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COPD



CHEST

Matrix Metalloproteinase-2 Protein in Lung Periphery Is Related to COPD Progression*

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Background: There is increasing evidence that matrix metalloproteinases (MMPs) may contribute to the pathogenesis of COPD, but their role in humans is not completely understood. We performed this study to quantify the expression of MMP-2 in a population of COPD patients at different stages of severity.

Methods: We collected surgical specimens from 46 subjects, as follows: 10 smokers with severe COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stage III-IV); 13 smokers with mild/moderate COPD (GOLD stage I-II); 12 control smokers; and 11 nonsmoking control subjects. We quantified MMP-2 expression in alveolar macrophages, alveolar walls, peripheral airways, and pulmonary arterioles by immunohistochemistry.

Results: In all compartments, MMP-2 expression was increased both in smokers with severe COPD and in smokers with mild/moderate COPD compared to control smokers and nonsmokers (p < 0.05 for all comparisons). Only in alveolar macrophages was MMP-2 expression increased in smokers with severe COPD compared to smokers with mild/moderate COPD (p = c0.002). Moreover, MMP-2 expression was inversely related to values of FEV₁/FVC ratio (p < 0.0001; r = -0.71) and Pao₂ (in millimeters of Hg) [p = 0.005; r = -0.49], and was positively related to emphysema score (p = 0.01; r = 0.65) and residual volume percent predicted (p = 0.04; r = 0.49). A stepwise increase in the total number of alveolar macrophages was observed in the four groups of subjects examined, with the highest value in those with severe COPD.

Conclusion: This study shows that MMP-2 expression in the lung periphery progressively increases as lung function worsens and the degree of emphysema increases. These results suggest that MMP-2 may be a key mediator of the mechanisms leading to lung tissue remodeling and inflammation in patients with severe COPD. *(CHEST 2007; 132:1733–1740)*

Key words: airflow limitation; cigarette smoking; emphysema; extracellular matrix; inflammation

 $\label{eq:abbreviations: GOLD = Global Initiative for Chronic Obstructive Lung Disease; MMP = matrix metalloproteinase; RV = residual volume$

COPD is a major clinical disorder that is usually associated with cigarette smoking. The disease is currently the fourth leading cause of death in the world, and further increases in its prevalence and mortality can be predicted in the coming decades.¹ The defining feature of COPD is a chronic, slowly progressive airflow limitation that results from the combination of pulmonary emphysema and small airways obstruction.

Inflammation, airway remodeling, and parenchy-

mal destruction are established features of COPD.^{2–4} Despite intensive research, the precise mechanisms involved in the development and progression of

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airflow limitation remain unclear. There is evidence that the connective tissue plays an important role in the homeostasis of the respiratory system by modulating the activity of numerous cytokines and growth factors. It has therefore been proposed⁵ that alterations in the connective tissue skeleton may represent an important step in the pathogenesis of emphysema.

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent and calcium-dependent proteolytic enzymes that are mainly derived from macrophages and neutrophils, which can degrade most of the components of the extracellular matrix. Under normal physiologic conditions, they promote remodeling of the extracellular matrix, thus facilitating cell migration, and are involved in immune responses by cleaving the inactive forms of cytokines and chemokines.⁶ Under pathologic conditions, a switch in MMP expression and activity occurs, which may lead to excessive lung inflammation and tissue destruction.^{7,8}

It is widely recognized that many MMPs are expressed in the human lung; however, very few studies have addressed the direct quantification of these proteases in lung tissue. When considering clinical and animal studies together,⁹⁻²¹ there is compelling evidence linking MMP-1, MMP-2, MMP-8, MMP-9, and MMP-12 to emphysema. In particular, MMP-12 is thought to be essential to cigarette smoke-induced pathology in mouse disease,^{9,10} but may not be equally critical in human disease.6,11 MMP-9 has been the focus of interest with several studies^{12–19} reporting an increase of this MMP in patients with COPD. Since MMP-9 is largely produced by neutrophils, which accumulate preferentially in the airway lumen rather than in the tissue, it is better investigated in induced sputum and BAL fluid.

MMP-2 belongs to the same gelatinase family as MMP-9, but it has been less extensively studied in

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COPD patients.^{14–17} MMP-2 is expressed by structural cells (*ie*, endothelial cells and smooth muscle cells)^{22,23} as well as by macrophages and lymphocytes,^{24,25} which are the main cell types infiltrating the lung tissue in COPD patients. In addition to extracellular matrix degradation, MMP-2 may act as a tuner and amplifier of the immune/inflammatory response.^{26,27} In particular, there is evidence that MMP-2 may amplify type 1 responses,²⁸ and, in fact, a predominant type 1 profile has been reported in patients with COPD.^{29,30}

We therefore decided to focus on MMP-2 because, among MMPs, it is the one more likely to influence inflammatory changes occurring specifically in the lung tissue and to have direct effects on lung remodeling. To quantify the expression of MMP-2 in patients with COPD at different stages of disease severity, we examined surgically resected specimens from the following four groups of subjects: smokers with severe COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stage III-IV; n = 10; smokers with mild/moderate COPD (GOLD stage I-II; n = 13); asymptomatic smokers with normal lung function (n = 12); and asymptomatic nonsmoking subjects with normal lung function (n = 11). In these subjects, MMP-2 expression was quantified by immunohistochemistry in alveolar macrophages, in alveolar walls, in peripheral airways, and in pulmonary arterioles.

MATERIALS AND METHODS

Study Population

To quantify the expression of MMP-2, we collected lung tissue from 46 subjects undergoing surgery for appropriate clinical indications (*ie*, lung volume reduction surgery for the treatment of severe emphysema or lung resection for a solitary peripheral carcinoma). The subjects were categorized into the following four groups: smokers with severe COPD (GOLD stage III-IV [*ie*, FEV₁/FVC ratio, <70%; FEV₁, < 50% predicted]; n = 10); smokers with mild/moderate COPD (GOLD stage I–II [*ie*, FEV₁/FVC ratio, <70%; FEV₁, >50% predicted]; n = 13); asymptomatic smokers with normal lung function (n = 12); and asymptomatic nonsmoking subjects with normal lung function (n = 11).

Subjects with COPD did not experience any exacerbation, and all recruited subjects had been free of acute upper respiratory tract infections during the month preceding the study. The subjects were nonatopic (*ie*, they had negative skin test results for common allergen extracts), and had no history of asthma or allergic rhinitis. The study conformed to the Declaration of Helsinki, and informed written consent was obtained for each subject undergoing surgery.

Clinical Assessment

Each patient underwent an interview, pulmonary function tests, ECG, and routine blood tests in the week before surgery. Pulmonary function tests were performed as previously de-

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scribed.³¹ Briefly, they included measurements of FVC, FEV₁, and, in a subset of 15 patients, complete pulmonary function tests (*ie*, functional residual capacity, residual volume [RV], total lung capacity, and diffusing capacity of the lung for carbon monoxide). The predicted normal values used were those from the Communité Europeenne du Carbon e de l'Acier.³² In subjects with normal lung function, inhalation challenge with methacholine was performed, and all subjects had reactivity within the normal range (provocative dose of methacholine causing a 20% fall in FEV₁, > 1.44 mg).

Chest radiographs were obtained within 48 h of the lung function studies. In a subset of 15 patients, scores for emphysema were calculated on the basis of criteria for the evaluation of roentgenographic signs of emphysema, as previously described.³³ A detailed description is present in Web-only material.

Samples Collection and Analysis

Four to six randomly selected tissue blocks were taken from the subpleural parenchyma at surgery (avoiding areas affected by tumor in patients who underwent lung resection for carcinoma). Samples were processed with immunohistochemical methods, and MMP-2 expression was quantified in macrophages, alveolar walls, peripheral airways, and pulmonary arterioles (see Webonly material). Group data were expressed as the mean and SEM or as the median and range where appropriate. Differences between groups were analyzed using the nonparametric Kruskal-Wallis *U* test for morphologic data, and analysis of variance for clinical data. The Mann-Whitney *U* test was performed after the Kruskal-Wallis test, where appropriate. Correlation coefficients were calculated using the Spearman rank method. p Values of < 0.05 were accepted as significant.

Results

Table 1 shows the clinical characteristics of the subjects examined. The complete analysis of clinical and functional parameters is reported in Web-only material.

As for MMP-2 immunoreactivity, a prominent staining was observed in alveolar macrophages and alveolar walls (Fig 1), as well as in peripheral airways, and pulmonary arterioles, where it was mainly associated with smooth muscle bundles. The percentage of MMP-2⁺ macrophages differed significantly among the four groups of subjects examined (p < 0.0001) [Fig 2]. In particular, the percentage of MMP-2⁺ macrophages was increased in smokers with severe COPD when compared to smokers with mild/moderate COPD (p = 0.002), to control smokers (p = 0.01), and to nonsmokers (p = 0.0001). The percentage of MMP-2⁺ macrophages was also increased in smokers with mild/moderate COPD when compared to both smoking and nonsmoking control subjects (p = 0.03and 0.001, respectively).

In alveolar walls, increased MMP-2 expression was observed in smokers with severe COPD compared to both smoking and nonsmoking control subjects (p = 0.008 and 0.02, respectively) [Fig 3]. Smokers with severe COPD tended to have an increased MMP-2 expression even when compared to smokers with mild/moderate COPD, but this difference did not reach the level of statistical significance (p = 0.07). Finally, smokers with mild/moderate COPD had an expression of MMP-2 in alveolar walls similar to that of smoking and nonsmoking control subjects.

In bronchiolar smooth muscle, MMP-2 expression was increased to a similar degree in both smokers with severe COPD (median, 2; range, 0.75 to 3) and smokers with mild/moderate COPD (median, 2; range, 1 to 3) compared to control smokers (median, 0.6; range, 0 to 3; p = 0.03 and 0.01, respectively) and nonsmokers (median, 0.35; range, 0 to 2.2; p = 0.003 and 0.0008, respectively). A similar pattern of expression was also observed in the tunica media of pulmonary arte-

	<i></i>			
Characteristics	Patients With Severe COPD	Patients With Mild/ Moderate COPD	Control Smokers	Nonsmokers
Subjects examined, No.				
Male	8	12	12	5
Female	2	1	0	6
Age, yr	63 ± 3	65 ± 2	63 ± 2	62 ± 4
Smoking history, pack-yr	48 ± 9	47 ± 5	45 ± 7	
FEV ₁ , % predicted	$33 \pm 3^{\dagger}_{\pm}$	73 ± 4 ‡	101 ± 3	106 ± 5
FEV ₁ /FVC ratio, %	$36 \pm 4^{\dagger}_{12}$	65 ± 1 ‡	78 ± 2	79 ± 1
PaO ₂ , mm Hg	$65 \pm 6^{\dagger}_{12}$	80 ± 2	88 ± 2	88 ± 6
PaCO ₂ , mm Hg	40 ± 2	41 ± 1	41 ± 3	38 ± 1
DLCO, % predicted	$30 \pm 5^{\dagger}$	90 ± 15		
RV, % predicted	$170 \pm 17^{\dagger}$	94 ± 15		
Emphysema score	$10 \ (7-15)^{\dagger}$	5(1-5.5)		

Table 1—Clinical Characteristics of the Patients*

*Values are given as the mean \pm SEM or median (range), unless otherwise indicated.

 \dagger Significantly different from patients with mild/moderate COPD (p < 0.005).

 \pm Significantly different from control smokers and nonsmokers (p < 0.0001).



FIGURE 1. Microphotograph showing MMP-2 staining (in red) in lung tissue from a smoker with severe COPD (top left, a), a smoker with mild/moderate COPD (bottom left, b), a control smoker (top center, c), and a nonsmoker (bottom center, d). [original ×630]. Representative examples of MMP-2 staining in peripheral airways (top right, e) and pulmonary arterioles (bottom right, f) from a smoker with severe COPD (original ×200). Arrowheads indicate the specific immunostaining as identified with the monoclonal antibody antihuman MMP-2 (Calbiochem; San Diego, CA).

rioles, with increased MMP-2 expression in both smokers with severe COPD (median, 2.5; range, 1 to 3) and smokers with mild/moderate COPD (median, 2.5; range, 0 to 3) compared to control smokers (median, 1.3; range, 0.25 to 2.5; p = 0.005 and 0.01, respectively) and nonsmokers (median, 1; range, 0 to 2; p = 0.001 and 0.008, respectively).

p= 0.02



p= 0.008 p= 0.07 15 MMP-2+cells/mm 10 5 0 Mild/Moderate Severe COPD Control Non (GOLD III-IV) COPD Smokers Smokers (GOLD I-II)

FIGURE 2. Individual counts for the percentage of MMP-2⁺ macrophages (over total macrophages) in smokers with severe COPD, smokers with mild/moderate COPD, control smokers, and nonsmokers. Horizontal bars represent median values. The p values represent the results of Mann-Whitney *U* test analyses. Overall comparison was made using the Kruskal-Wallis test (p < 0.0001).

FIGURE 3. Individual counts for the number of MMP-2⁺ cells in alveolar walls in smokers with severe COPD, smokers with mild/moderate COPD, control smokers, and nonsmokers. Horizontal bars represent median values. The p values represent the results of Mann-Whitney U test analyses. Overall comparison was made using the Kruskal-Wallis test (p < 0.05).

In none of the compartments examined was MMP-2 expression different between smokers with normal lung function and nonsmoking control subjects. The expression of MMP-2 was not influenced by current smoking status and was not correlated with cumulative smoking history.

The total number of macrophages in alveolar spaces increased stepwise. Indeed, the macrophage score was significantly increased in smokers with severe COPD (median, 2; range, 0.5 to 3) compared to smokers with mild/moderate COPD (median, 1; range, 0.3 to 2; p = 0.01), to control smokers (median, 0.5; range, 0.2 to 1.5; p = 0.0005), and to nonsmokers (median, 0.3; range, 0.2 to 0.5; p = 0.0001). The macrophage score was also significantly increased in smokers (p = 0.02) and nonsmokers (p = 0.0066). Finally, the macrophage score was significantly increased in control smokers compared to nonsmokers (p = 0.04).

Considering all smokers as one group, the percentages of MMP2⁺ macrophages were inversely related to the values of FEV₁ percent predicted (p = 0.0001; r = -0.66) [Fig 4], FEV₁/FVC ratio (p < 0.0001; r = -0.71), and PaO₂ millimeters of Hg (p = 0.005; r = -0.49). Moreover, the number of MMP2⁺ cells in alveolar walls was inversely related to the values of FEV₁ percent predicted (p = 0.05; r = -0.33) and FEV₁/FVC ratio (p = 0.01; r = -0.42). Finally, even in peripheral airways, MMP-2 expression was inversely related to the value of FEV₁/ FVC ratio (p = 0.02; r = -0.43), while in pulmonary arterioles it was related to values of both FEV₁/FVC ratio (p = 0.007; r = -0.46) and PaO₂ millimeters of Hg (p = 0.03; r = -0.35).

When the analysis was limited to smokers with COPD, the only correlations to remain significant



FIGURE 4. Relationship between the values of FEV₁ (percent predicted [% pred]) and the percentage of MMP-2⁺ macrophages in all of the smoking subjects included in the study. Spearman rank correlation yield p = 0.0001 and r = -0.66. The correlation remained significant when only smokers with COPD were considered (p = 0.01; r = -0.53).



FIGURE 5. Relationship between the values of emphysema score and the percentage of MMP-2⁺ macrophages in the subset of patients in whom a radiologic evaluation of emphysema was performed. Spearman rank correlation yielded p = 0.01 and r = -0.65.

were those between the percentages of MMP2⁺ macrophages and FEV₁ percent predicted (p = 0.01; r = -0.53) or FEV₁/FVC ratio (p = 0.003; r = -0.62). In the subset of patients in whom complete pulmonary function test results and radiologic scores of emphysema were determined, the percentages of MMP2⁺ macrophages were positively correlated with the values of residual volume (p = 0.04; r = 0.49) and emphysema score (p = 0.01; r = 0.65) [Fig 5].

DISCUSSION

This study shows that in patients with COPD there is a marked up-regulation of MMP-2 in the lung periphery that is related to the degree of lung function impairment and emphysema. These results suggest that MMP-2 may be a key mediator of the mechanisms leading to lung tissue destruction in patients with severe COPD.

A number of animal models have illustrated the potential role of MMPs in the development of emphysema. In guinea pigs, exposure to cigarette smoke produces emphysematous changes that are associated with increased levels of collagenase messenger RNA, resulting in collagen loss.³⁴ In mice exposed to cigarette smoke, the lack of MMP-12 activity prevents emphysema development and macrophage infiltration.^{9,10} In humans, an analysis of protease production by alveolar macrophages in vitro has shown an increased expression of MMP-9 and MMP-12 in subjects with emphysema.^{12,13,35} However, studies of alveolar macrophages in culture have raised questions about the relationship of these findings to what is actually occurring *in vivo*, a limitation that may be overcome with the direct analysis of lung tissue.

Our study has provided evidence of MMP-2 up-

regulation within the lung tissue in a population of smokers with COPD at different stages of disease severity, extending the findings of previous reports^{14,15} that examined smaller groups of subjects with a narrow range of airflow limitation. Moreover, by performing immunohistochemical analysis on morphologically preserved lung samples, we could quantify MMP-2 in the different compartments of the lung. Unfortunately, we could not directly measure enzyme activity and, therefore, correlate it with MMP-2 content. Of interest, Ohnishi and coworkers,15 who analyzed frozen samples from patients with emphysema, found that the increase in MMP-2 content was paralleled by a significant increase in enzyme activity. The authors¹⁵ suggested that the increased MMP-2 expression, which was related to increased proteolytic activity, may significantly contribute to the destruction of the alveolar walls. This hypothesis is supported by the significant correlation observed in our study between MMP-2 expression and the degree of radiologic evidence of emphysema.

In our study, immunostaining for MMP-2 was more prominent in alveolar macrophages than in all the other compartments examined. At variance with the traditional view,^{36,37} there is recent evidence that macrophages have the ability to produce MMP-2 in *vitro*²⁵ and our observations suggest that they may do so *in vivo* as well. It should be highlighted however that, even if we are assuming that most of the immunoreactivity is due to endogenous MMP-2, we cannot rule out the possibility that some may be due to extracellular MMP-2 sequestered by macrophages. Of interest in our own, as well as in previous reports,^{38,39} the total number of macrophages significantly increases as COPD progresses toward the most severe stages. The combination of this remarkable macrophage expansion and MMP-2 up-regulation must result in a dramatic increase in proteolytic activity, which may account for tissue destruction.

Of note, MMPs may also be involved in orchestrating the amplification of the inflammatory response, which occurs in the lung tissue of smokers with severe COPD.^{4,38,39} For example, it has been suggested²⁸ that an unrestrained expression of MMP-2 may contribute to the aberrant type 1 immune response that promotes tissue destruction in autoimmune diseases. This observation is particularly relevant to the present study, since an increased type 1 response has been reported in patients with COPD,^{29,30} and COPD has been proposed to be an autoimmune disorder in which the aberrant response is driven by the products of proteolytic activity.^{40–42}

It is presently unclear what cell type is driving the degradation of the extracellular matrix in patients with emphysema. In our study, we examined MMP-2 expression by alveolar macrophages; however, we

cannot rule out the possibility that other inflammatory cells may also be involved. In particular, lymphocytes, which are increased in the alveolar walls of smokers with COPD,43,44 and neutrophils, which are abundant in patients with more advanced stages of the disease,⁴⁵ can secrete a wide array of proteases. Finally, even lung structural cells may secrete MMPs; indeed, in our study the great majority of the MMP-2 expression within peripheral airways and pulmonary arterioles was not observed in inflammatory cells (as it was in the alveolar compartment), but was mainly associated with smooth muscle cells. Increased smooth muscle mass is one of the most important pathologic lesions observed in the peripheral airways of smokers with COPD, where it may contribute to airway wall thickening and, therefore, to the development of airflow limitation. Evidence has been gathered²³ that human airway smooth muscle cells can secrete MMP-2 and that the inhibition of this MMP reduces airway smooth muscle proliferation. These observations support the hypothesis that MMP-2 may have an important role in the progression of airflow limitation. Conversely, whether the presence of airway obstruction itself could influence the retention of MMP-2 in the airway wall still remains to be investigated.

It is noteworthy that, in our study, although tobacco smoking enhanced the recruitment of macrophages to the alveolar spaces, it did not affect MMP-2 expression. Indeed, this protease was not up-regulated in smokers with normal lung function compared to nonsmoking control subjects. Furthermore, the expression of MMP-2 was not influenced by current smoking status or cumulative smoking history, suggesting that smoking per se did not influence MMP-2 expression. This scenario is different from that observed in animal models, where exposure to tobacco smoke was sufficient to induce MMPs, and raises the hypothesis that up-regulation of MMP-2 in humans is not a response to cigarette smoking in general, but is strictly related to the presence of COPD.

Of interest, it has been shown that MMP-2 is involved in the pathogenesis of lung cancer, which is a frequent comorbidity in patients with COPD,^{46,47} by promoting tumor invasion and metastasis.^{48,49} While it is true that the presence of lung cancer in some of our patients may have influenced the results, we tried to avoid this bias by collecting lung tissue from areas not affected by tumor and by having included subjects with lung cancer in the control groups. Furthermore, since the highest MMP-2 expression was observed in the group without concomitant cancer (*ie*, smokers with severe COPD undergoing lung volume reduction surgery), we feel rather confident that our findings of an increased MMP-2 expression in COPD patients are valid.

Another potential limitation of our report is the low power of the study because of the small number of subjects in each group. Although we cannot exclude the possibility of a type 2 statistical error (*ie*, the inability to detect subtle differences between groups), we are rather confident that the significant differences observed were not influenced by the relatively small size of our groups. Moreover, we should acknowledge that, in our study, some of the MMP-2 content may have been masked by binding to other proteins, such as tissue inhibitors of metalloproteinases. However, previous reports⁵⁰ as well as our experience in Western blot experiments, suggest that anti-MMP-2 antibodies identify not only free MMP-2, but also MMP-2/tissue inhibitor of metalloproteinase complexes, which are not completely dissociated by sodium dodecyl sulfate.

In conclusion, in patients with COPD, MMP-2 expression in the lung periphery is progressively enhanced as lung function worsens and the degree of emphysema increases. These observations suggest that MMP-2 may have a crucial role in the development and progression of COPD, possibly by promoting parenchymal destruction and the remodeling of airways and vasculature.

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