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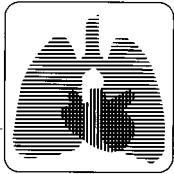
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laboratory and animal investigations

Matrix Metalloproteinases Inhibition Attenuates Tobacco Smoke-Induced Emphysema in Guinea Pigs*

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Study objective: To evaluate the effect of CP-471,474 (Pfizer Global Research and Development; Groton, CT), a broad-spectrum inhibitor of matrix metalloproteinases (MMPs) in an experimental model of emphysema.
Design: Randomized, double-blinded, controlled experiment.
Setting: Biochemistry and morphology laboratories and animal research facility.
Methods: Guinea pigs were exposed to cigarette smoke over 1 month, 2 months, and 4 months, and half of the animals received CP-471,474. Age-matched guinea pigs exposed to room air were used as control animals. After death, the lungs were lavaged with saline solution, and MMPs in the lavage fluid were determined by zymography and immunoblot. Lungs were fixed for histology, immunohistochemistry, and morphometry.
Results: Following a 1-month exposure to tobacco smoke, semiquantitative histologic assessment showed moderate lung inflammation, which progressed in extent and severity and reached a peak at 2 months. CP-471,474 significantly reduced both the extent (p < 0.002) and severity (p < 0.05) of inflammation at 2 months. At 4 months, a spontaneous reduction of the inflammatory response was observed in both treated and untreated animals, and consequently no difference was observed between both. Emphysematous changes, revealed by a significant increase in the average size of alveoli, were detected at 2 months and 4 months of tobacco smoke exposure. The inhibitor significantly decreased the destructive lesions mainly at 2 months (p < 0.0001) and also at 4 months (p < 0.02). Smoking increased MMP-9 and MMP-1 activities as shown by zymography and immunoblot. Immunoreactive MMP-9 was mainly localized in alveolar and bronchiolar epithelial cells, macrophages, and airways smooth-muscle cells.
Conclusion: These findings support a role for MMPs in the early inflammatory response and in the emphysematous lesions provoked by cigarette smoking.
                                                        (CHEST 2003; 123:1633–1641)

Key words: collagenases; COPD; gelatinases; pulmonary emphysema

Abbreviation: MMP = matrix metalloproteinase

Lung emphysema is a major component of tobacco-smoke induced COPD. The condition is characterized by enlargement of respiratory regions of the lung distal to the terminal bronchioles and is accompanied by the destruction of the alveolar septa, leading to progressive and irreversible respiratory insufficiency.[1] The prevalent theory for the patho-

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genesis of emphysema is that the disease results from an excessive neutrophil elastase burden in the lower respiratory tract.[1–3] In recent years, however, a growing body of evidence strongly suggests that other enzymes, primarily matrix metalloproteinases (MMPs), play a role in the disruption of the alveolar walls.

The first important contribution suggesting a role for interstitial collagenase (MMP-1) in the pathogenesis of this disease was reported by D'Armiento et al,[4] who demonstrated that mice expressing a collagenase transgene in their lungs had morphologic changes strikingly similar to human emphysema. This finding was later confirmed in an experimental model induced by tobacco smoke in guinea pigs.[5] In this model, animals exposed to cigarette smoke exhibited progressive lung inflammatory lesions of mononuclear predominance, and after 6 to 8 weeks a varied degree of emphysematous changes. The progression of bronchiolar and alveolar inflammation was coincident with the development of emphysematous lesions, expression of MMP-1, and increased collagenolytic activity. The up-regulation of MMP-1 has been confirmed in human emphysematous lung tissues obtained from individuals during lung reduction surgery or necropsy and collagenase activity has been detected in BAL fluid of patients with pulmonary emphysema.[6–8]

In addition to MMP-1, other MMPs could contribute to disruption of the alveolar walls. For example, immunohistochemical and zymographic analysis of COPD lungs have revealed marked increases in expression of collagenase-2 (MMP-8), gelatinase A (MMP-2), and gelatinase B (MMP-9).[7] Alveolar macrophages obtained from BAL of patients with emphysema produce elevated quantities of MMP-1 and MMP-9.[9] Additionally, macrophage metalloelastase (MMP-12) may be important since emphysematous lesions did not develop in metalloelastase-deficient mice in response to long-term exposure to cigarette smoke.[10]

There is currently no therapy for the treatment of lung emphysema. Theoretically, inhibition of the enzymes responsible for the continuous disruption of the pulmonary extracellular matrix could arrest the progression of the disease. Therefore, the aim of this work was to examine the efficacy of the broad-spectrum MMP inhibitor CP-471,474 (Pfizer Global Research and Development; Groton, CT)[11] in the progression of tobacco-smoke induced emphysema in guinea pigs.

Materials and Methods

Experimental Model

Three groups of 16 guinea pigs (Hartley; Harlan Sprague Dawley; Madison, WI) weighing 400 to 450 g were exposed to the whole smoke of 20 commercial cigarettes per day, 5 days per week, for 1 month, 2 months, and 4 months through a whole-body exposure chamber.[5] Eight animals in each group received CP-471,474. Eight age-matched guinea pigs exposed to room air were used as control animals at 1 months, 2 months, and 4 months. The protocol was approved by the Committee on Use and Care of Animals from the National Institute of Respiratory Diseases.

CP-471,474 Dosing Schedule

Guinea pigs were pretreated 30 min prior to smoke exposure with CP-471,474 (20 mg/kg subcutaneously in 20% ethanol/80% polyethylene glycol with molecular weight of 300) or an equivalent volume of vehicle. This treatment was administered once a day during the entire course of the experiment. The range of CP-471,474 plasma levels following subcutaneous injection were 2,020 ng/mL (1 h postdose) to 160 ng/mL (6 h postdose). In addition, CP-471,474 was also supplemented daily by the diet (200 mg/200 g powdered chow), which maintain plasma levels of 180 to 500 ng/mL during feeding times.

Histology

Groups of eight control, tobacco-smoke exposed, and tobacco-smoke plus CP-471,474-treated animals were anesthetized with sodium pentobarbital 50 mg/kg intraperitoneally at 1 month, 2 months, or 4 months of treatment. Lungs were instilled with 10% formaldehyde for histology, immunohistochemistry, and morphometry.[5] The lungs were inflated at an initial pressure of 50 cm H_2O followed by a continuous pressure infusion at 25 cm H_2O.

Semiquantitative Histologic Assessment

The extent and severity of the inflammatory lesions was examined in a blind fashion as described previously for human samples.[12,13] Briefly, the assessment was done on the slide scanned completely in zigzag fashion, at $\times 25$ magnification in approximately 8 to 10 fields. In all cases, two slides from each lung stained with hematoxylin-eosin were analyzed. The percentage of lung inflammation (extent of the lesion) was first determined and twice evaluated by the same pathologist with a 4-month difference. The intraclass correlation coefficient was 0.8 (p < 0.01). The severity of the inflammatory lesions was qualitatively evaluated as none, slight, moderate, or severe (0, 1, 2, and 3, respectively). Some slides were stained with Gomori iron staining since lung burden of iron is increased in cigarette smoking, and sequestered within alveolar macrophages.[14]

Evaluation of Air Spaces by Digital Image

Ten areas of hematoxylin-eosin–stained sections with maximum alveoli present were sampled. Photographs were taken with a 10 $\times$ objective on a Nikon Microphot FXA (Nikon; Melville, NY) interfaced to a Sony DKC-5000 digital camera system (Sony; New York, NY). The images were collected directly into an image analysis program (Optimas 6.5; MediaCybernetics; Carlsbad, CA). A threshold unique to each image was set on the air spaces, and the sample areas were set automatically around that threshold. Each image was inspected for accuracy of the samples, and any bronchioles, blood vessels, and edges around the lung were manually removed from the data set. Approximately the same number of air spaces were counted and measured for all conditions (from 19,765 to 39,922). Area, perimeter, and circularity measurements were exported to Microsoft Excel (Microsoft;

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Redmond, WA). Analysis was done in both Excel and Stat View 5.0.1 (SAS Institute; Cary, NC).

BAL Gelatin Zymography

In a parallel experiment, six animals per group underwent lavage for zymography adjusting BAL fluid samples to contain 5 μg of protein in 15 μL of loading buffer. Samples from control, tobacco smoke-treated, and tobacco smoke plus CP-471,474– treated animals at 2 months and 4 months were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (8.5%) containing gelatin (1 mg/mL) as described elsewhere.[15] For the detection of collagenases, a final concentration of 0.3 mg/mL heparin was included in the loaded samples.[16] Serum-free conditioned media from human lung fibroblasts was used as an MMP-2 marker, conditioned medium from human lung fibroblasts stimulated with fibroblast growth factor-1/heparin was used as an MMP-1 marker,[17] and conditioned from PMA stimulated U2-OS cells was utilized as an MMP-9 marker. Gelatinolytic activities were quantified using image analysis software (ID; Eastman Kodak Company; Rochester, NY), which quantifies the surface and intensity of lysis bands. Results were expressed in relative units.

Western Blot Analysis

BAL samples were concentrated four times, and protein content was determined using BioRad protein assay (Bio-Rad Laboratories; Hercules, CA). Total proteins (20 μg per lane in 15 μL volume) were resolved by 8.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis under reduced conditions and transferred by semi-dry electrophoretic transfer at 15 V for 20 min to nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech; Amersham, UK). Nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline solution Tween buffer (10 mM Tris base, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 1 h, and then incubated at 4°C overnight with goat polyclonal antibody against human MMP-1 (Santa Cruz Biotechnology; Santa Cruz, CA) [1:350] in blocking buffer. After washing with Tris-buffered saline solution Tween buffer, the membrane was incubated with horseradish peroxidase donkey anti-goat IgG 1:5000 for 1 h at room temperature, washed twice, and developed with the Enhanced Chemiluminescence detection system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Radiograph film (X-OMAT; Eastman Kodak Company) was exposed to chemiluminescence reaction for 1 min and then developed. The image was digitalized with a DC-120 digital camera (Eastman Kodak Company) and quantified as mentioned for zymography.

Immunohistochemistry

Immunohistochemical evaluation was performed as described elsewhere.[18] Tissue sections were deparaffinized, rehydrated, and then blocked with 3% H_2O_2 in methanol for 30 min followed by antigen retrieval performed with citrate buffer (10 mM, pH 6.0) for 5 min in a microwave. Tissue sections were treated with an antibody diluent with background reducing components (Dako; Carpinteria, CA) diluted 1/100 in phosphate-buffered saline solution for 45 min, and then incubated with anti–MMP-9 (Chemicon International; Temecula, CA), at 4°C overnight. A secondary biotinylated anti-Ig followed by horseradish peroxidase-conjugated streptavidin (BioGenex; San Ramon, CA) was used according to instructions of the manufacturer. 3-amino-9-ethyl-carbazole (AEC; BioGenex) in acetate buffer containing

0.05% H_2O_2 was used as substrate. The sections were counterstained with hematoxylin. The primary antibody was replaced by nonimmune serum for negative control slides.

Statistical Analysis

Results are expressed as mean ± SD. Comparisons were made using an unpaired Student t test. The Tukey test was used for multiple comparisons. For digital image analysis of air spaces, the Dunnett test was used. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

CP-471,474 was synthesized at Pfizer Global Research and Development (Fig 1). Characteristics and the selectivity of this compound have been reported previously.[11] A summary of its activity against different MMPs is shown in Table 1.

Morphologic Study

Lungs derived from guinea pigs exposed for 1 month to tobacco smoke showed a moderate peribronchiolar, alveolar, and interstitial inflammation (Fig 2, *top left*, *A*) that progressed both in extent and severity as compared to control animals (Fig 2, *bottom right*, *F*). These effects reached a peak after 2 months of exposure, at which time emphysematous lesions were also evident (Fig 2, *top center*, *B*). Interstitial inflammation consisted mainly of lymphocytes and macrophages, although polymorphonuclear cells were also observed. Macrophages were
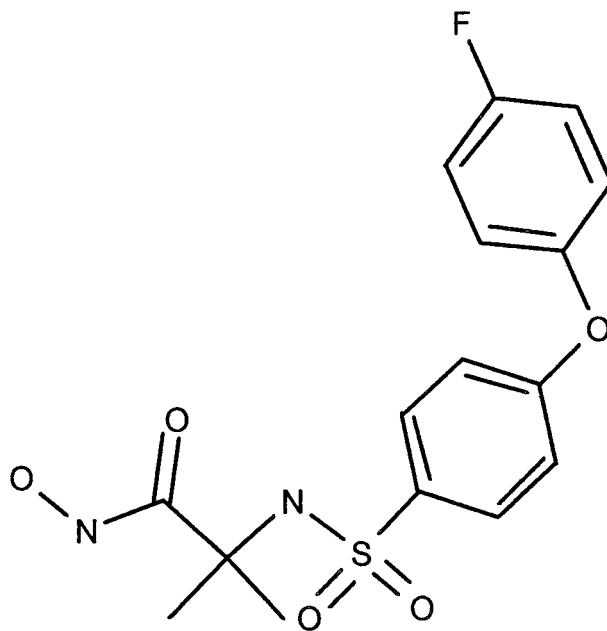

FIGURE 1. Structure of CP-471,474 (2-[4-(4-fluoro-phenoxy)-benzenesulfonylamino]-N-hydroxy-isobutyramide).

Table 1—*Inhibitory Activity of CP-471474 on Various MMPs**

MMP	IC_{50}, ng/mL
1	190
2	0.4
3	5.9
8	3.3
9	4.0
12	0.5
13	0.1

*Fifty percent inhibitory concentration (IC_{50}) values were calculated using recombinant human enzymes and fluorescent peptide substrates, as outlined previously.[11] Each value is the average of a minimum of three independent replicates.

the primary intra-alveolar inflammatory cells and often contained pigmented cytoplasm with finely granular iron-stained particles as shown following 2 months of exposure (Fig 2, *top right*, *C*). After 4 months of tobacco smoke exposure, inflammatory lesions were diminished although emphysematous lesions persisted (not shown). A decrease in the inflammatory response was observed in the lungs of tobacco smoke-exposed animals treated with the MMP inhibitor CP-471,474, following both 1 month and 2 months of cigarette smoke inhalation (Fig 2, *bottom left*, *D*, and *bottom center*, *E*). Semiquantita-

tive analysis of the histopathologic lesions (Table 2) revealed a marginal but significant decrease in the severity of inflammation in tobacco smoke-exposed guinea pigs receiving drug administration for 1 month (p = 0.05). A significant reduction in both extent (p < 0.002) and severity (p < 0.05) of inflammation was observed at 2 months of tobacco smoke exposure plus CP-471,474, corresponding to approximately 80% and 40% of protection. At 4 months, tobacco smoke-exposed animals not receiving CP-471,474 showed a reduction of lung inflammation, and no differences with the treated group were noted.

Emphysematous Changes

Lung parenchymal destruction was evaluated using digital imaging and was expressed as mean area of alveolar air spaces (Fig 3). At 1 month of smoke exposure, there were no differences between the lungs from control guinea pigs and tobacco smoke-injured animals receiving vehicle or CP-471,474. At 2 months and 4 months, however, tobacco smoke-exposed guinea pigs exhibited a significant increase in the average size of alveoli, indicating that the lungs were emphysematous. Lungs derived from smoking animals treated with CP-471,474 demonstrated a statistically significant reduction in the destructive lesions. This effect was most
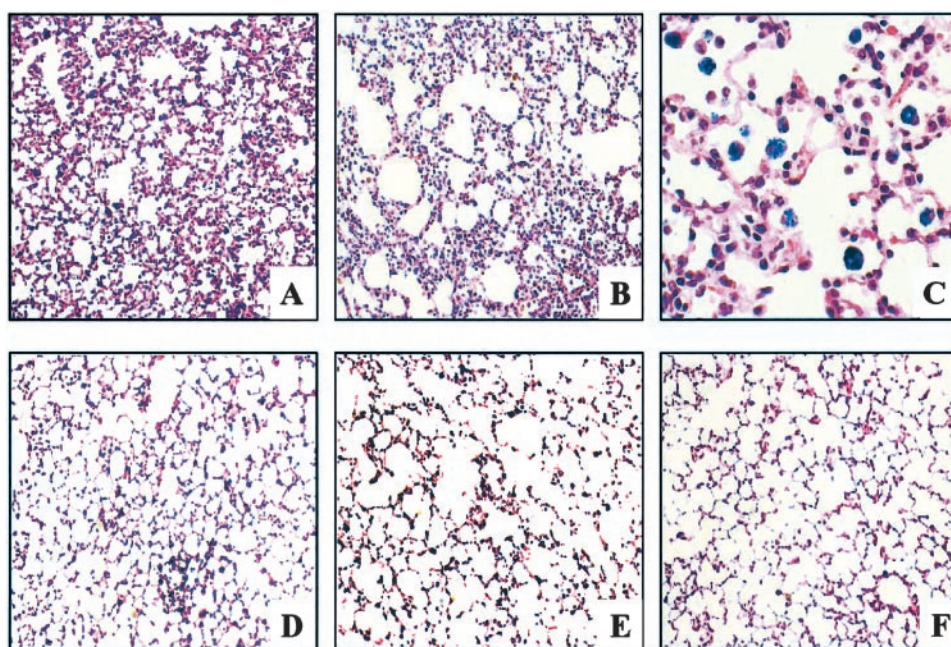

FIGURE 2. Representative photomicrographs of hematoxylin-eosin–stained sections of experimental and control guinea pig lung tissues. *Top left*, *A*: lung after 1 month of tobacco smoke exposure ($\times$ 10). *Top center*, *B*: tobacco smoke-exposed lung after 2 months of exposure ($\times$ 10). *Top right*, *C*: several iron-positive macrophages with a Gomori reaction staining in an 2-month tobacco smoke-exposed guinea pig lung ($\times$ 40). *Bottom left*, *D*: tobacco smoke plus CP-471,474 at 1 month ($\times$ 10). *Bottom center*, *E*: tobacco smoke plus CP-471,474 at 2 months ($\times$ 10). *Bottom right*, *F*: control lung ($\times$ 10).

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Table 2—Semiquantitative Histologic Assessment of Inflammation*

Months of Exposure, No.	Control	Smoking Plus Vehicle	Smoking Plus CP-471,474
Extent of lesion, %			
1	3.8 ± 2.1	19.3 ± 15.9	13.6 ± 6.3
2	2.3 ± 2.3	34.3 ± 18.6†	6.9 ± 7.2
4	2.4 ± 1.8	10.8 ± 6.5	9.6 ± 7.5
Severity of inflammation			
1	0.8 ± 0.3	1.6 ± 0.8	1.0 ± 0.0
2	0.7 ± 0.5	2.4 ± 0.5‡	1.4 ± 0.9
4	0.7 ± 0.6	1.5 ± 0.9	1.7 ± 0.5

*Data are mean ± SD from eight animals per group per time point.
†Significant difference from cigarette smoke exposure plus CP-471,474 (p < 0.002).
‡Significant difference from cigarette smoke exposure plus CP-471,474 (p < 0.05).

evident at 2 months (p < 0.0001), at which time the lung alveoli size in the CP-471,474-treated animals was equivalent to those of control animals, thus showing a 100% protection. At 4 months, the reduction of the size of alveoli in the tobacco smoke-exposed animals treated with CP-471,474 was less marked, reaching only approximately 30% of protection, which was still statistically different from the tobacco smoke-exposed animals not receiving CP-471,474 (p < 0.02). The size of alveolar spaces in lungs from control guinea pigs was consistent throughout the study.

Zymography of BAL

BAL fluid from six animals of the different groups was analyzed by gelatin zymography with heparin. A representative zymogram obtained from three pooled samples of different animals is illustrated in Figure 4, *top*, *A*. BAL fluid samples from tobacco smoke-exposed animals (2 months and 4 months) with and without CP-471,474 showed an increase of both pro–MMP-9 and its active form (lanes 5 to 8) as compared with control animals (lane 4). Densitometric analysis of total MMP-9 (proenzyme plus active form) revealed a 8.4-fold (2 months) and 12.6-fold (4 months) increase in tobacco smoke-exposed animals over control animals. Inhibitor treatment reduced this effect by 50% (Fig 4, *center*, *B*). Pro–MMP-2 and MMP-2 activities were slightly increased from 1.4-fold to 2.6-fold in experimental animals as compared to control animals. No differences were observed between CP-471474-treated or untreated tobacco-smoke exposed animals.

Because MMP-1 is difficult to detect at low levels in conventional gelatin zymography, heparin was used to enhance the signal.[16] MMP-1 produced by human lung fibroblasts stimulated with fibroblast growth factor-1 plus heparin was used as a positive control.[17] MMP-1 in active and latent forms is expected to be in the range of 40 to 60 kd. Bands corresponding to these molecular weights were clearly seen in samples from guinea pigs exposed to
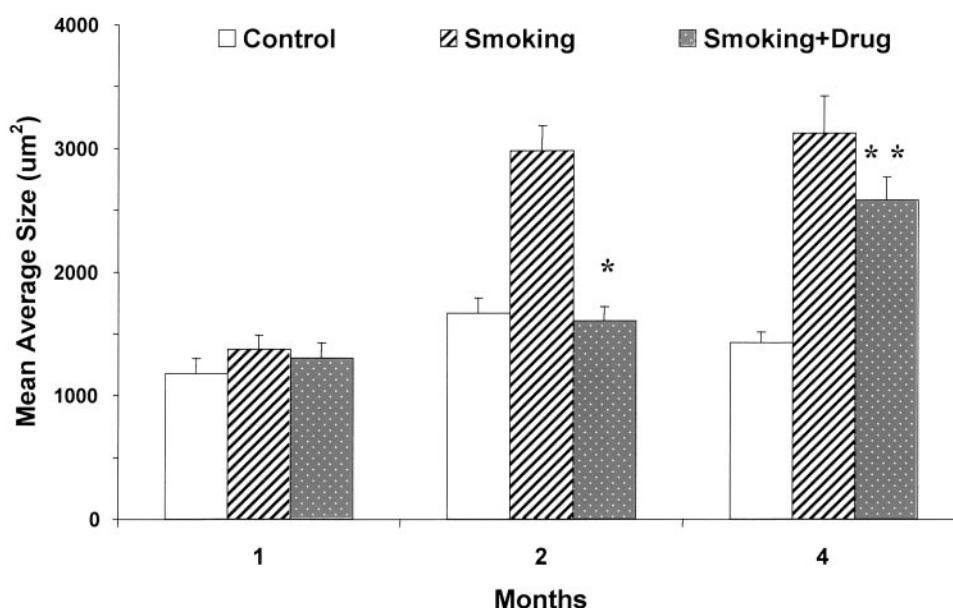

FIGURE 3. Evaluation of airspace changes by digital image analysis in hematoxylin-eosin–stained sections. A significant decrease in the mean size of the emphysematous lesions was observed at 2 months and 4 months in tobacco smoke-exposed guinea pigs receiving CP-471,474 (*p < 0.0001 and **p < 0.02, respectively, compared with the untreated tobacco smoke-exposed animals).
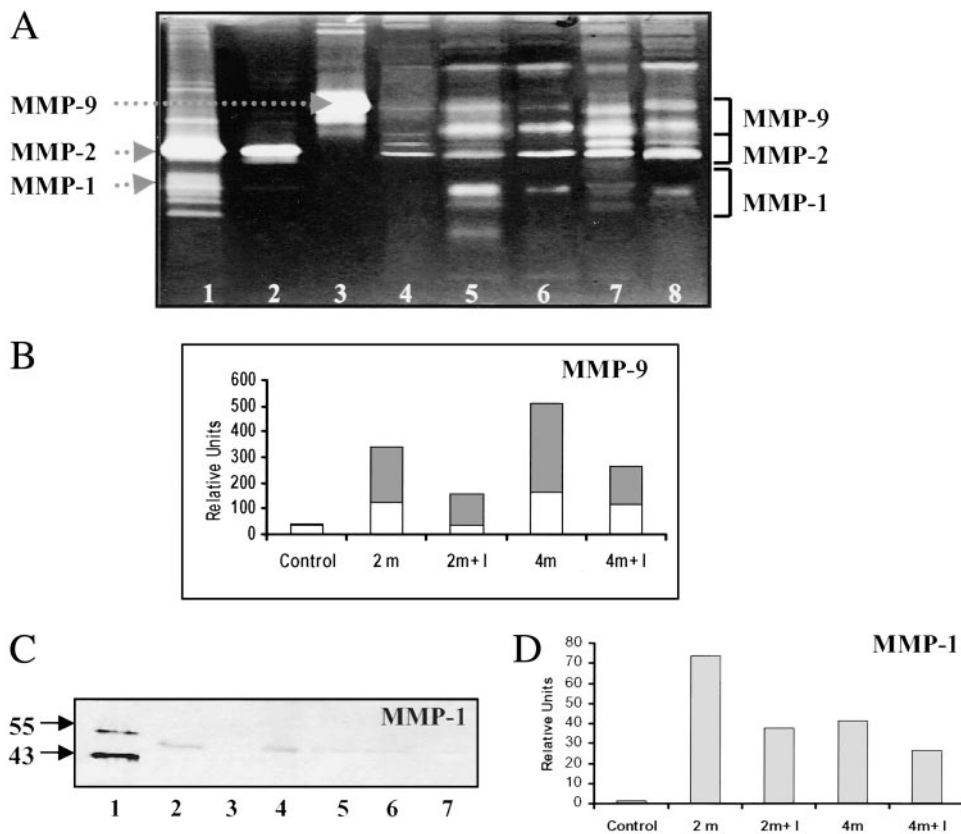
FIGURE 4. Identification of gelatinases and MMP-1 by gelatin zymography enhanced with heparin and Western blot analysis. *Top*, *A*: zymography of BAL fluids. Lane 1 shows conditioned media from lung fibroblasts stimulated with fibroblast growth factor-1. Arrows indicate MMP-1 and MMP-2. Lane 2 shows conditioned media from lung fibroblasts showing MMP-2. Lane 3 shows conditioned media from PMA-stimulated U2-OS cells showing MMP-9. Also shown are control animals (lane 4), 2-month and 4-month untreated tobacco smoke-exposed animals (lanes 5 and 7), and 2-month and 4-month tobacco smoke-exposed animals receiving CP-471,474 (lanes 6 and 8). *Center*, *B*: densitometric analysis of pro–MMP-9 and activated form showed in the zymogram. □ represents proenzyme, and shading indicates activated MMP-9. *Bottom left*, *C*: Western blot analysis of BAL fluids with MMP-1 antibody. Also shown are positive control for MMP-1 (AG770; Chemicon International; [lane 1]) and conditioned media derived from fibroblast growth factor-1/heparin stimulated lung fibroblasts (lane 2); control guinea pigs (lane 3); 2-month and 4-month untreated tobacco smoke-exposed animals (lanes 4 and 6); and 2-month and 4-month tobacco smoke-exposed animals receiving CP-471,474 (lanes 5 and 7). *Bottom right*, *D*: densitometric analysis of Western MMP-1.

cigarette smoke for both 2 months and 4 months (Fig 4, *top*, *A*, lanes 5 and 7). CP-471474 treatment reduced the MMP-1 activity at 2 months and 4 months (lanes 6 and 8).

Western Blot Analysis

To further support the observations related with MMP-1 in zymograms, BAL fluid samples from control, and 2-month and 4-month tobacco smoke-exposed guinea pigs with and without the drug were analyzed by Western blotting. In accordance with the zymogram findings, a more intense immuno-reactive MMP-1 band was observed in the sample from guinea pigs exposed to cigarette smoke for 2 months compared with 4 months (Fig 4, *bottom left*,

C, lanes 4 and 6). CP-471474 treatment reduced 50% MMP-1 protein at 2 months and 4 months (Fig 4, *bottom left*, *C*, lanes 5 and 7). BAL findings from control animals were negative for MMP-1 (lane 2). Densitometric analysis is illustrated in Figure 4, *bottom right*, *D*.

Lung Localization of MMP-9 by Immunohistochemistry

Since an increase in MMP-9 gelatinolytic activity was observed in BAL fluid from tobacco smoke-exposed animals, the cellular source was investigated by immunohistochemistry. Tobacco smoke-exposed guinea pigs killed at 2 months and 4 months with and without CP-471,474 treatment were examined for

the cellular source of lung MMP-9. A strong signal for MMP-9 was observed in cells located in the corners of alveoli, protruding into the alveolar spaces. These cells were putatively identified as type II pneumocytes (Fig 5, *top left*, A). A positive cytoplasmic label was also observed in macrophages, and in bronchiolar epithelium and smooth-muscle cells (Fig 5, *top right*, B). No differences in cell localization were observed among CP-471,474-treated and untreated animals. MMP-9 was barely detectable in lung sections from control animals (Fig 5, *bottom left*, C). Controls using nonspecific antisera showed no reactivity (Fig 5, *bottom right*, D).

DISCUSSION

Among the problems faced in the treatment of pulmonary emphysema are its complex pathogenesis and the irreversible destruction of the alveolar walls. The disease was initially attributed to excessive lung elastolytic activity resulting from either an increase of neutrophil elastase and/or genetic or functional deficiency of α_1-proteinase inhibitor.[2,19]

More recently, however, a number of studies, both in the human disease and experimental models, have supported a pivotal role for MMPs.[4–9] Immunoreactivity for MMP-2 and MMP-9 has been shown to be up-regulated in lung tissues from patients with
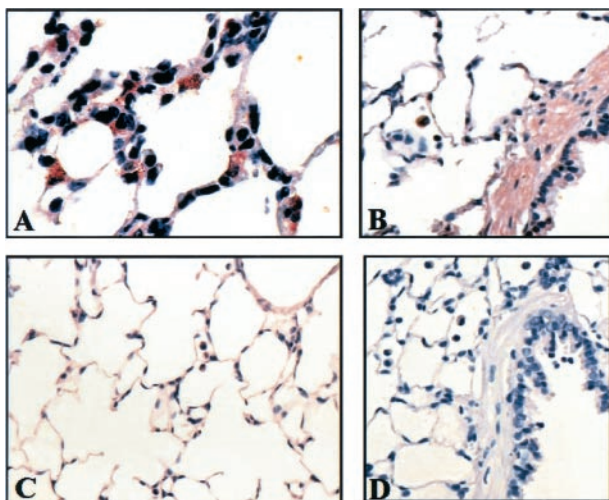

FIGURE 5. Immunohistochemical localization of MMP-9 in lung tissue sections. Immunoreactive gelatinase B was revealed by peroxidase reaction. Sections were lightly counterstained with hematoxylin. *Top left*, A: 2-month tobacco smoke-exposed guinea pig showing positive labeled alveolar epithelial cells (original × 100). *Top right*, B: 4-month tobacco smoke-exposed guinea pig exhibiting signal in free alveolar macrophages, epithelial bronchiolar cells, and smooth-muscle cells around the bronchiole (original × 40). *Bottom left*, C: normal lung (original × 40). *Bottom right*, D: the primary antibody was replaced by nonimmune serum for negative control slides (original × 40).

COPD and emphysema.[7,20] Elastin-degrading activities corresponding to both MMP-2 and MMP-9 but not MMP-12 were elevated in tissue extracts from patients with emphysema compared to smoking control subjects. In an experimental murine model of pulmonary emphysema, it was shown that MMP-9 was up-regulated on overexpression of IL-13 and that genetic ablation of the MMP abrogated this effect.[21,22] Evidence supporting a role for macrophage metalloelastase was found in mice genetically deficient in MMP-12.[10]

In this context, our rationale was that if MMPs were actively participating in the destruction of the alveolar septa, then the use of an MMP inhibitor could represent a potential therapeutic strategy for lung emphysema. Our results demonstrated that the daily administration of the broad-spectrum MMP inhibitor, CP-471474, attenuated both the initial inflammatory response and the emphysematous lesions induced by chronic exposure to cigarette smoke in guinea pigs. However, it is important to emphasize that a spontaneous reduction of the inflammatory response was observed at 4 months in tobacco smoke-exposed animals not receiving CP-471,474, and no differences with the treated guinea pigs were found at this time. Likewise, the attenuation of the emphysematous lesions was evident after 2 months of treatment, coinciding with the peak of the inflammatory response; however, the protective effect of the anti-MMPs drug marginally persisted at 4 months. The reason for these findings is unclear, but it can be speculated that most of the emphysematous lesions at 2 months were related to MMPs secreted by inflammatory cells. The persistence of alveolar septal destruction at 4 months and the lower effect of the drug at this stage may suggest that other enzymes not inhibited by the drug are playing a role.

The ability of CP-471,474 to inhibit lung parenchymal inflammation may be partially explained by the fact that some MMPs, primarily gelatinases, appear to be involved in leukocyte transendothelial migration and subsequent trafficking of leukocytes into the injured tissues. There is a growing body of evidence suggesting that these enzymes participate in lymphocyte and macrophage migration.[23–27] Thus, for example, MMP-2 is induced in T lymphocytes by binding to adhesion molecules on endothelial cells, which in turn facilitates T-cell migration into perivascular tissue.[23] Likewise, IL-2 both induces gelatinase B expression by T cells and enhances their migration across basement membrane *in vitro*.[24] MMP-2 and MMP-9 have been shown to actively participate in the airway infiltration of lymphocytes and eosinophils in a murine model of allergic asthma.[25] In addition, monocytes infected by live, virulent *Mycobacterium tuberculosis* secrete higher amounts of

MMP-9, which seems to facilitate leukocyte migration across the blood-brain barrier. In fact, MMP-9 concentrations per cerebrospinal fluid leukocyte have been found elevated in fatal tuberculous meningitis and in patients with signs of cerebral tissue damage[26]; however, there is some controversy regarding the role of MMP-9 in neutrophil migration.[28–30] In addition, since collagen fragments have been shown to both to be chemotactic and activate alveolar macrophages,[31] it is possible that the compound could also provide direct protection against inflammation by inhibiting the collagenase activity due to MMP-8, MMP-13, and MMP-14.

CP-471474 also reduced the emphysematous changes, mainly those observed at 2 months, suggesting a protective effect on extracellular matrix degradation. The two major extracellular matrix components of the lung interstitial compartment are fibrillar collagens and elastin that are natural substrates for a number of MMPs. In the present study, BAL fluid revealed an upregulation of MMP-9 in tobacco smoke-exposed guinea pigs, an enzyme that may contribute to elastin degradation. MMP-9 was localized in alveolar and bronchiolar epithelial cells as well as in macrophages. The ability of CP-471474 to reduce MMP-9 activity in BAL fluid is consistent with its *in vitro* activity against this enzyme. Additionally, the finding of less MMP-9 activity on the zymograms of the inhibitor treated guinea pigs might be attributed both to fewer inflammatory cells primarily at 2 months, and/or either to the covalently linkage of the inhibitor to the enzyme. In addition, CP-471474 might provide protection against elastin degradation by blocking the MMP component of elastase activity due to MMP-2 and MMP-12. Likewise, since several MMPs are able to degrade α_1-proteinase inhibitor, the neutrophil elastase inhibitor,[32] MMP inhibition might protect against elastin degradation by this enzyme.

CP-471474 is also a potent inhibitor of MMPs able to degrade fibrillar collagens, including MMP-8, MMP-13, and MMP-14, although it is a weak inhibitor of MMP-1 and would not be expected to inhibit this enzyme under the dosing regimen used in this study. In the present study, in addition to MMP-2 and MMP-9, we detected a gelatinolytic band in heparin-enhanced gelatin zymograms of BAL samples putatively corresponding to MMP-1, which appeared to decrease in drug-treated guinea pigs at 2 months, a result that was confirmed by immunoblotting. Thus, although the drug does not directly inhibit MMP-1 under the conditions of this study, it might be indirectly decreasing this enzyme through a reduction of infiltrating macrophages that are the main source of MMP-1 in this model.[5]

In conclusion, the pathogenesis of emphysema likely involves a complex network of excessive MMPs and serine proteases activities in the lung leading to alveolar wall disruption. In this context, new directions for effective therapy may arise from a better understanding of these mechanisms. Our study shows that the use of a synthetic MMP inhibitor reduced the inflammatory response and the emphysematous-derived lesions mainly at 2 months in a cigarette smoke-induced model of lung disease, and suggests that probably other enzymes produced by noninflammatory cells not inhibited by the drug are also playing a role.

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