to moderate reaction is observed in the cytoplasm of epithelial cells and myofibroblasts. A strong reaction is localized in the basement membranes of alveolar epithelial and capillary endothelial cells in thickened septa. Focal discontinuities are evident in the basement membranes of the epithelial cells* (arrows). *The matrix is unreactive. Magnification,* × *560.* E: *TIMP-2. A strong reaction is localized in the basement membranes of myofibroblasts and epithelial cells* (arrows) *of an alveolar bud. The cytoplasm of these cells shows a weak reaction. The matrix is unreactive. Magnification,* × *560.* F: *Type IV collagen. The reaction is limited to the basement membranes of epithelial and endothelial cells. This staining pattern is similar to that of TIMP-2* (*compare with* D). *Note the discontinuities in the staining of the epithelial basement membranes. Magnification,* × *560.*

septa showed strong reactions (Figure 2F). The epithelial basement membranes of alveolar buds showed a strong but sometimes focally discontinuous reaction. A focal reaction was also seen on the surface of myofibroblasts in the interstitium and alveolar buds. Some masses of fibrin were partially lined by epithelial cells, the basement membranes of which were moderately reactive. In acute DAD, the alveolar capillaries and the epithelial basement membranes next to hyaline membranes showed a thin, discontinuous, and uneven reaction.

IPF Patients

MMP-2: The reaction for MMP-2 (Figure 3A) in alveolar epithelial cells, myofibroblasts, and epithelial cells covering alveolar buds was generally less intense than in DAD. The ECM of the alveolar buds and interstitium was negative whereas endothelial cells in the buds were positive. Squamous metaplastic and cuboidal epithelial cells showed a moderate reaction. The reactions in bronchial and bronchiolar epithelial cells, endothelial cells, smooth muscle cells, alveolar epithelial cells, lymphocytes, neutrophils, and alveolar and tissue macrophages were similar to those observed in normal lung, as was the reaction in large vessels. However, treatment with pepsin resulted in increased staining of focal areas of the surfaces of some of the smooth muscle cells and myofibroblasts but without change in the intensity of their cytoplasmic staining. As in control lungs, the ECM did not stain for MMP-2.

MMP-9: In general, the reactions for MMP-9 were similar in normal and IPF lung. Nevertheless, in IPF (Figure 3B), the epithelial cells of the alveolar buds were more strongly reactive than those of normal and fibrotic alveoli. In addition, the type II alveolar epithelial cells frequently were more reactive than those in normal lung. Squamous metaplastic and cuboidal epithelial cells showed moderate reactivity. Myofibroblasts in interstitium and alveolar buds showed weak to moderate reactivity. The staining of connective tissue cells for MMP-9 was not altered by pepsin pretreatment. Thick bundles of collagen were weakly positive, but other components of the ECM in normal and fibrotic areas of interstitium and in alveolar buds were unreactive. Alveolar macrophages also reacted more intensely than in normal lung.

TIMP-1: In comparison with normal lung (Figure 1D), the staining for TIMP-1 also was increased (Figure 3C). The epithelial cells lining the alveolar buds were more strongly reactive for TIMP-1 than those lining both normal and fibrotic alveoli. Squamous metaplastic epithelium and myofibroblasts showed a weak to moderate reaction. Bronchial ciliated epithelial cells, bronchiolar cuboidal epithelial cells, and smooth muscle cells also showed a stronger reaction than in control lung. Basal epithelial cells showed a weak reaction (this reaction was negative in normal lung). Treatment with pepsin reduced the cytoplasmic staining of myofibroblasts, epithelial cells, and endothelial cells but also caused focal reactivity to appear in endothelial basement membranes of larger vessels. Lymphocytes showed a weak reaction, in contrast to a negative reaction in normal lung. Macrophages showed only a minimal increase in reactivity. Thick bundles of collagen fibers in less cellular fibrotic septa and around large vessels showed weak reactions. The matrices in fibrotic but cellular septa and alveolar buds were negative.

TIMP-2: Bronchial epithelial cells and smooth muscle cells showed a mild increase in reactivity, whereas bronchiolar cuboidal cells showed a decrease. The epithelial cells lining the alveolar buds were more reactive than the other epithelial cells in other areas. Myofibroblasts showed a negative to weak cytoplasmic reaction. After treatment with pepsin, the myofibroblast, particularly those in alveolar buds, developed a strong reaction on their surfaces, and the epithelial basement membranes in alveolar buds became reactive (compare Figure 3, D and E). In sections not treated with pepsin, the endothelial cells showed weak, focal cytoplasmic reactivity and the basement membranes were unreactive; after treatment with pepsin, the cytoplasmic staining became more diffuse and the basement membranes became intensely reactive. Smooth muscle cells in bronchi, bronchioles, and larger vessels also showed similar but less marked changes in reactivity after pepsin treatment. The ECM of the buds did not stain, in contrast to a weak and diffuse reaction in the interstitial matrix. However, a weak reaction was also observed in the basal portions of buds undergoing fibrotic changes.

Type IV Collagen: In fibrotic septa, remnants of old basement membranes were seen in addition to basement membranes of epithelial and endothelial cells. In alveolar buds (Figure 3F), the epithelial basement membranes were uneven, sometimes multilayered, and often focally disrupted, in contrast to their even and continuous appearance in unaltered alveolar walls. In the matrix of alveolar buds, remnants of basement membranes were seen in addition to the basement membranes of myofibroblasts.

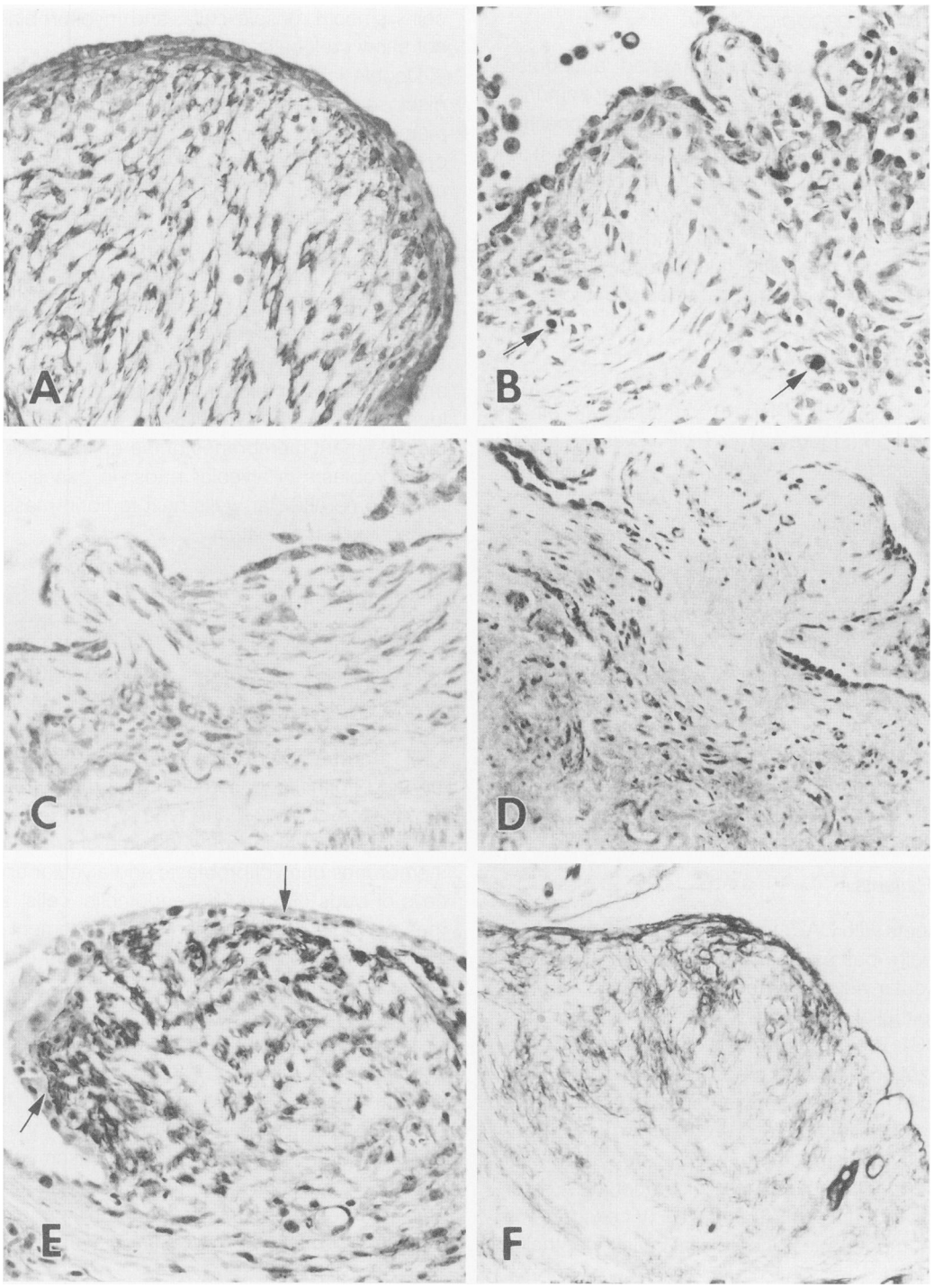


Figure 3. *Immunoperoxidase reactions in alveolar buds in IPF. All sections except those shown in* C *and* D *were pretreated with pepsin.* A: *MMP-2. The epithelial cells lining the bud show a moderate reaction. Myofibroblasts show a strong surface reaction and a weak to moderate cytoplasmic reaction. The matrix is negative. Magnification,* ×*560.* B: *MMP-9. The epithelial cells of the alveolar bud are moderately reactive. The myofibroblasts in the bud show only weak cytoplasmic reactivity and the matrix is unreactive. Intensely reactive neutrophils* (arrows) *are present near the base of the bud. Magnification,* ×*560.* C: *TIMP-1. The reaction is moderate in epithelial cells, weak in the cytoplasm of myofibroblasts, and negative in the matrix. Magnification,* ×*560.* D: *TIMP-2. Myofibroblasts and epithelial cells lining an alveolar bud show a weak cytoplasmic reaction. The matrix at the base of the bud* (lower left) *and in surrounding interstitial tissue shows a weak and diffuse reaction; however, the matrix of the remainder of the bud* (upper right) *is unreactive. Note that this section was stained without pretreatment with pepsin. Therefore, it does not show surface staining such as that evident in* E, *in which the tissue was treated with pepsin before immunostaining. Magnification,* ×*220.* E: *TIMP-2. A strong reaction is seen on the surfaces of the myofibroblasts and focally in the epithelial basement membranes* (arrows). *The cytoplasm of the epithelial cells and myofibroblasts shows weak reactions. The matrix is negative. Magnification,* ×*560.* F: *Type IV collagen. The epithelial basement membranes are uneven, multilayered, and focally disrupted. In the matrix of an alveolar bud, remnants of basement membranes are seen in addition to the basement membranes of myofibroblasts. Magnification,* ×*560.*

Confocal Microscopic Study

Staining with fluorochrome-conjugated antibodies gave results similar to those observed after immunoperoxidase staining. On examination by confocal microscopy, a green fluorescence was indicative of type IV collagen; a red fluorescence identified either the MMPs or their corresponding TIMPs; a yellow fluorescence indicated co-localization of the red and green colors; and a blue fluorescence (4′,6-diamidino-2-phenylindole stain) revealed the nuclei.

Control Patients

No co-localization of type IV collagen with either MMP-2, MMP-9 (Figure 4A), or TIMP-1 was observed in control tissue. Co-localization of type IV collagen and TIMP-2 was observed in the basement membranes of the alveolar epithelial cells and endothelial cells (Figure 4B). A weak red fluorescence for TIMP-2 was also observed in the cytoplasm of endothelial cells as well as in type I and type II alveolar epithelial cells. In the arteries, the endothelial cells showed a weak red fluorescence (TIMP-2) and the basement membranes showed green fluorescence (type IV collagen) and focal yellow fluorescence for co-localization of these two components. The basement membranes of the bronchiolar smooth muscle cells and epithelial cells showed no co-localization.

DAD Patients

In patients with DAD, type IV collagen and MMP-2 were focally but extensively co-localized in thickened alveolar septa and alveolar buds and in disrupted epithelial and capillary basement membranes (Figure 4C). The myofibroblasts did not show surface co-localization; however, focal co-localization was observed in the basement membranes of the epithelial cells and myofibroblasts in mature buds, endothelial cells, and smooth muscle cells of larger vessels and smooth muscle cells of bronchioles. After double labeling for MMP-9 and type IV collagen, the basement membranes of epithelial cells, endothelial cells, smooth muscle cells, and myofibroblasts did not show co-localization (Figure 4D).

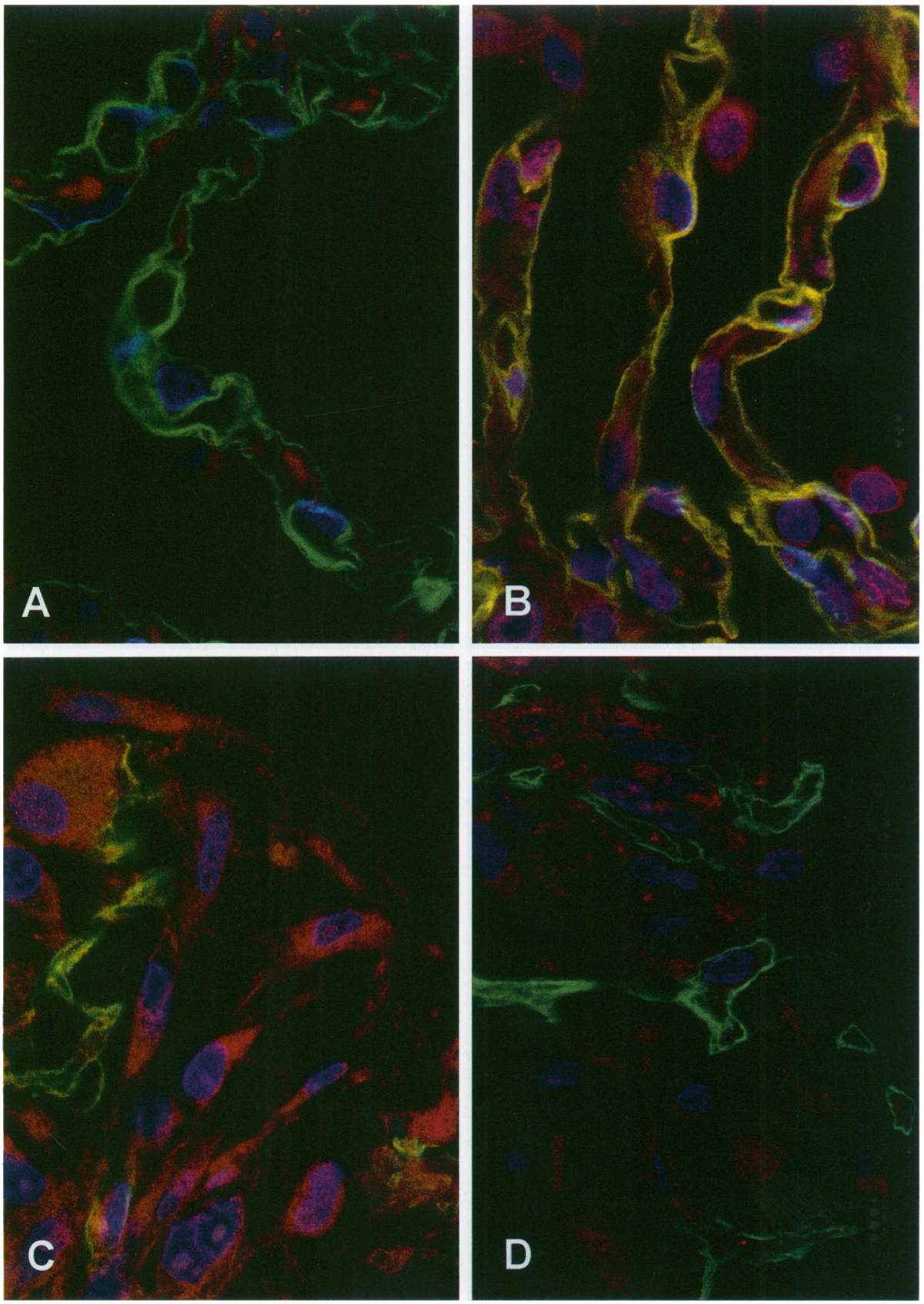
Double labeling for TIMP-1 and type IV collagen disclosed focal co-localization in the basement membranes of capillary endothelial and epithelial cells in thickened alveolar walls and of endothelial and smooth muscle cells of larger vessels. No co-localization was observed in the basement membranes of bronchiolar smooth muscle cells and epithelial cells. TIMP-2 and type IV collagen were diffusely co-localized (Figure 5A) in the basement membranes of endothelial cells, epithelial cells, and smooth muscle cells. The basement membranes of myofibroblasts in thickened septa showed focal co-localization of type IV collagen and TIMP-2, as did the basement membranes of the epithelial cells and myofibroblasts in alveolar buds. In cases of acute DAD, some alveolar walls next to fibrin masses did not show co-localization.

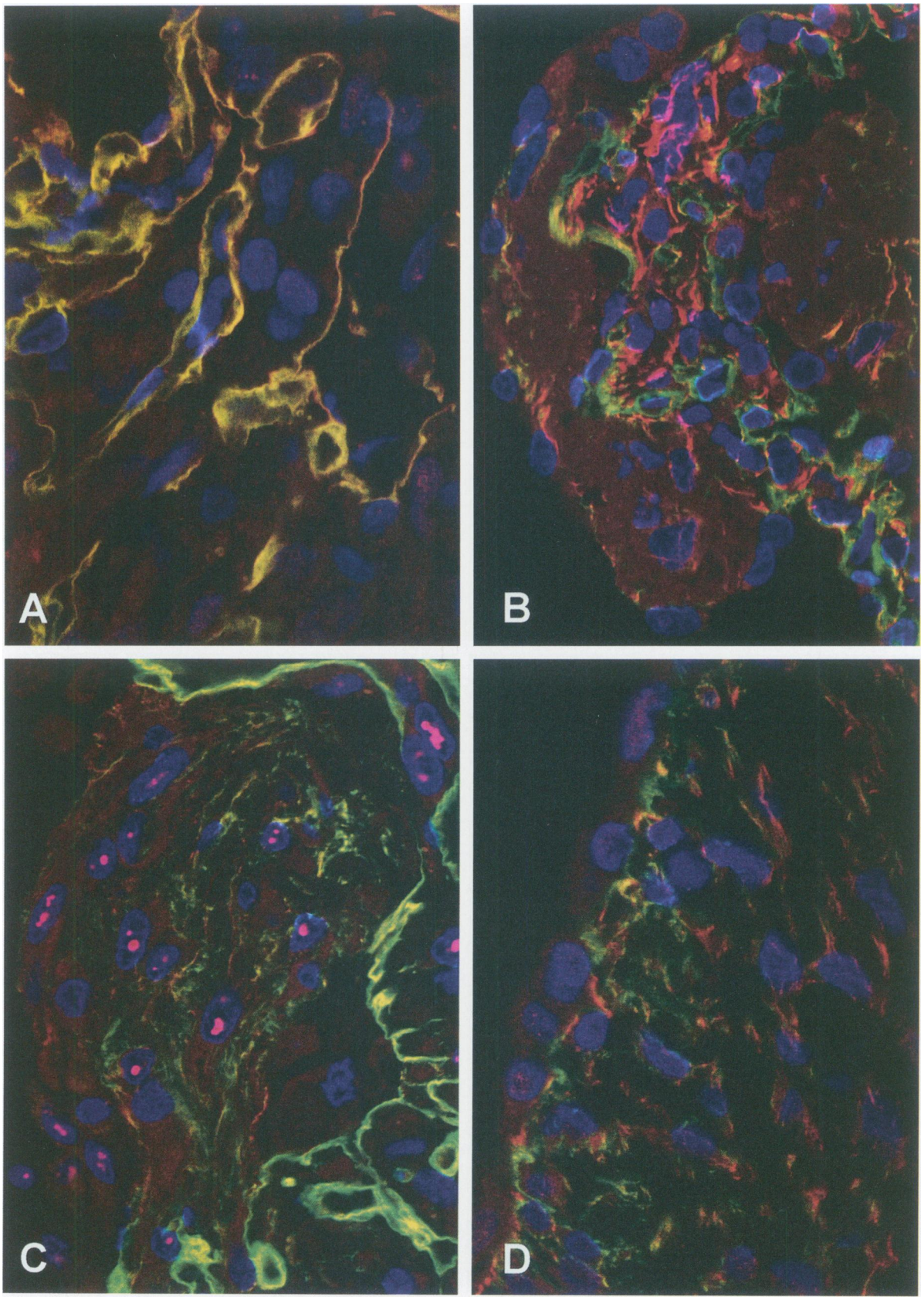
IPF Patients

Nonfibrotic alveoli showed the same patterns of co-localization of type IV collagen with MMP-2, MMP-9, and TIMP-1 as in normal lung. Fibrotic alveolar walls frequently showed co-localization of MMP-2 and type IV collagen in focal areas of capillary and epithelial basement membranes. Focal co-localization of MMP-2 and type IV collagen was observed to a variable extent in some of the basement membranes of myofibroblasts and alveolar epithelial cells of buds (Figure 5B), endothelial cells, smooth muscle cells, and remnants of basement membranes in the interstitium. Co-localization of type IV collagen and TIMP-1 was very focal and was limited to portions of the basement membranes of epithelial cells and endothelial cells in capillaries in fibrotic septa.

Much more extensive co-localization of type IV collagen was observed with TIMP-2 than with MMP-2, MMP-9, or TIMP-1. The basement membranes of capillary endothelial cells and epithelial cells in fibrotic septa and of endothelial and smooth muscle cells in larger vessels showed diffuse co-

Figure 4. *Confocal microscopic images showing fluorescent staining of nuclei (blue), type IV collagen (green), MMPs or TIMPs (red), and areas of co-localization of type IV collagen with either a MMP or a TIMP (yellow).* A: *Normal lung stained for MMP-9 and type IV collagen. Alveolar endothelial and epithelial basement membranes show green fluorescence (type IV collagen). Epithelial cells and endothelial cells show a weak reaction (red fluorescence) for MMP-9. No co-localization is observed. Magnification, ×1200.* B: *Normal lung stained for TIMP-2 and type IV collagen. The basement membranes of the alveolar epithelial cells and endothelial cells show yellow fluorescence indicative of co-localization. A weak red fluorescence (TIMP-2) is observed in the cytoplasm of endothelial cells and of type I and type II alveolar epithelial cells. Magnification, ×1200.* C: *DAD stained for MMP-2 and type IV collagen. Proliferative lesion shows discontinuities in the staining of the epithelial basement membranes for type IV collagen (green fluorescence). Focal areas of yellow fluorescence, indicative of co-localization, are seen in the basement membranes of the epithelial and endothelial cells. The cytoplasm of myofibroblasts shows red (MMP-2) fluorescence. Magnification, ×1200.* D: *DAD stained for MMP-9 and type IV collagen. Endothelial and epithelial basement membranes show type IV collagen (green fluorescence). Disruption of the epithelial basement membranes is evident in the form of discontinuities in this green fluorescence. Myofibroblasts show cytoplasmic red (MMP-9) fluorescence. No co-localization is evident. Magnification, ×1200.*


A
B
C
D


A
B
C
D

localization. In addition, the basement membranes of myofibroblasts in the buds and fibrotic septa, the epithelial cells in the buds (Figure 5, C and D), the remnants of basement membranes in fibrotic foci, and the basement membranes of bronchial and arterial smooth muscle cells showed focal areas of co-localization.

Discussion

The present study provides the first systematic evaluation of the histochemical localization of MMPs and TIMPs in interstitial lung disorders. Two novel approaches were utilized in this study: 1) the use of pepsin digestion, which enhances detection of certain antigens,[33] and 2) the application of confocal microscopy to evaluate the co-localization of type IV collagen with the MMPs and TIMPs.

MMPs and TIMPs in Normal Lung

In normal lung, the immunoreactivity for MMP-2 was more intense and more widely distributed than that for MMP-9. These observations are consistent with the notion that MMP-2 activity is regulated at the level of proenzyme activation, whereas MMP-9 can be transcriptionally regulated.[1,2] The mRNA for MMP-2 is known to be present in pulmonary fibroblasts and endothelial cells.[23] Cultured lung fibroblasts synthesize MMP-2.[34,35] Collagenases and TIMPs are produced by cultured pulmonary fibroblasts from patients with IPF.[13,14,36] Moloney et al[37] found collagenase and collagenase inhibitors in bronchoalveolar lavage fluid from patients with IPF and other interstitial lung diseases. A large proportion of the collagenase may have been derived from neutrophils and macrophages. Neutrophils express MMP-8[38] and MMP-9.[39] Macrophages showed weak reactions for MMP-2 and MMP-9 and negative to weak reactions for TIMP-1 and TIMP-2. These observations are in agreement with the results of other studies showing that these cells produce MMPs and TIMPs. Rat alveolar macrophages stimulated with bleomycin secrete larger amounts of MMP-9 than do nonstimulated macrophages.[24] Alveolar macrophages from patients with active pulmonary sarcoidosis release greater amounts of type IV collagenase than do macrophages from patients with inactive disease and control subjects.[40] Cultured alveolar macrophages from cigarette smokers secrete TIMP-2; however, when stimulated, they also secret TIMP-1 but decrease their secretion of TIMP-2.[26] Thus, both the parenchymal cells and the inflammatory cells participate in the regulation of ECM turnover in pulmonary disorders.

In many areas of normal lung, the presence of MMP-2 within the cytoplasm of epithelial and endothelial cells correlated with the presence of TIMP-2 in both the cytoplasm and basement membranes of these cells. The co-expression of MMP-2 and TIMP-2 in endothelial cells suggests that this inhibitor (which was present in the basement membranes adjacent to these cells but without being associated with MMP-2 at these sites) may prevent MMP-2 from causing damage to the basement membranes. The lack of TIMP-2 expression in the basement membranes of smooth muscle cells and bronchiolar epithelial cells indicates potential differences between the basement membranes at these two sites.

MMPs and TIMPS in DAD

After lung injury, the expression of MMPs and TIMPs is enhanced to allow for increased collagen deposition or breakdown, or both. In DAD, the injury initiates inflammatory and fibroproliferative processes that can either progress to end-stage fibrosis or resolve completely. In IPF, there seems to be a continuing series of processes of tissue injury and repair, in which the altered balance of MMPs and TIMPs leads to irreversible fibrosis. Our data do not provide information concerning whether or not the co-localization of MMPs and TIMPs is related to the formation of specific enzyme-inhibitor complexes at the cell surfaces. The MMPs in such complexes undergo activation by a membrane-type MMP.[41] We found abundant reactivity for TIMP-2 in basement membranes in which no reactivity for MMP-2 was demonstrable,

Figure 5. *Confocal microscopic images showing fluorescent staining of nuclei (blue), type IV collagen (green), MMPs or TIMPs (red), and areas of co-localization of type IV collagen with either a MMP or a TIMP (yellow).* A: *DAD stained for TIMP-2 and type IV collagen. The basement membranes of endothelial and epithelial cells show diffuse co-localization (yellow fluorescence). Magnification, × 1200.* B: *IPF stained for MMP-2 and type IV collagen. Focal co-localization (yellow) is observed in the basement membranes of epithelial cells covering two alveolar buds* (lower left *and* center right). *The epithelial basement membranes of a mildly thickened alveolar wall adjacent to the buds also show focal co-localization (yellow), but most of the alveolar basement membranes show green fluorescence (no co-localization). The cytoplasm of the endothelial cells shows red (MMP-2) fluorescence. Red and yellow fluorescence is observed in remnants of basement membranes at the base of the buds. Magnification, × 1200.* C: *IPF stained for TIMP-2 and type IV collagen. Epithelial and endothelial basement membranes of a fibrotic alveolar wall show co-localization. The myofibroblasts in an alveolar bud show red (TIMP-2) fluorescence. Areas of green (type IV collagen) and yellow (co-localization) fluorescence are present in the basement membranes of the myofibroblasts and the epithelial cells of the bud. Magnification, × 1200.* D: *IPF stained for TIMP-2 and type IV collagen. Surface epithelial cells and myofibroblasts show cytoplasmic red (TIMP-2) fluorescence. Orange and yellow fluorescence, indicative of co-localization, is observed in the basement membranes of epithelial cells and myofibroblasts in the bud. Magnification, × 1200.*

suggesting that TIMP-2 is bound to extracellular sites that are not related to complexing with MMP-2.

The most important immunohistochemical difference observed in DAD was the more intense staining of myofibroblasts for MMP-2, MMP-9, TIMP-1, and TIMP-2. This probably reflects the very active role of these cells in the changes that take place in the ECM during the proliferative phase of DAD. These findings are similar in some respects to those reported in wound healing.[42–49] The abundance of MMPs in the proliferating myofibroblasts may provide an explanation for the rapid evolution of the pulmonary morphological changes in DAD, as increased activity of these enzymes would favor ECM proteolysis in alveolar septa and in alveolar buds.

An important difference between DAD and IPF was the accentuated staining of the basement membranes of endothelial cells for TIMP-2 in areas of cellular proliferation in DAD. The accumulation of TIMPs could protect the alveolar capillaries from the lytic effects of MMPs on their basement membranes. Both MMPs and TIMPs appear to play critical roles in angiogenesis. MMP-2 promotes the formation of tubes in cultures of endothelial cells, and TIMP-2 inhibits both endothelial proliferation and tube formation.[50,51] It is not known to what extent these effects of TIMP-2 modulate the angiogenic response to acute lung injury. An increase in TIMPs also may promote vascular fibrosis in the pulmonary hypertension associated with fibrotic lung disorders.

An important similarity between the fibrotic phase of DAD and that of IPF concerned the presence of TIMP-1 and TIMP-2 in areas of dense fibrosis in both disorders. This may contribute to stabilizing the collagen and other matrix components deposited in these areas.

MMPs and TIMPs in IPF

IPF is characterized not only by progressive deposition of collagen in the pulmonary interstitium but also by lysis and remodeling of lung tissue. Therefore, it is not surprising that increased expression of both MMPs and TIMPs was found in IPF. Two sites were of particular importance with respect to the development of pulmonary fibrotic changes: alveolar buds and alveolar septa.[29,30] Alveolar buds are lined by a layer of epithelial cells and contain myofibroblasts scattered in a loosely arranged matrix. This matrix is composed of abundant amounts of proteoglycans and, depending upon its stage of organization, variable quantities of collagen.[29] Damage to the alveolar basement membranes allows the migration of myofibroblasts into the alveolar lumina to form alveolar buds.[29] These buds can either undergo regression (as in resolving DAD) or become fibrotic and incorporated into the adjacent fibrotic alveolar walls (as in progressive DAD and in IPF).[29] In IPF, the myofibroblasts and the epithelial cells of the buds showed moderate to strong reactions for MMP-2 and MMP-9. A moderate reaction for TIMP-1 and TIMP-2 was also observed in the epithelial lining cells of the buds. The basement membranes of these cells often appeared discontinuous and multilayered and also were reactive for TIMP-2. This reflects the ongoing destruction of these basement membranes and the attempted protective effect of TIMP-2. The surfaces of the myofibroblasts in alveolar buds usually were more strongly reactive for TIMP-2 in IPF than in DAD. This is consistent with the more frequent development of fibrotic changes in alveolar buds in IPF. The ECM in many alveolar buds showed no reaction either for MMPs or TIMPs, in contrast to the reactivity for these components in fibrotic alveolar septa. An excess of MMP-2 and MMP-9 in relation to the TIMPs reflects an overall proteolytic effect in the buds, consistent with the concept that these structures represent the advancing edge of tissue destruction and can undergo involution without residual fibrosis.

The conclusions of the present study can be summarized as follows. First, pretreatment with pepsin was necessary to disclose reactive sites for MMPs and TIMPs, particularly in basement membranes, that were considered to be important and were not evident without this procedure. Second, confocal microscopy was a valuable method to demonstrate the co-localization of the MMPs or TIMPs with type IV collagen. Third, in normal lung, MMP-2, MMP-9, TIMP-1, and TIMP-2 were localized in bronchial and bronchiolar ciliated cells, endothelial cells, pneumocytes, and smooth muscle cells; fibroblasts were strongly positive only for MMP-2, and goblet cells and Clara cells were negative for both MMPs and TIMPs. MMP-9 and TIMPs were also localized in the collagenous matrix. Only TIMP-2 showed co-localization with type IV collagen in the alveolar endothelial and epithelial basement membranes, suggesting a protective role of TIMP-2 in these structures. Fourth, myofibroblasts and epithelial cells lining the proliferative lesions in DAD and IPF were the main cell types that expressed increased reactivity for MMPs and TIMPs in both conditions. Fifth, the reactivities for MMPs and TIMPs were stronger in DAD than in IPF or normal tissue. These findings correlated with the rapidly evolving changes in DAD. Sixth, alveolar buds showed increased reactivity for MMPs and TIMPs in their lining epithelial cells, myofibroblasts, and their basement membranes; how-

ever, their matrices were unreactive, except for the presence of TIMP-2 in association with early fibrotic changes in some buds. Seventh, in DAD and IPF, MMP-2 showed focal co-localization with type IV collagen in capillary endothelial and disrupted epithelial basement membranes, suggesting activation of this protease and subsequent proteolysis of basement membrane components; however, a protective effect against collagen breakdown was suggested by the extensive co-localization of TIMP-2 with type IV collagen and with dense fibrillar collagen in both disorders.

References

1. Stetler-Stevenson WG, Aznavoorian S, Liotta LA: Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993, 9:541–573
2. Ray JM, Stetler-Stevenson WG: The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis, and angiogenesis. Eur Respir J 1994, 7:2062–2072
3. Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He CS: Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc Natl Acad Sci USA 1989, 86:8207–8211
4. Stetler-Stevenson WG, Krutzsch HC, Liotta LA: Tissue inhibitor of metalloproteinase (TIMP-2): a new member of the metalloproteinase inhibitor family. J Biol Chem 1989, 264:17374–17378
5. Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI: SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages [published erratum appears in J Biol Chem 1990, 265(36):22570]. J Biol Chem 1989, 264:17213–17221
6. Carmichael DF, Sommer A, Thompson RC, Anderson DC, Smith CG, Welgus HG, Stricklin GP: Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. Proc Natl Acad Sci USA 1986, 83:2407–2411
7. Cawston TE, Galloway WA, Mercer E, Murphy G, Reynolds JJ: Purification of rabbit bone inhibitor of collagenase. Biochem J 1981, 195:159–165
8. Welgus HG, Stricklin GP: Human skin fibroblast collagenase inhibitor: comparative studies in human connective tissues, serum, and amniotic fluid. J Biol Chem 1983, 258:12259–12264
9. Stetler-Stevenson WG, Brown PD, Onisto M, Levy AT, Liotta LA: Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J Biol Chem 1990, 265:13933–13938
10. Ganser GL, Stricklin GP, Matrisian LM: EGF and TGF-α influence *in vitro* lung development by the induction of matrix-degrading metalloproteinases. Int J Dev Biol 1991, 35:453–461
11. McGowan SE: Extracellular matrix and the regulation of lung development and repair. FASEB J 1992, 6:2895–2904
12. Minoo P, Penn R, deLemos DM, Coalson JJ, deLemos RA: Tissue inhibitor of metalloproteinase-1 mRNA is specifically induced in lung tissue after birth. Pediatr Res 1993, 34:729–734
13. Pardo A, Selman M, Ramírez R, Ramos C, Montaño M, Stricklin G, Raghu G: Production of collagenase and tissue inhibitor of metalloproteinases by fibroblasts derived from normal and fibrotic human lungs. Chest 1992, 102:1085–1089
14. Pardo A, Selman M: Decreased collagenase production by fibroblasts derived from idiopathic pulmonary fibrosis. Matrix Suppl 1992, 1:417–418
15. D'Armiento J, Dalal SS, Okada Y, Berg RA, Chada K: Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. Cell 1992, 71: 955–961
16. Sepper R, Konttinen YT, Sorsa T, Koski H: Gelatinolytic and type IV collagenolytic activity in bronchiectasis. Chest 1994, 106:1129–1133
17. Piedboeuf B, Johnston CJ, Watkins RH, Hudak BB, Lazo JS, Cherian MG, Horowitz S: Increased expression of tissue inhibitor of metalloproteinases (TIMP-I) and metallothionein in murine lungs after hyperoxic exposure. Am J Respir Cell Mol Biol 1994, 10:123–132
18. Hurewitz AN, Zucker S, Mancuso P, Wu CL, Dimassimo B, Lysik RM, Moutsiakis D: Human pleural effusions are rich in matrix metalloproteinases. Chest 1992, 102: 1808–1814
19. Urbanski SJ, Edwards DR, Maitland A, Leco KJ, Watson A, Kossakowska AE: Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. Br J Cancer 1992, 66:1188–1194
20. Zucker S, Lysik RM, Malik M, Bauer BA, Caamano J, Klein-Szanto AJ: Secretion of gelatinases and tissue inhibitors of metalloproteinases by human lung cancer cell lines and revertant cell lines: not an invariant correlation with metastasis. Int J Cancer 1992, 52:366–371
21. Zucker S, Wieman J, Lysik RM, Imhof B, Nagase H, Ramamurthy N, Liotta LA, Golub LM: Gelatin-degrading type IV collagenase isolated from human small cell lung cancer. Invasion Metastasis 1989, 9:167–181
22. Ohori NP, Yousem SA, Griffin J, Stanis K, Stetler-Stevenson WG, Colby TV, Sonmez-Alpan E: Comparison of extracellular matrix antigens in subtypes of bronchioloalveolar carcinoma and conventional pulmonary adenocarcinoma: an immunohistochemical study. Am J Surg Pathol 1992, 16:675–686
23. Soini Y, Pääkkö P, Autio-Harmainen H: Genes of laminin B1 chain, α1 (IV) chain of type IV collagen, and 72-kd type IV collagenase are mainly expressed by the stromal cells of lung carcinomas. Am J Pathol 1993, 142:1622–1630
24. Denholm EM, Rollins SM: Alveolar macrophage secretion of a 92-kDa gelatinase in response to bleomycin. Am J Physiol 1993, 265:L581–L585

25. Weiland JE, Garcia JG, Davis WB, Gadek JE: Neutrophil collagenase in rheumatoid interstitial lung disease. J Appl Physiol 1987, 62:628–633
26. Shapiro SD, Kobayashi DK, Welgus HG: Identification of TIMP-2 in human alveolar macrophages: regulation of biosynthesis is opposite to that of metalloproteinases and TIMP-1. J Biol Chem 1992, 267:13890–13894
27. Hembry RM, Murphy G, Reynolds JJ: Immunolocalization of tissue inhibitor of metalloproteinases (TIMP) in human cells: characterization and use of a specific antiserum. J Cell Sci 1985, 73:105–119
28. Canete-Soler R, Litzky L, Lubensky I, Muschel RJ: Localization of the 92-kd gelatinase mRNA in squamous cell and adenocarcinomas of the lung using *in situ* hybridization. Am J Pathol 1994, 144:518–527
29. Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, Crystal RG: Intraluminal fibrosis in interstitial lung disorders. Am J Pathol 1986, 122:443–461
30. Katzenstein AL: Idiopathic interstitial pneumonia: classification and diagnosis. The Lung: Current Concepts. Edited by A Churg, AL Katzenstein. Baltimore, Williams & Wilkins, 1993, pp 1–31
31. Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG: Independent expression and cellular processing of M_r 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res 1990, 50:6184–6191
32. Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM: Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes. J Biol Chem 1992, 267:515–519
33. Swanson PE, Hagen KA, Wick MR: Avidin-biotin-peroxidase-antiperoxidase (ABPAP) complex: an immunocytochemical method with enhanced sensitivity. Am J Clin Pathol 1987, 88:162–176
34. Goldberg GI, Collier IE, Eisen AZ, Grant GA, Marmer BL, Wilhelm SM: Mosaic structure of the secreted ECM metalloproteases and interaction of the type IV collagenases with inhibitors. Matrix Suppl 1992, 1:25–30
35. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA, Goldberg GI: H-*ras* oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 1988, 263:6579–6587
36. Montano M, Ramos C, Gonzalez G, Vadillo F, Pardo A, Selman M: Lung collagenase inhibitors and spontaneous and latent collagenase activity in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis. Chest 1989, 96:1115–1119
37. Moloney D, O'Connor CM, Fitzgerald MX: Bronchoalveolar lavage collagenase and collagenase inhibitory capacity in interstitial lung disease. Biochem Soc Trans 1992, 20:79S
38. Fosang AJ, Last K, Knauper V, Neame PJ, Murphy G, Hardingham TE, Tschesche H, Hamilton JA: Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. Biochem J 1993, 295:273–276
39. Ståhle-Bäckdahl M, Parks WC: 92-kd gelatinase is actively expressed by eosinophils and stored by neutrophils in squamous cell carcinoma. Am J Pathol 1993, 142:995–1000
40. Agostini C, Garbisa S, Trentin L, Zambello R, Fastelli G, Onisto M, Cipriani A, Festi G, Casara D, Semenzato G: Pulmonary alveolar macrophages from patients with active sarcoidosis express type IV collagenolytic proteinase: an enzymatic mechanism for influx of mononuclear phagocytes at sites of disease activity. J Clin Invest 1989, 84:605–612
41. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI: Mechanism of cell surface activation of 72-kDa type IV collagenase: isolation of the activated form of the membrane metalloprotease. J Biol Chem 1995, 270:5331–5338
42. Ågren MS: Gelatinase activity during wound healing. Br J Dermatol 1994, 131:634–640
43. Matsubara M, Girard MT, Kublin CL, Cintron C, Fini ME: Differential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the remodelling cornea. Dev Biol 1991, 147:425–439
44. Matsubara M, Zieske JD, Fini ME: Mechanism of basement membrane dissolution preceding corneal ulceration. Invest Ophthalmol Vis Sci 1991, 32:3221–3237
45. Oikarinen A, Kylmaniemi M, Autio-Harmainen H, Autio P, Salo T: Demonstration of 72-kDa and 92-kDa forms of type IV collagenase in human skin: variable expression in various blistering diseases, induction during re-epithelialization, and decrease by topical glucocorticoids. J Invest Dermatol 1993, 101:205–210
46. Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H, Larjava H: Expression of matrix metalloproteinase-2 and -9 during early human wound healing. Lab Invest 1994, 70:176–182
47. Stricklin GP, Li L, Jancic V, Wenczak BA, Nanney LB: Localization of mRNAs representing collagenase and TIMP in sections of healing human burn wounds. Am J Pathol 1993, 143:1657–1666
48. Stricklin GP, Li L, Nanney LB: Localization of mRNAs representing interstitial collagenase, 72-kda gelatinase, and TIMP in healing porcine burn wounds. J Invest Dermatol 1994, 103:352–358
49. Stricklin GP, Nanney LB: Immunolocalization of collagenase and TIMP in healing human burn wounds. J Invest Dermatol 1994, 103:488–492
50. Murphy AN, Unsworth EJ, Stetler-Stevenson WG: Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. J Cell Physiol 1993, 157:351–358
51. Schnaper HW, Grant DS, Stetler-Stevenson WG, Fridman R, D'Orazi G, Murphy AN, Bird RE, Hoythya M, Fuerst TR, French DL: Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis *in vitro*. J Cell Physiol 1993, 156:235–246