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Cigarette Smoke Induces Activation of Polymorphonuclear Leukocytes

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Abstract

Introduction Cigarette smoking is a major risk factor for chronic obstructive pulmonary disease (COPD). Exposure to cigarette smoke may stimulate inflammatory response and activate polymorphonuclear leukocytes (PMN) thus resulting in secretion of cellular proteases. The aim of our study was to explore the effect of cigarette smoke extract (CSE) on the release of matrix metalloproteinase-9 (MMP-9) from PMN.

Methods The study included 23 patients with stable COPD and 9 healthy controls. PMN were isolated from blood of all participants and exposed to 4% CSE or basal culture medium (0% CSE) for 20 h. MMP-9 concentration in PMN culture media was measured using the ELISA method.

Results Exposure of PMN to 4% CSE did not cause cytotoxic effects, as determined by no changes in lactate dehydrogenase (LDH) activity in PMN culture media when compared to untreated PMN ($P=0.689$). In basal conditions, PMN of COPD patients released significantly more MMP-9 compared with PMN of healthy controls ($P=0.016$). However, concentration ratio of MMP-9 released from PMN exposed to 4% CSE or 0% CSE of each participant was significantly higher for healthy subjects than for COPD patients ($P=0.025$).

Conclusion Cigarette smoke induces activation of PMN in healthy controls. However, chronically activated PMN in COPD patients could not be further stimulated by in vitro exposure to CSE. Constantly raised amount of MMP-9 released into the tissues may be involved in the degradation of extracellular matrix in the lungs as seen in COPD patients.

Keywords Chronic obstructive pulmonary disease · Cigarette smoke · Matrix metalloproteinase-9 · Polymorphonuclear leukocytes

Abbreviations

COPD Chronic obstructive pulmonary disease
CSE Cigarette smoke extract

FEV₁ Forced expiratory volume in 1 second
FVC Forced vital capacity
GOLD Global initiative for chronic obstructive lung disease
IL Interleukin
LDH Lactate dehydrogenase
MMP Matrix metalloproteinase
PMN Polymorphonuclear leukocytes

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common respiratory disease characterized by persistent airflow limitation associated with abnormal chronic inflammatory response to noxious particles and gases. Chronic inflammation may induce parenchymal destruction and disrupt normal repair and defence mechanisms [1]. Pathologic changes and clinical symptoms in COPD result from interaction of chronic exposure to the various risk factors, such as cigarette

smoke and other inhalatory pollutants, and genetic predisposition [2].

Many studies show association between pathological process in COPD and dysregulation of protease activity, either because of the higher activity of proteases or dysfunction of protease inhibitors. Proteases are released from various cells (activated macrophages, neutrophils, epithelial cells, fibroblasts) in response to inflammation, the presence of cytokines or external factors, such as cigarette smoke. Once released, proteases may cause a degradation of extracellular matrix in the lungs, leading to the production of peptide fragments that have a chemotactic effect on leukocytes. This creates a vicious cycle that leads to the progression of the disease into a chronic form [3, 4].

Matrix metalloproteinase (MMP)-9 plays an important physiological role in lung extracellular matrix remodeling and repair. Several studies showed increased levels of MMP-9 in alveolar macrophages, neutrophils, sputum, and lung epithelial cells of COPD patients and healthy smokers [5–8]. In addition to increased concentrations of MMP-9 in sputum of patients with COPD, Beeh et al. [9] found a correlation of MMP-9 with the number of neutrophils in sputum and negative correlation of MMP-9 with a FEV₁/FVC ratio. It is known that stimulation of neutrophils with different agents may lead to the rapid degranulation and release of MMP-9 [10]. The aim of our study was to explore the release of MMP-9 from polymorphonuclear leukocytes (PMN) isolated from COPD patients and healthy controls in basal conditions and after exposure to cigarette smoke extract (CSE).

Methods

Study Design

Twenty-three patients with clinically stable COPD and 9 healthy controls were included in this study. Inclusion criterion for patients was a clinical diagnosis of COPD according to GOLD (Global Initiative for Chronic Obstructive Lung Disease) report [1]. COPD was diagnosed by a pulmonology specialist based on clinical examination (chronic and progressive dyspnea, cough and sputum production) and spirometry results. Fixed ratio FEV₁/FVC < 0.70 was common for all COPD patients. Based on FEV₁ (% predicted), there were 12 patients with GOLD 2 stage, 6 patients with GOLD 3 stage, and 5 patients with GOLD 4 stage. Healthy subjects had good general health status and normal spirometry results. Exclusion criteria for both COPD patients and healthy subjects were the presence of other pulmonary disease, infective and inflammatory diseases, neoplastic pathologies, hepatic diseases, and excessive alcohol consumption. All participants were classified according to their

smoking status as smokers, ex-smokers, or non-smokers. The study was approved by medical ethics committees of the University Hospital Centre Zagreb, Clinical Hospital for Lung Diseases “Jordanovac” (Zagreb, Croatia) and the University of Zagreb, Faculty of Pharmacy and Biochemistry (Zagreb, Croatia). The study design was made according to the Declaration of Helsinki. Informed consent was signed by all subjects in the study.

Isolation of Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMN) from blood of all participants were isolated using the modified method developed by Bøyum [11]. Briefly, fresh blood (drawn into the tube with lithium heparin) was mixed with dextran (Sigma-Aldrich; 5% in 7 g/L NaCl) in 5:1 ratio and left aside at room temperature for 60 min. The upper layer of leukocyte-rich plasma was layered over the Histopaque-1077 (Sigma-Aldrich) in 1:1 ratio and centrifuged at 400×g during 30 min at room temperature. Supernatant was carefully removed and a pellet of PMN was resuspended in ice-cold 9 g/L NaCl. The residual erythrocytes were removed by lysis with ice-cold distilled water. PMN were then resuspended in complete RPMI 1640 cell culture medium (containing 5% fetal bovine serum). Viability and number of PMN were determined by staining with 0.4% trypan blue (Sigma-Aldrich). Viability of the isolated PMN has always been higher than 95%.

Cigarette Smoke Extract Preparation and Standardization

Cigarette smoke extract (CSE) was freshly prepared just before each experiment. 100% CSE was made by bubbling the smoke from two 3R4F Kentucky Research Cigarettes (University of Kentucky) without filter through the 25 mL of complete RPMI 1640 cell culture medium with the use of vacuum pump [12]. CSE preparation was standardized by measuring the absorbance of 100, 20, 10, 5, 2.5, and 1.25% CSE at 320 nm using the UV/VIS spectrophotometer (Cecil Aquarius CE 7200). Mean absorbance of 100% CSE at 320 nm was 1.717 ± 0.386 ($N=6$). The absorbance of freshly prepared CSE for each independent experiment matched the obtained values.

Lactate Dehydrogenase Activity Measurement

The cytotoxicity of CSE towards PMN was determined by measuring the catalytic activity of lactate dehydrogenase (LDH) released into the medium over the PMN. PMN isolated from a randomly selected healthy subject were cultured into the 24-well plate (100 000 PMN in 1 ml of complete RPMI 1640 cell culture medium per well). PMN were

treated with freshly prepared CSE at a concentration of 0% (control) or 4% during 1, 4, 20, or 22 h (in triplicate). After the incubation, cell culture media above the PMN were centrifuged at $1000\times g$ for 5 min at $+4\text{ }^{\circ}\text{C}$. LDH activity was immediately determined in the resulting supernatants using the spectrophotometric method with L-lactate as substrate and measurement of produced NADH at 340 nm (Herbos Dijagnostika reagent; Trace 30 analyzer). Results are shown as mean values of LDH activity (U/L) \pm standard error.

Exposure of Polymorphonuclear Leukocytes to Cigarette Smoke Extract

200 000 PMN in 2 mL of complete RPMI 1640 cell culture medium were cultured in duplicate into the 6-well plate for each participant. PMN in one well were not stimulated (control PMN, 0% CSE), while PMN in another well were exposed to 4% CSE for 20 h. After the incubation, cell culture medium above PMN was centrifuged at $1000\times g$ for 5 min at $+4\text{ }^{\circ}\text{C}$ and the resulting supernatant was aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ for the analysis of MMP-9 concentration.

MMP-9 Concentration Measurement

MMP-9 concentration was measured in the incubation medium above PMN using the commercially available ELISA kit (Quantikine, R&D Systems) and microtiter plate reader (VICTOR³ Multilabel Counter 1420, Wallac, Perkin-Elmer). The detection limit was declared as 0.156 ng/mL. Results are shown as mean values (ng/mL) \pm standard error.

Statistical Analysis

Data analysis was performed using the statistical software SigmaStat for Windows Version 3.00 (SPSS Inc.). All data were tested for normal distribution by Kolmogorov–Smirnov test. Data were presented as absolute numbers, percentages, median (interquartile range), or mean \pm standard error. Categorical variables were compared by Chi-square or Fisher's Exact test. The difference between two groups was tested using the non-parametric Mann–Whitney Rank Sum test and between more than two groups using the Kruskal–Wallis One-Way Analysis of Variance on Ranks. Data were considered statistically significant when $P < 0.05$.

Results

Relevant clinical and demographic characteristics of the study groups are shown in Table 1. In comparison with the group of healthy controls, the group of COPD patients showed statistically significant differences in age ($P = 0.046$)

Table 1 Clinical and demographic characteristics of the study groups

	Healthy controls ($N = 9$)	COPD ($N = 23$)	P
Gender (male/female)	5/4	19/4	0.176
Age (years) ^a	53 (32–66)	59 (43–76)	0.046
Smokers/ex-smokers/non-smokers (%)	22/45/33	20/70/10	<0.001
FEV ₁ (% predicted)	102 (97–107)	52 (34–68)	<0.001
FEV ₁ /FVC (%)	78 (75–83)	46 (39–62)	<0.001

^aShown as median (range)

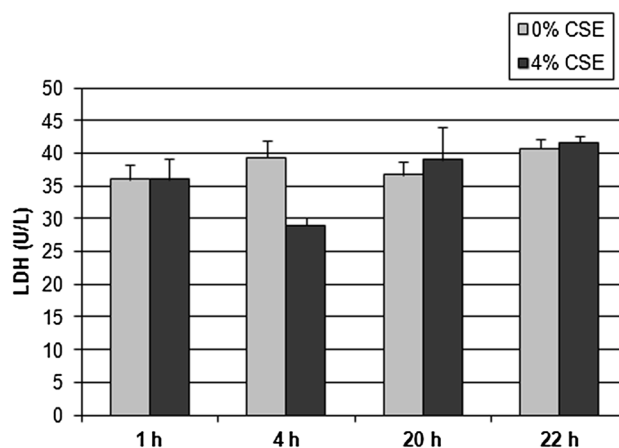


Fig. 1 LDH catalytic activities (mean \pm standard error) in the media above PMN exposed to 4% CSE or cell culture medium (0% CSE) for 1, 4, 20, or 22 h ($N = 3$). There were no statistically significant differences for any of the tested conditions ($P > 0.05$)

and smoking habits ($P < 0.001$), but not in gender. As expected, we found significant differences in parameters of pulmonary function (FEV₁ and FEV₁/FVC) between COPD patients and healthy controls ($P < 0.001$).

Stimulation of PMN with different agents may cause a release of the content of their granules into the environment. However, it is important to establish whether the CSE is cytotoxic to the cells. Therefore, based on our preliminary results, we examined the effect of 4% CSE on viability of PMN over time (1, 4, 20, and 22 h) by measuring the catalytic activity of LDH released into the medium above PMN (Fig. 1). Results showed that incubation of PMN with 4% CSE does not alter the release of LDH into the medium in comparison with untreated PMN ($P = 0.086$), suggesting no cytotoxic effect of 4% CSE during the tested times.

Hence, we isolated PMN from blood of patients with COPD and healthy subjects, exposed them to cell culture medium without CSE (0% CSE) or to medium containing 4% CSE during 20 h and measured the release of MMP-9

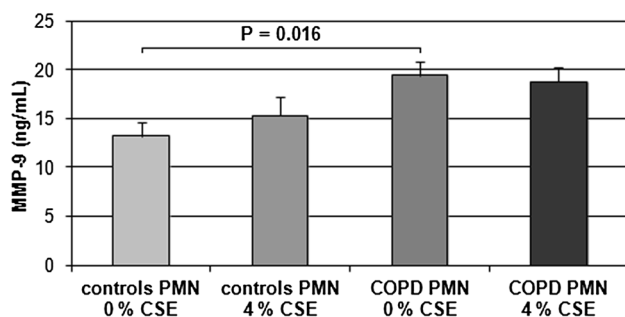


Fig. 2 MMP-9 concentrations (mean ± standard error) in the media above PMN of healthy controls ($N=9$) and COPD patients ($N=23$) exposed to 4% CSE or cell culture medium (0% CSE) during 20 h. Statistically significant difference is highlighted

into the media above PMN. Results showed that MMP-9 concentrations in the media above PMN from COPD patients that were exposed to cell culture medium without CSE (0% CSE) were significantly higher (19.4 ± 1.4 ng/mL) when compared to PMN of healthy subjects (13.2 ± 1.4 ng/mL) treated in the same way ($P=0.016$) (Fig. 2). However, PMN of healthy subjects exposed to 4% CSE released slightly higher (but not significantly, $P=0.373$) amounts of MMP-9 into the medium (15.3 ± 1.8 ng/mL) when compared with untreated PMN of healthy controls. These amounts were also similar to the amounts of MMP-9 released by PMN of COPD patients exposed to 4% CSE (18.8 ± 1.5 ng/mL) ($P=0.216$). There were no significant differences between PMN of COPD patients in both cases, either unexposed or following exposure to 4% CSE ($P=0.750$) (Fig. 2). Furthermore, concentration ratio of MMP-9 released into the media from PMN exposed to 4% CSE or 0% CSE was calculated for all the subjects. The results showed that the ratio is significantly higher for the group of healthy subjects (1.17 ± 0.07) comparing to COPD patients (0.97 ± 0.05) ($P=0.025$) (Fig. 3). However, there were no differences in MMP-9 release between the COPD patients subdivided into GOLD 2, 3, and 4 stage of the disease. Similarly, no effect of smoking habits was recorded for any group of the participants, probably due to the small number of subjects in each subgroup (data not shown).

Discussion

This is one of the first studies showing a direct effect of cigarette smoke on the release of MMP-9 from PMN isolated from patients with COPD and healthy subjects. We showed that basal levels of MMP-9 released into the media above PMN of COPD patients were significantly higher when compared to PMN of healthy controls. However, exposure of PMN to 4% CSE resulted in similar production of MMP-9

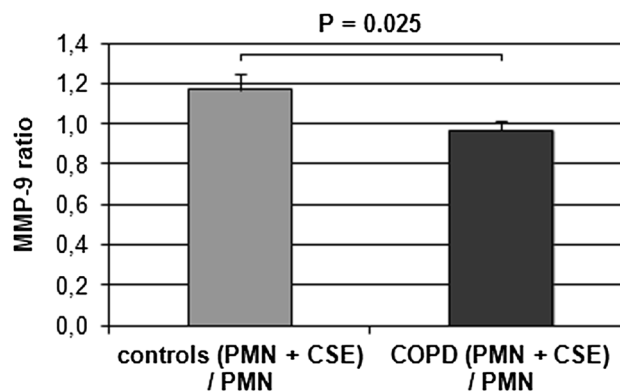


Fig. 3 Concentration ratio of MMP-9 released from PMN exposed to 4% CSE or 0% CSE (mean ± standard error) for healthy controls and COPD patients

from PMN of COPD patients and healthy controls. PMN of COPD patients stimulated with 4% CSE could not produce more MMP-9 when compared to PMN exposed to cell culture medium alone (0% CSE). Furthermore, the degree of MMP-9 release into the medium above PMN exposed and unexposed to CSE was presented by the concentration ratio that was calculated for each subject. Results showed that stimulation of PMN from healthy controls with CSE lead to the significantly higher release of MMP-9 compared to patients with COPD. Taken together, these results are showing chronic activation of PMN from COPD patients.

Previously published research on granulocytes of patients with COPD, asthmatic patients, and healthy controls showed that granulocytes may release MMP-9 spontaneously in basal conditions, but without significant differences between these three groups [13]. Overbeek et al. [4] showed that stimulation of PMN with CSE for 9 h resulted in release of IL-8, MMP-8, and MMP-9, without differences between healthy donors and COPD patients in MMP-9 production. A recently published study demonstrated considerable activation of neutrophils upon the exposure to CSE, as shown by the release of MMP-8, MMP-9, and lactoferrin, without differences between smokers and non-smokers [14]. The increased release of MMP-9, MMP-2, and elastase from PMN of healthy non-smokers stimulated with cigarette smoke cannot be prevented by prior incubation of PMN with salmeterol and/or fluticasone propionate, drugs that are normally used for treatment of COPD [15]. It is known that cigarette smoke activates airway macrophages to release the proinflammatory mediators, such as IL-8, and matrix metalloproteinases, such as MMP-9 [16]. Furthermore, IL-8 has a chemotactic effect on neutrophils and stimulates a release of MMP-9, which cleaves the IL-8 (1–77), thus creating an active IL-8 (7–77), and making a positive feedback loop [17]. In addition, it was shown that cigarette smoke extract induces the release of IL-8 from neutrophils themselves [18].

Therefore, it is possible that cigarette smoking is one of the causes of increased release of proteases from cells present in the lungs (demonstrated in this study by an increased release of MMP-9 from PMN of healthy controls exposed to cigarette smoke), which leads to degradation of the extracellular matrix. At the same time, peptide fragments which act as chemoattractants and activators of leukocytes are released, generating a vicious circle that leads to the further development of inflammation and progression of the disease into a chronic form [3, 4]. PMN isolated from patients with COPD, that release high levels of MMP-9 even in the basal conditions in our study, might be the proof for this phenomenon.

In conclusion, cigarette smoke may lead to the high release of MMP-9 from PMN. Chronically activated PMN of COPD patients suggest their indirect role in degradation of extracellular matrix in the lungs and potential as a therapeutic target for COPD treatment.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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