ORIGINAL ARTICLES

Markers of Airway Remodeling in Induced Sputum From Healthy Smokers

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OBJECTIVE: Airway remodeling in chronic obstructive pulmonary disease (COPD) has been linked to the equilibrium between matrix metalloproteinase (MMP) 9 and its inhibitor, tissue inhibitor of metalloproteinase (TIMP) 1. However, that equilibrium has not been analyzed in healthy smokers. The aim of this study was to assess the equilibrium between MMP-9 and TIMP-1 in induced sputum from healthy smokers, healthy nonsmokers (controls), and patients with COPD.

PATIENTS AND METHODS: Samples of induced sputum were obtained from 35 individuals: 12 healthy smokers, 12 controls, and 11 patients with COPD. In each sample, a differential cell count was performed and enzyme-linked immunosorbent assays were used to analyze the concentrations of MMP-9 (total and active fraction) and TIMP-1.

RESULTS: Compared with controls, healthy smokers were found to have a higher mean (SD) concentration of total MMP-9 (273 [277] ng/mL vs 128 [146] ng/mL) and a higher ratio of total MMP-9 to TIMP-1 (0.16 [0.14] vs 0.08 [0.06]). However, the ratio of active MMP-9 to TIMP-1 was similar in the 2 groups. Samples from patients with COPD had the highest concentrations of total MMP-9 (477 [262] ng/mL) and active MMP-9 (178 [126] ng/mL) and the lowest concentrations of TIMP-1 (1.044 [1.036] μg/mL). When all groups were considered together, there was an inverse relationship between the MMP-9/TIMP-1 ratio and the forced expiratory volume in the first second (FEV_1). The relationship between the active MMP-9/TIMP-1 ratio and FEV_1 was even stronger, and the relation of both ratios with FEV_1 became stronger still when smoking was considered.

CONCLUSIONS: Healthy smokers had a higher concentration of total MMP-9 and that concentration was correlated with their exposure to tobacco smoke. Maintenance of the active MMP-9/TIMP-1 ratio in healthy smokers may explain the absence of progressive airway obstruction. Measurement of active MMP-9 concentration could be useful for assessment of airway remodeling.

Key words: *Matrix metalloproteinase 9. Airway remodeling. Healthy smokers.*

Marcadores de remodelado bronquial en el esputo inducido de fumadores sanos

OBJETIVO: El remodelado bronquial en la enfermedad pulmonar obstructiva crónica (EPOC) se ha relacionado con el equilibrio entre la metaloproitenasa (MMP) 9 y su inhibidor, el inhibidor tisular de MMP tipo 1 (TIMP-1). Dicho equilibrio no se ha analizado en fumadores sanos. Nuestro objetivo ha sido estudiar dicho equilibrio en el esputo inducido de fumadores sanos respecto a sanos no fumadores (controles) y pacientes con EPOC.

PACIENTES Y MÉTODOS: Se obtuvieron 35 muestras de esputo inducido, de las que 12 provenían de fumadores sanos, otras 12 de controles y 11 de pacientes con EPOC. Se estudiaron la celularidad de las muestras y la concentración de MMP-9 (total y fracción activa) y TIMP-1 mediante enzimoinmunoanálisis.

RESULTADOS: Los fumadores sanos mostraron mayor concentración media (± desviación estándar) de MMP-9 total (273 ± 277 ng/ml) y una ratio mayor (0,16 ± 0,14) que los controles (128 ± 146 ng/ml y 0,08 ± 0,06, respectivamente). Sin embargo, la ratio MMP-9 activa/TIMP-1 fue equiparable en ambos grupos. Los pacientes con EPOC mostraron los valores más altos de MMP-9 total (477 ± 262 ng/ml) y activa (178 ± 126 ng/ml) y los más bajos de TIMP-1 (1.044 ± 1.036 ng/ml). Globalmente, la ratio mostró una relación inversa con el volumen espiratorio forzado en el primer segundo. Dicha relación fue aún superior con la MMP-9 activa y con el grado de tabaquismo.

CONCLUSIONES: Los fumadores sanos presentaron una mayor concentración de MMP-9 total en relación con el grado de exposición tabáquica. Una ratio MMP-9 activa/TIMP-1 conservada en fumadores sanos podría explicar la ausencia de obstrucción progresiva de la vía aérea. La medida de la MMP-9 activa puede ser útil en la determinación del remodelado bronquial.

Palabras clave: *Metaloproteinasa 9 (MMP-9). Remodelado bronquial. Fumadores sanos.*

Introduction

Mortality due to smoking-related respiratory disease, not including lung cancer, has increased in recent years to become a significant health problem.[1] However, the effects and time course of airway pathology caused by exposure to tobacco smoke are still not known with any

certainty. Various studies have demonstrated that exposure to tobacco smoke causes cellular oxidative stress and the release of inflammatory mediators in the airways of healthy subjects, and that these effects can be both acute and chronic.[2,3] In susceptible individuals, these processes would lead to structural changes in the small airway that would cause progressive loss of lung function. The appearance of chronic progressive airflow limitation in part reflects the lung remodeling that characterizes chronic obstructive pulmonary disease (COPD).[4] Some enzymes, such as matrix metalloproteinase (MMP) 9, have been linked to COPD, in which an increasing number of studies have described a local overproduction of MMP-9 and an altered ratio between this enzyme and its inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1).[5,6] The usefulness of this enzyme as a marker of remodeling has also been described.[7,8]

MMPs—in particular MMP-1, MMP-2, and MMP-9—are collagenases that regulate the homeostasis of the lung matrix, which is made up principally of collagen and other proteins.[9,10] The direct effect of tobacco smoke on these enzymes is poorly understood. Finlay et al[11] were the first to describe an increase in the levels of MMP-9 associated with COPD, finding elevated levels of the enzyme in bronchoalveolar lavage fluid from a subgroup of individuals within a small sample of smokers. More recently, Kang et al[12] described increased expression of MMP-9 seen in bronchial biopsies from smokers compared with nonsmokers; that increase was correlated with the number of cigarettes smoked. It has recently been suggested that the equilibrium between these proteases and their inhibitors would better reflect or display a stronger correlation with the intensity of the disease.[7] Very little data is available on TIMP-1 in smokers with which to assess the effect of smoking on structural changes.

The aim of this study was to analyze the ratio between MMP-9 and TIMP-1 in samples of induced sputum from healthy smokers and healthy nonsmokers. A third group of subjects with pulmonary disease caused by smoking (COPD group) was included as a reference. In addition, the fraction of active MMP-9 was analyzed and compared with results provided by analysis of the total concentration of the enzyme.

Patients and Methods

Selection of Patients

A total of 37 volunteers were recruited: 26 healthy subjects, of whom 13 were smokers and 13 nonsmokers, and 11 patients with COPD. Healthy nonsmokers were mostly volunteers from among hospital staff. Healthy smokers were recruited from subjects who had registered with the smoking cessation clinic but had yet to begin the cessation program. Patients with COPD were consecutively recruited in the outpatient pneumology department. All patients gave signed informed consent for inclusion in the study, which was approved by the hospital's ethics committee. Volunteers were

considered to be healthy nonsmokers if they had no prior history of significant respiratory disease, their results for forced spirometry and bronchodilator test were within the limits of reference values from the Spanish Society of Pulmonology and Thoracic Surgery (SEPAR),[13] and they had never smoked. Volunteers were considered healthy smokers if they met all of the above criteria, except the last, and were also active smokers.

Criteria for inclusion in the COPD group were as follows: chronic airflow limitation according to the definition of the Global Initiative for Chronic Obstructive Lung Disease[1] and a negative bronchodilator test (<12%) in the stable phase (recorded in the patient's chart). All recruited patients continued with their normal treatment, which had not changed for at least 1 month prior to the study. Exclusion criteria for patients with COPD were exacerbation of the disease or the presence of extensive infiltrates, bronchiectasis, or pleural inflammation and thickening in chest radiographs, severe alcoholism, cancer, inability to cooperate, or severe heart, liver, or kidney failure. Table 1 shows the clinical characteristics and lung function for all groups.

Study Design and Methods

A cross-sectional, observational study was designed to compare the 3 groups of subjects: healthy nonsmokers (control group), healthy smokers, and patients with COPD. In all subjects a history was taken and a physical examination performed, and sputum was induced according to the method described below. Samples were sent to the cytology laboratory for immediate processing. The supernatant was frozen at –80ºC and sent to the biochemistry laboratory for processing.

Sputum induction. Sputum was induced in healthy subjects according to a slightly modified version of the technique described by Belda et al.[14,15] A 3% saline solution was nebulized for 7 minutes, 10 minutes after administration of salbutamol (200 µg) through a spacer chamber. If sputum was not obtained, the concentration of the saline was progressively increased (4%, 5%, etc) every 7 minutes. In the case of patients with stable COPD, isotonic saline was used initially. If it was well tolerated and a valid sputum sample was not obtained, nebulization was continued for 7 minutes with hypertonic saline at a concentration of 3%, and this was repeated again at the same concentration if no result was obtained. Droplets with a mean diameter of 7 µm were produced at a rate of 3 mL/s using an ultrasound nebulizer (Omron NE U07, Omron Healthcare Europe, Hoofddorp, Holland). Spirometry was performed according to SEPAR guidelines[13] as a safety measure at the beginning of each inhalation period and at the end of sputum induction. The patient was then instructed in the best maneuvers to achieve effective expectoration. All patients rinsed their mouth and blew their nose to limit, as far as possible, contamination of the induced sputum, which was collected in a sterile container.

Sputum processing. Within a maximum of 2 hours, mucus plugs were separated from saliva in the expectorated samples. Mucus plugs were homogenized in a volume of fresh 0.1% dithiothreitol equivalent to 4 times the weight of the plugs. Later, an equal volume of phosphate buffered saline was added and the solution was filtered through a 41 µm nylon mesh. A 10 µL aliquot of this solution was taken for analysis of cell viability and the remainder was centrifuged for 10

minutes at 1500 rpm to separate the cell pellet from the supernatant. The cell pellet was spread on a slide for subsequent analysis and the supernatant was frozen at –80ºC.

Differential cell count. Cell viability was assessed in unfixed cells by trypan blue staining and hemocytometry. A differential cell count of neutrophils, macrophages, eosinophils, basophils, lymphocytes, and bronchial epithelial cells was then performed using the May-Grünwald-Giemsa stain.

Analysis of supernatants. The concentrations of total and active MMP-9 and of TIMP-1 were determined by enzyme-linked immunosorbent assay (ELISA) using the Biotrak ELISA system (reference RPN 2614, RPN 2634, and RPN 2611, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Statistical Analysis

Statistical calculations were performed using SPSS version 10.0 (1999). Data are shown as means (SD). Comparisons of the means between the 3 groups were made using the Kruskal-Wallis nonparametric test for independent samples and pairwise comparisons were made using the Mann-Whitney U test. The Spearman linear correlation coefficient was used to assess correlations between variables.

Results

An inadequate sample was obtained from 2 of the 37 recruited subjects. The anthropometric and functional characteristics of the 35 subjects who were finally included are shown in Table 1.

Analysis of Total and Active MMP-9

Concentrations of MMP-9 in sputum samples are shown in Table 2. Patients with COPD had significantly higher concentrations of total and active MMP-9 than healthy smokers or control nonsmokers (Figure 1). Likewise, healthy smokers had higher concentrations of total MMP-9 than the control group; however, this result was not statistically significant. Within the group of smokers, when total MMP-9 was analyzed only for subjects whose cigarette consumption was at least 20 packet-years (n=9), the initially observed tendency achieved statistical significance (F=7.65; $P<.002$); furthermore, a positive correlation was observed between the concentration of MMP-9 and the extent of smoking habit ($r=0.63$; $P<.001$).

The concentration of active MMP-9 was similar in both groups of healthy individuals. The results for the active form of MMP-9 were generally less variable than
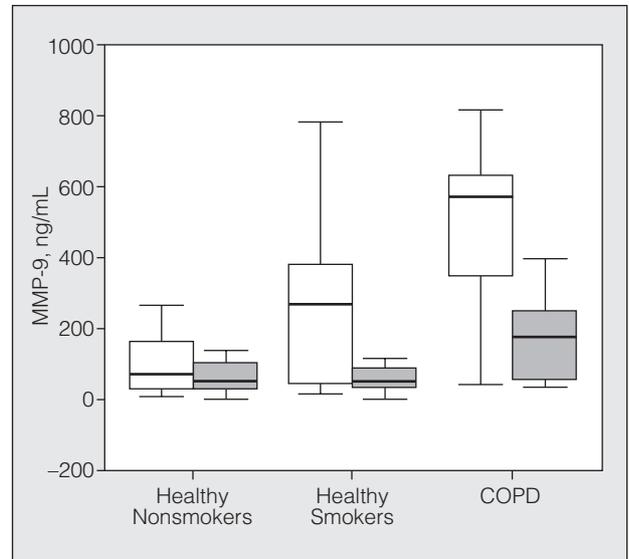

Figure 1. Distribution of total and active MMP-9 in the different study groups. White bars show total MMP-9 and shaded bars active MMP-9. COPD indicates chronic obstructive pulmonary disease.

those for total MMP-9. The nonactive fraction (essentially the proform and MMP-9 bound by inhibitors) differed between the different groups and showed a gradual increase in concentration from control subjects to patients with COPD. The concentration of the nonactive fraction was also correlated with the extent of smoking habit ($r=0.57$; $P=.001$).

Analysis of TIMP-1

The concentration of TIMP-1 displayed a progressive reduction from healthy subjects to patients with COPD; however, the differences were not statistically significant.

MMP-9/TIMP-1 Ratio

The equilibrium between markers of airway remodeling can be defined by the ratio of their concentrations. Patients with COPD displayed a higher ratio of total MMP-9 to TIMP-1 than healthy subjects. The group of healthy smokers had an intermediate MMP-9/TIMP-1 ratio between that of the control and COPD groups (Figure 2). When the active MMP-9/TIMP-1 ratio was measured a different pattern was found. Healthy individuals—smokers or nonsmokers—displayed similar ratios, while the COPD group continued to display higher ratios (Table 2).

TABLE 1
Clinical Characteristics of the Study Subjects*

	Age, Years	Sex, M/W	FEV$_1$, L	FEV$_1$, % of Theoretical	Smoking Habit, Pack-Year	Active Smokers
Healthy controls	32 (3)	6/6	3.9 (0.9)	106 (11)	0	0
Healthy smokers	34 (8)	5/7	4.5 (1.1)	103 (11)	18 (9)	12/12
COPD	61 (11)	9/2	1.1 (0.7)	38 (21)	54 (22)	5/11

*Data are shown as the mean (SD) except for sex, which is shown as the ratio of men to women.
COPD indicates chronic obstructive pulmonary disease; M, men; W, women; FEV$_1$, forced expiratory volume in the first second.
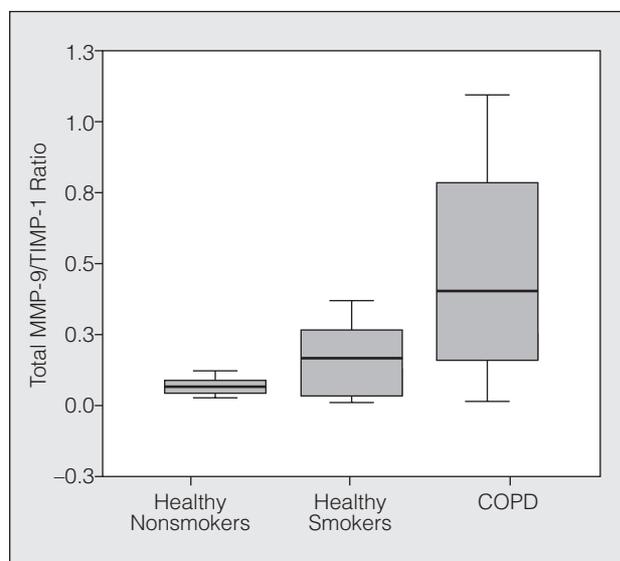
Figure 2. Distribution of the ratio of MMP-9 to TIMP-1 in the different study groups. COPD indicates chronic obstructive pulmonary disease.

Relationship Between Markers of Remodeling and Bronchial Obstruction

In all groups, there was a significant inverse correlation between the MMP-9/TIMP-1 ratio and the forced expiratory volume in the first second (FEV_1) expressed as a percentage of the theoretical value (r=–0.3; P=.02).

When we analyzed the COPD group in isolation, the correlation was stronger (r=–0.56; P=.002), and an even stronger correlation was observed when the extent of smoking habit was considered (r=–0.81; P<.0001). Correlations between the MMP-9/TIMP-1 ratio and FEV_1 were stronger than those found between MMP-9 concentration and FEV_1.

Relationship Between Markers of Remodeling and Bronchial Inflammation

The differential cell count did not display significant differences between healthy smokers and the control group (Table 2). Patients with COPD did display significant differences in the number of neutrophils and macrophages compared with the other 2 groups (Table 2).

The number of polymorphonuclear leukocytes was correlated with the concentration of both active MMP-9 (r=0.5, P=.003) and total MMP-9 (r=0.68, P<.001). The correlation between total MMP-9 concentration and the number of polymorphonuclear leukocytes increased with increases in the extent of smoking habit (r=0.82; P<.001).

Discussion

To our knowledge, this is the first study to analyze the concentrations of an MMP and its inhibitors in induced sputum from smokers without airway obstruction, compared with healthy controls and patients with COPD. The concentration of MMP-9 in sputum was higher in healthy smokers than in the control group. The highest concentrations of MMP-9 were found in patients with COPD. In contrast, the concentration of TIMP-1 was lower in that group. The equilibrium or ratio between MMP-9 and its inhibitor displayed a progressive increase from the healthy control group to the group of patients with COPD.

In this study, patients with COPD presented elevated concentrations of MMP-9, a finding that is consistent with the results of previous studies analyzing the presence of MMPs in the lungs of patients with COPD.[16,17] In addition, healthy smokers presented a higher concentration of MMP-9 than control subjects. We only found 2 studies in the literature that addressed the presence of MMP-9 in healthy smokers. Kang et al[12] used immunochemical methods to analyze homogenates of lung biopsies and observed an increase in the level of MMP-9 in smokers. Finlay et al[11] analyzed bronchoalveolar lavage fluid in a group of healthy smokers and found increased levels of MMP-9 in some of the subjects. In addition, Lim et al[18] performed in vitro studies in which they found that alveolar macrophages from smokers released higher concentrations of MMP-9 than those from healthy subjects. The data obtained in this study revealing an increase in the concentration of MMP-9 in induced sputum from healthy smokers and COPD patients are consistent with the results of those earlier studies. However, in this study a simple technique was used that is available in any laboratory and that allows samples to be obtained noninvasively.[19]

TABLE 2
Cytology and Markers of Remodeling in Induced Sputum Samples*

	Total No. of Cells, ⬚10⁶/g	Viability, %	PMN, %	Macrophages, %	Eosinophils, %	MMP-9, ng/mL	
						Total	Active
Control	5990 (4363)	59 (25)	38 (15)	59 (15)	0.2 (0.4)	128 (146)	75 (66)
Healthy smokers	9914 (9301)	69 (14)	53 (18)	40 (17)	1.7 (3.1)	273 (277)	88 (106)
COPD	20 257 (25 612)	72 (35)	82 (18)† (P<.001)	14 (17)† (P=.001)	1.1 (0.8)	477 (262)† (P=.015)	178 (124)† (P=.032)

*Data are shown as means (SD).
PMN indicates polymorphonuclear leukocytes; MMP-9, matrix metalloproteinase 9; TIMP-1, tissue inhibitor of metalloproteinase 1.
†Statistically significant compared with the other groups.

Few studies have analyzed the concentration of TIMP-1 and the results obtained have been inconsistent. In our study, the concentrations of TIMP-1 found in patients with COPD were lower than in the group of healthy smokers, and the concentrations in healthy smokers were lower than in the control group. These results are consistent with the results of Kang et al[12] using immunochemical methods in biopsy material, where the level of TIMP-1 was found to be lower in smokers and, likewise, further reduced with the appearance of airway obstruction. Russell et al[20] observed lower synthesis of TIMP-1 following in vitro stimulation of alveolar macrophages from patients with COPD than in cells from healthy subjects. In contrast to the results obtained in this study, Vignola et al[8] reported that the production of TIMP-1 in sputum samples from patients with COPD was higher than in healthy subjects. This difference may be accounted for by differences in sputum processing that would affect the results obtained from analysis of the supernatant.[21] Furthermore, the patients included in the study by Vignola et al had less severe airway obstruction (median FEV_1 as a percentage of reference, 71%) and differences in the extent of smoking habit.

The few studies to have analyzed the ratio between these markers agree that it is more representative of the extent of remodeling than any of the measurements performed separately.[8,22] In the present study, the ratio of MMP-9 to TIMP-1 was higher in patients with COPD than in controls, and was inversely correlated with FEV_1. This lower ratio is indicative of a reduced availability of TIMP-1 per molecule of MMP-9 and, therefore, a greater predisposition to tissue damage,[23,24] which would favor remodeling.

When the ratio of active MMP-9 to TIMP-1 was calculated, the group of patients with COPD continued to display a higher ratio compared with the other groups. However, that ratio in healthy smokers was reduced to a level similar to that found in the control group of nonsmokers. This may indicate that healthy smokers, despite the differences in the total concentrations of the enzymes, maintain the same equilibrium as the control group. This would protect individuals against the appearance of airflow obstruction.

The active form of MMP-9 in samples of lung tissue has generally been studied semiquantitatively by zymography.[25] Veernoy et al,[26] who performed a quantitative estimation of the active form using specific immunocapture assays, reported that this measure may be more appropriate since it is the active fraction that is ultimately involved in destruction of tissue. The results obtained in our study using ELISA to analyze active MMP-9 concentration were more homogeneous than those obtained for total MMP-9 concentration and were more tightly correlated with indicators of bronchial obstruction.

Analysis of the effect of smoking on markers of bronchial remodeling revealed a strong correlation between the extent of smoking habit and the expression of MMP-9. This suggests that smoking could act as a direct stimulus in the pathogenesis of bronchial remodeling.[27]

In summary, the results of this study show that healthy smokers present intermediate values for the concentrations of MMP-9 and TIMP-1 between those found for patients with COPD and for healthy nonsmokers and that those values are correlated with the number of cigarettes smoked. The increased presence of enzymes in the airway could play a role in the histologic changes seen in smokers. However, the balance between these proteases and their inhibitors in healthy smokers is similar to that of healthy nonsmokers. This finding may explain the absence of sufficient changes to cause chronic airflow obstruction. Further studies, preferably longitudinal, should determine whether these changes are linked to disease prognosis.

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TIMP-1, ng/mL	Ratio MMP-9/TIMP-1	Ratio Active MMP-9/TIMP-1
1579 (1015)	0.08 (0.06)	0.05 (0.04)
1927 (1306)	0.16 (0.14)	0.06 (0.06)
1444 (1036)	0.56 (0.6)† (P=.017)	0.17 (0.13)† (P=.005)

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