Eur Respir J 2008; 32: 644–650
DOI: 10.1183/09031936.00121507

Impact of acute exposure to tobacco smoke on gelatinases in the bronchoalveolar space

P. Glader*, B. Eldh*, S. Bozinovski#,¶, K. Andelid*, M. Sjöstrand*, C. Malmhäll*, G.P. Anderson#,¶, G.C. Riise*, I. Qvarfordt* and A. Lindén*

ABSTRACT: Clinical studies have indicated increased gelatinase activity in the airways of patients suffering from chronic obstructive pulmonary disease caused by tobacco smoke. The present study aimed to determine whether acute exposure to tobacco smoke *per se* causes a substantial and lasting impact on gelatinases and their inhibitors in the peripheral airways of atopic and nonatopic human subjects.

Bronchoscopy with bronchoalveolar lavage (BAL) was performed on occasional smokers with and without atopy before and after smoking 10 cigarettes over a 48-h period. Samples from a group of never-smokers not exposed to tobacco smoke served as controls. Gelatinase identity and activity were measured using zymography, and gelatinase activity assay and concentrations of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of MMP (TIMP)-1 and TIMP-2 were measured using ELISA.

The results revealed no pronounced changes in identity, net activity or concentration of the gelatinases or changes in concentrations of TIMP-1 and TIMP-2 in BAL fluid before and after acute exposure to tobacco smoke.

In conclusion, the present experimental study indicates that acute exposure to tobacco smoke does not cause any substantial impact on gelatinases or their inhibitors in the peripheral airways, irrespective of atopy status, a finding that is compatible with the fact that it takes many years of tobacco smoking to establish chronic obstructive pulmonary disease.

KEYWORDS: Chronic obstructive pulmonary disease, macrophage, matrix metalloproteinase 9, neutrophils, tissue inhibitor of matrix metalloproteinase

In the lungs and other organs, several matrix metalloproteinases (MMPs) are believed to be important for maintaining normal turnover of extracellular matrix. These MMPs can be functionally divided into several groups, including gelatinases, collagenases and membrane-type MMPs, depending on which specific molecules they degrade [1]. The gelatinases include MMP-2 and MMP-9, which, apart from gelatine, also degrade collagen, elastine and fibronectine, as well as other extracellular matrix proteins [2]. MMP-2 and MMP-9 have specific inhibitors: tissue inhibitors of MMP (TIMP)-2 and TIMP-1 [1]. As a consequence, the endogenous control of TIMP production might be as important as the control of MMPs to maintain gelatinase homeostasis.

Interestingly, previous clinical studies indicate an altered production of both gelatinolytic MMPs and their matching TIMPs in chronic lung disease caused by tobacco smoke. Thus, an upregulation of MMP-9 and MMP-2 has been demonstrated in the lungs of patients with manifest chronic obstructive pulmonary disease (COPD), and an upregulation of MMP-9 has been detected in the lungs of patients with emphysema [3]. This upregulation of MMP may lead to an imbalance in the gelatinase homeostasis, especially if occurring in combination with a downregulation of TIMPs [4, 5]. However, the course and order of proteolytic events preceding manifest COPD in tobacco smokers remain largely unknown. As a consequence, it is still uncertain to what extent acute exposure to tobacco smoke *per se* leads to an

AFFILIATIONS
*Lung Immunology Group, Dept of Internal Medicine/Respiratory Medicine and Allergology, Sahlgrenska Academy, University of Gothenburg, Göteborg, Sweden.
#Depts of Medicine, and
¶Pharmacology, Cooperative Research Centre for Chronic Inflammatory Disease, University of Melbourne, Melbourne, Australia.

CORRESPONDENCE
P. Glader
Lung Immunology Group
University of Gothenburg
Guldhedsgatan 10A
413 46 Göteborg
Sweden
Fax: 46 31413290
E-mail: Pernilla.Glader@gu.se

Received:
September 14 2007
Accepted after revision:
March 26 2008

STATEMENT OF INTEREST
A statement of interest for B. Eldh can be found at www.erj.ersjournals.com/misc/statements.shtml

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary data accessible from www.erj.ersjournals.com

imbalance in the gelatinase homeostasis in the peripheral airways of clinically healthy humans. A study on induced sputum from occasional smokers has shed some light on this by providing evidence that, in the proximal airways, acute exposure to tobacco smoke does cause a transient increase in the number of neutrophils, an inflammatory cell that constitutes a potentially important source of the gelatinase MMP-9 [6]. However, that study did not indicate any differences in MMP-9 or TIMP-1 in the proximal airways after smoke exposure. Moreover, in addition to reflecting the proximal and not the peripheral airways, the sputum technique is problematic in that it induces neutrophil accumulation *per se*, if used repeatedly [7]. Thus, induced sputum introduces a confounding factor already at baseline, since it may cause even more neutrophils to become exposed to the locally accumulated stimuli associated with exposure to tobacco smoke. In addition, sputum contents are likely to reflect mainly proximal and not peripheral airways, as opposed to bronchoalveolar lavage (BAL) fluid [8–10].

Yet another issue of clinical interest in the context of COPD is the importance of atopy for the gelatinase homeostasis in human lungs. Previous studies have demonstrated increased MMP-9 activity in the airways of patients with asthma, but the impact of acute exposure to tobacco smoke has not previously been addressed in the peripheral airways of atopic subjects [11].

The current study was designed to determine whether an acute exposure to tobacco smoke exerts a substantial and lasting impact on gelatinases and their inhibitors in the lower airways of clinically healthy subjects. The study also addressed the potential importance of atopy in this specific context. Specifically, in addition to assessing the gelatinase:gelatinase inhibitor ratio in terms of concentrations of MMP-2 and -9 and TIMP-2 and -1, the present study ascertained the identity and the net activity of the dominant gelatinases in the peripheral airways and controlled for the examination procedure, *i.e.* BAL.

MATERIALS AND METHODS

Study subjects

Three groups of study subjects with normal lung function were recruited for this study: nonatopic-occasional smokers, atopic-occasional smokers and never-smokers. All subjects gave their written informed consent to participate in the study which was approved by the Ethics committee at the University of Gothenburg (Gothenburg, Sweden). All three groups had been free from smoking and respiratory infections for $\geqslant$4 weeks prior to participating in the study. Spirometry was performed to measure and confirm normal lung function in each individual. All atopic occasional smokers had a history of subjective symptoms from the upper and/or lower airways. The history of atopy was objectively confirmed through Phadiatop™ testing (Phadia AB, Uppsala, Sweden) of specific immunoglobulin (Ig)E and by assessing total IgE levels in blood. All subjects underwent two bronchoscopies including BAL; the first at day 1 (termed BAL1) and a second at day 14 (termed BAL2). On days 12 and 13, all occasional smokers smoked, in total, 10 filter cigarettes of a commercial brand (tar 10 mg, nicotine 0.8 mg), purchased commercially (not given as a gift). To be considered as an occasional smoker, the subjects had to habitually smoke cigarettes on at least one occasion per month and a maximum of four occasions per month. The dose (number) of cigarettes was chosen based upon the clinical observation that none of the recruited occasional smokers habitually smoked >20 cigarettes over a 48-h period. It was reasoned that it would be unethical to exceed the number of cigarettes the recruited subjects would habitually smoke on average; therefore, a dose of 10 cigarettes over a 48-h period was chosen and considered as ethically impregnable. The smoking status for each subject was controlled by measuring the urine cotinine level at the time of each of the two bronchoscopies. To be included, all subjects had to display cotinine levels <100 ng·mL^{-1} prior to BAL1. For the continued inclusion of occasional smokers at the time of BAL2 (*i.e.* as a confirmation of the intervention smoke exposure), these subjects had to display cotinine levels at least five-fold of those obtained at the first bronchoscopy. Subjects were excluded if they suffered from any infection between the two bronchoscopies.

In total, 29 occasional smokers were recruited and underwent the two bronchoscopies. Seven of these were later excluded from further analysis as they did not meet the study criteria for cotinine levels in urine. Of the remaining 22 occasional smokers, 13 were nonatopic and nine were atopic. In total, 18 never-smokers were recruited, of which three were subsequently excluded due to infections during the study. The patient characteristics of the 13 nonatopic occasional smokers, nine atopic occasional smokers and 15 never-smokers are shown in table 1. Age and lung function did not differ markedly between the groups. Cotinine levels in urine at the time of BAL1 and BAL2 are shown in table 2 of the supplementary data.

Bronchoscopy, BAL sampling and handling

Bronchoscopies were performed according to standard procedure using a flexible bronchoscope. A bronchial wash of 20 mL PBS preceded the BAL to avoid contamination from the proximal airways in BAL. BAL was subsequently performed in the right middle lobe utilising 3× 50 mL of PBS. BAL fluid was collected in a polypropylene tube and kept on ice until it reached the lab. The total cell count and trypan blue exclusion on the BAL cells was carried out and the BAL samples were centrifuged to separate cells from BAL fluid. Cells were resuspended in buffer and put on cytospins that were subsequently stained with May-Grünwald-Giemsa staining and subsequent differential counting of 600 cells per sample according to standard morphological criteria. The BAL fluid was aliquoted and kept at -80°C until further analysis.

Gelatinases and gelatinase inhibitors in BAL fluid

Identity of gelatinases

Zymography was used to identify MMP-2 and MMP-9 bands and to screen for total MMP-2 and MMP-9 activity in the BAL fluid of the first five subjects in each study group. Since the process of zymography may physically disassociate bound TIMPs from gelatinases, the present authors utilised representative bands for densitometry (Kodak-1D image analysis; Eastman Kodak Company, Rochester, NY, USA) as a quantitative assessment of the total gelatinase activity. Identical areas around individual bands were scanned and a background subtracted net intensity value was generated. The assay was performed as described previously [12]. Briefly, gelatin

TABLE 1 Patient characteristics

	Never-smokers	Occasional smokers	Atopic occasional smokers
Subjects	15	13	9
Female/male	8/7	7/6	5/4
Age yrs	23 (21–36)	26 (22–44)	23 (21–26)
FEV_1 % pred	108 (96–129)	105 (95–130)	107 (85–125)
FEV_1/FVC	89 (74–99)	85 (75–99)	91 (83–99)

Data are presented as n or median (range). FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity.

(2 mg·mL^{-1}; Labchem, Pittsburgh, PA, USA) was added to a 10% SDS gel before casting. Under nonreducing conditions, 10 μL of neat BAL samples were run through the gel at a constant voltage (200 V). After electrophoresis, the gel was washed twice in 2.5% Triton X-100, incubated overnight at 37°C in zymography buffer (50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 0.01% NaN_3) and subsequently stained with Brilliant Blue (B7920; Sigma-Aldrich, Saint Louis, MO, USA).

Quantity of gelatinases and gelatinase inhibitors

The concentrations of MMP-2 (pro plus active forms), MMP-9 (pro plus active forms), TIMP-1 and TIMP-2 were determined in all of the BAL fluid samples using commercial ELISA kits from R&D Systems (Abingdon, UK) and carried out according to the manufacture's recommendations. For MMP-2 analysis, BAL samples were concentrated as required for detection (15- to 30-fold) prior to ELISA. Amicon Ultra tubes with a 5 kDa cut-off (Millipore, Billerica, MA, USA) were used for the concentration procedure. According to the manufacturer of the ELISA kits, the sensitivities of the ELISAs were 0.16, 0.156, 0.08 and 0.011 ng·mL^{-1} for MMP-2, MMP-9, TIMP-1 and TIMP-2, respectively.

Net activity of gelatinases

The net gelatinase activity was measured in all of the BAL fluid samples using a fluorescence-conjugated gelatine substrate (D-12054 DQ gelatin from pig skin; Invitrogen, Mount Waverley, Australia). BAL fluid proteins were precipitated using acetone (2 mL BAL fluid in 8 mL ice cold acetone), the pellet was air dried and then re-suspended in 200 μL PBS, effectively concentrating the BAL fluid 10-fold. The concentrated BAL fluid (25 μL) was incubated with the gelatine substrate (5 μg) diluted in 175 μL gelatinase substrate (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% NaN_3) for 16 h, while gently rotating in an incubator at 37°C. PBS alone was used for background readings subtracted from all samples measurements. The fluorescence intensity of the digested substrate was then measured in a FlexStation II micro plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at the absorption/emission wavelength of 495/515 nm.

Statistical analysis

The Kruskal-Wallis test was used to detect differences between the three study groups. In case the test showed a statistical significant difference between the groups, the Mann–Whitney U-test was used to perform pairwise tests between the groups. The Wilcoxon signed-rank test was used to test differences between the paired BAL1 and BAL2 samples within each study group. Differences were considered statistically significant if the p-value was <0.05. Correlation analysis was performed by calculating Spearman's rank correlation coefficient.

RESULTS

BAL cell counts

BAL recovery, total cell count and cell viability did not differ markedly between BAL1 and BAL2 in any of the study groups or between the study groups (table 3 in the supplementary data). Likewise, the differential counts revealed no substantial differences in percentage of neutrophils or macrophages, two important producers of the investigated MMPs. In addition, no differences were found in the percentage of lymphocytes. The percentage of eosinophils, however, was higher in BAL2 than in BAL1 among never-smokers ($p=0.016$) and atopic smokers ($p=0.039$; table 3 in the supplementary data). A similar trend was observed in nonatopic smokers but this trend did not prove statistically significant.

Gelatinases and gelatinase inhibitors in BAL fluid

Identity of gelatinases

The identification of the dominant gelatinases in BAL fluid was ascertained by utilising zymography with gelatine as the substrate. BAL samples from the first five subjects in each group were studied. Three main bands were identified at ~70, 90 and 150 kDa in size (fig. 1). Based on their respective molecular weights, the 70 and 90 kDa bands were identified as pro-MMP-2 and pro-MMP-9. The upper (150 kDa) band was attributed to dimeric complexes retained under nonreducing conditions. No clear differences were found in the appearance or the density of the bands for any of the MMPs between never-smokers, smokers or atopic smokers. No differences were found between BAL1 and BAL2 in any of the groups in this either (table 4 in supplementary data).

Quantity of gelatinases and gelatinase inhibitors

To confirm the presence of pro-MMP-2 and pro-MMP-9, MMP-2 and MMP-9 were quantified in all BAL samples using ELISA to detect the pro plus the active forms of each MMP. The ELISA measurements confirmed the zymography results with no pronounced differences seen between BAL1 and BAL2 or between study groups (fig. 2a and b). TIMP-1 and TIMP-2 were also analysed with ELISA. No substantial differences were found between BAL1 and BAL2 within or between the study groups for any of the TIMPs (fig. 2c and d). Correlation analysis was performed to ensure that urine cotinine levels,

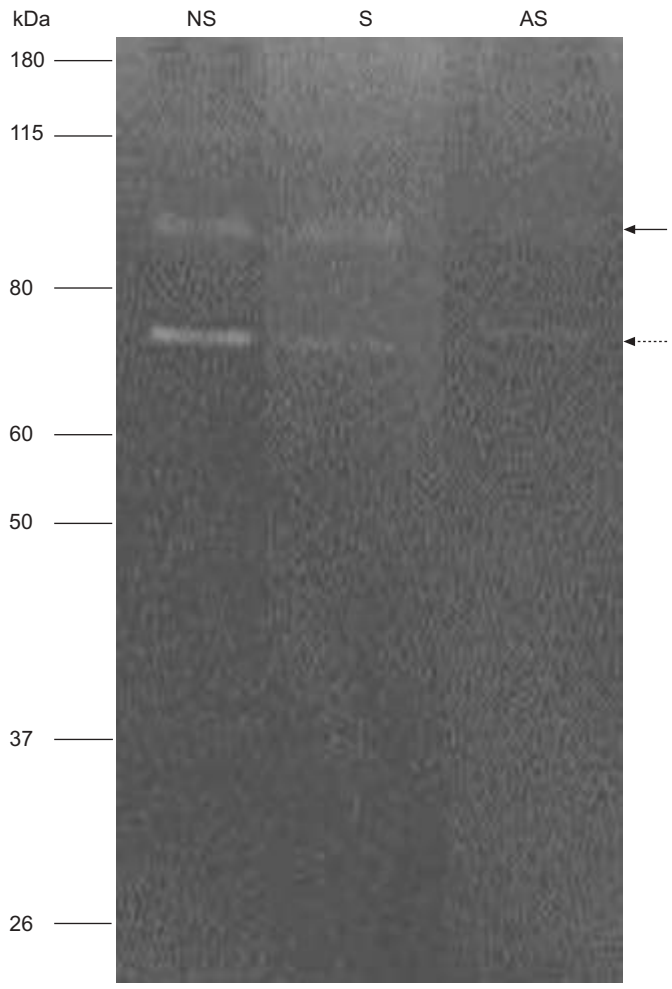

FIGURE 1. Qualitative analysis of identifying dominating gelatinases in human bronchoalveolar (BAL) fluid. The representative image shows a zymography gel with bands of 70 (dotted arrow), 90 (solid arrow) and 150 kDa corresponding to pro-matrix metalloproteinase (MMP)-2, pro-MMP-9 and (presumably) dimeric complexes, respectively, retained under nonreducing conditions. All samples were from the second BAL procedure carried out in each subject. NS: never-smoker not exposed to tobacco smoke; S: occasional smoker exposed to tobacco smoke; AS: atopic occasional smoker exposed to tobacco smoke.

which to some extent differed between the individuals after smoking, did not affect the level of MMPs and TIMPs. None of the concentrations of MMP-9, MMP-2, TIMP-1 or TIMP-2 correlated with cotinine levels.

Net activity of gelatinases

The net gelatinase activity in BAL fluid was also determined using fluorescent conjugated gelatine, which is detectable only upon its degradation. The present authors found no clear difference in net gelatinase activity for BAL1 and BAL2 in atopic or nonatopic smokers (fig. 3). However, in atopic smokers, the net gelatinase activity displayed a higher activity in BAL1 (baseline) samples, compared with never-smokers ($p=0.002$) and nonatopic smokers ($p=0.014$). A modest increase in gelatinase activity was observed in BAL2 compared with BAL1 in the group of never-smokers ($p=0.022$; fig. 3).

Gelatinase/gelatinase inhibitor ratios

When ELISA results were used to calculate ratios of MMP-9:TIMP-1, MMP-2:TIMP-2, MMP-9:TIMP-2 and MMP-2:TIMP-1, the present authors were unable to reveal any major differences within or between the study groups (table 5 in supplementary data).

DISCUSSION

The present study assessed the effects of acute exposure to tobacco smoke on the identity, quantity and net activity of local gelatinases, as well as the gelatinase:gelatinase inhibitor ratios in BAL fluid from both atopic- and nonatopic-occasional smokers. The results consistently indicated that under the conditions stipulated in the current study, acute exposure to tobacco smoke does not cause any pronounced and lasting impact on the local gelatinases or gelatinase inhibitors in the peripheral airways of humans. In addition, the results did not reveal any corresponding impact on the local number of macrophages or neutrophils. These results were found regardless of atopy status. Moreover, the current study presents observations of potential methodological importance as the results indicate that the BAL procedure itself can exert a certain impact on the accumulation of eosinophils and on the net gelatinase activity locally.

The results in the current study, on both gelatinases and neutrophil counts, correspond well with findings reported in a previous study on induced sputum from intermittent smokers, in spite of the fact that the current study reflects the peripheral and not the proximal airways [6]. The previous study on proximal airways indicated no substantial changes in sputum MMP-9, TIMP-1 or neutrophil counts 24 h after exposure to tobacco smoke [6]. However, in contrast to the current study, the study by VAN DER VAART *et al.* [6] did not address MMP-2 and TIMP-2.

The outcome of the present study does not support the results of previous studies in mouse models, in which it has been demonstrated that acute exposure to cigarette smoke increases MMP-9 levels and neutrophil counts in BAL fluid [13, 14]. One reason for this discrepancy may of course be species differences; another reason may be the fact that excessively high doses of tobacco smoke have been administered in the previous studies on mouse models. In the current study, the authors chose what was considered to be a moderate dose of tobacco smoke, *i.e.* 10 cigarettes during a 48-h period. This dose of tobacco smoke seemed clinically relevant and ethically defendable, considering that the occasional smokers who participated in the study reported that they habitually did not smoke >20 cigarettes over a 48-h period, a dose the present authors did not want to exceed. Due to this moderate dose of tobacco smoke, one could of course argue that the negative results in this study were due to an insufficient dose of tobacco smoke. Against this argument stands the clear and reproducible increase in the nicotine metabolite cotinine in urine, which was documented for all the included subjects. Apart from being important for the inclusion of study subjects, this documentation of cotinine in urine proves that the utilised dose of tobacco smoke was indeed sufficient to enable systemic dissemination of one key component of tobacco smoke, nicotine. As judged from oral information from the investigated occasional smokers (data not shown), 10–20 h passed

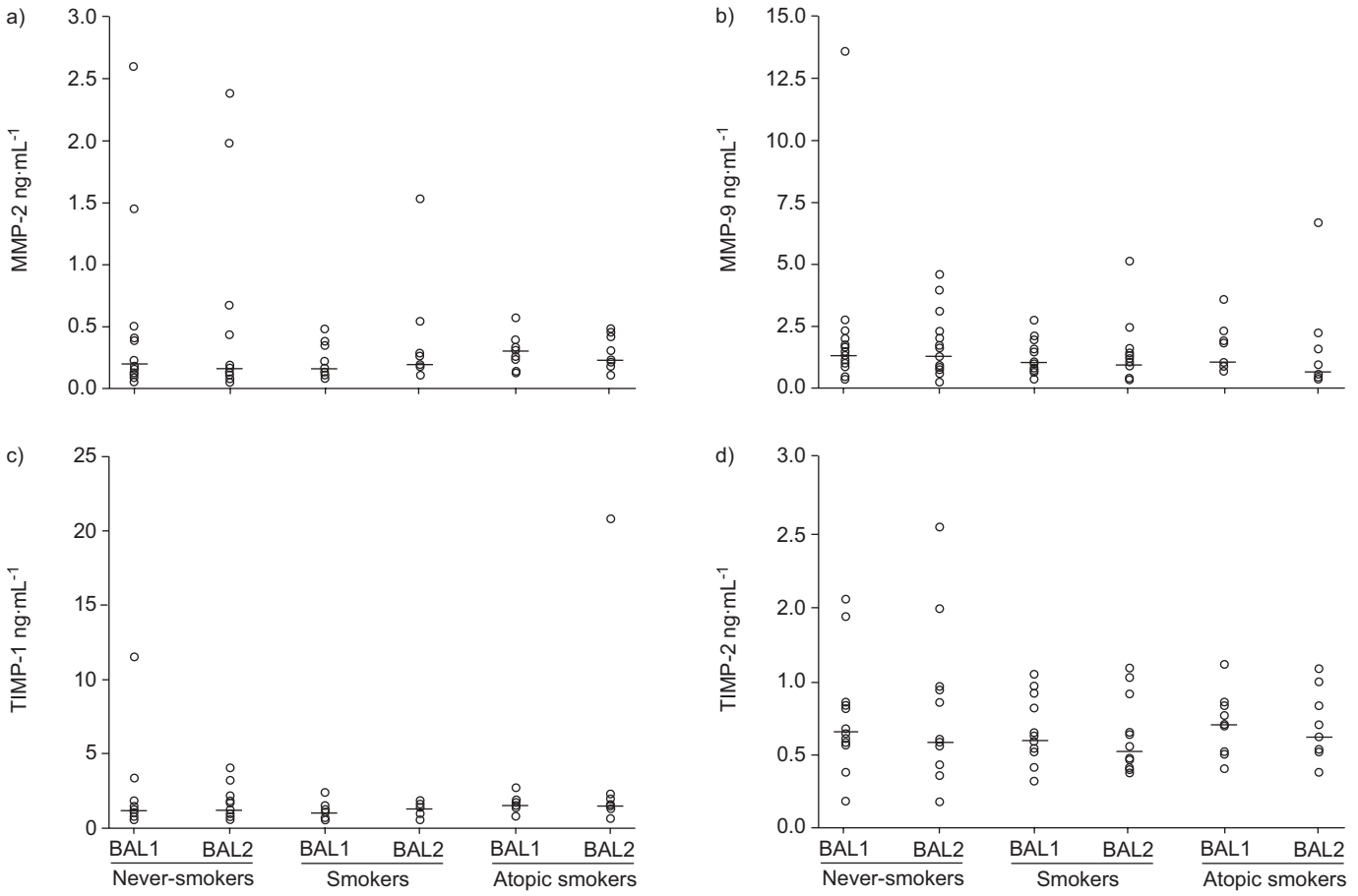

FIGURE 2. Quantitative analysis of gelatinases and gelatinase inhibitors in human bronchoalveolar lavage (BAL) fluid. ELISA was utilised to measure concentrations of total a) matrix metalloproteinase (MMP)-2, b) MMP-9, c) tissue inhibitors of MMP (TIMP)-1 and d) TIMP-2 in BAL samples before (BAL1) and after (BAL2) smoking in 13 nonatopic-occasional smokers and nine atopic occasional smokers, and in corresponding BAL samples from a control group of 15 never-smokers not exposed to tobacco smoke between bronchoscopies. No significant differences were found between study groups or before and after smoking.

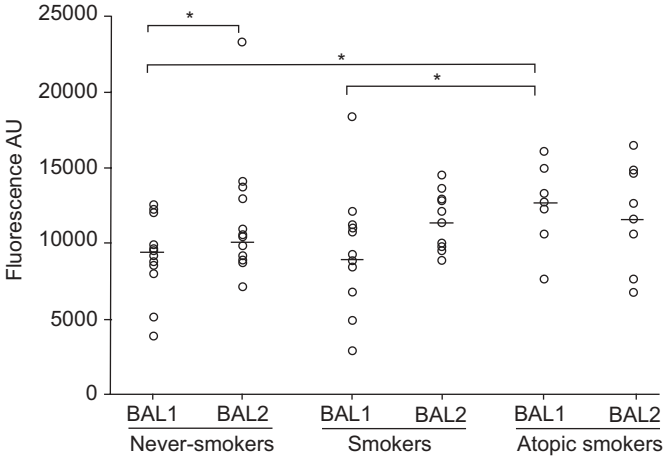

FIGURE 3. Net gelatinase activity in human bronchoalveolar lavage (BAL) samples. Samples were harvested before (BAL1) and after (BAL2) controlled smoking in 13 nonatopic-occasional smokers, nine atopic occasional smokers and a control group of 15 never-smokers. Baseline levels (BAL1) of net gelatinase activity was higher in atopic than in nonatopic occasional smokers ($p=0.014$) and never-smokers ($p=0.002$). In the control group of never-smokers alone, this net gelatinase activity was higher in BAL2 than in BAL1 ($p=0.022$). AU: arbitrary unit. *: $p<0.05$.

after the last cigarette smoked until the harvest of BAL fluid during BAL2. Therefore, a transient peak in gelatinase mobilisation within this time frame cannot be ruled out, based upon the results of the current study.

According to previous studies on local quantity and/or activity of MMP-9 and MMP-2 in human airways, gelatinases are increased in COPD and emphysema patients compared with nonsmokers [4, 15] and chronic smokers without COPD [3, 16], while the latter do not display significantly different MMP-9 protein levels in relation to never-smokers [17]. Notably, a recent study on smokers with and without COPD reveals that immunoreactivity for MMP-2 protein in peripheral lung tissue correlates with disease severity in patients with COPD rather than to cigarette smoking *per se* [18], which is fully in line with the current findings. Moreover, COPD patients display higher MMP-9 protein levels in the airways during exacerbations than during the stable phase of their disease [5] and ex-smokers with COPD colonised with bacteria display higher MMP-9 protein levels in the peripheral airways than noncolonised ex-smokers with COPD [19]. These observations, together with the observations in the current study, are all compatible with the idea that there is no pronounced impact on gelatinase mobilisation that occurs immediately

after tobacco exposure in naïve airways and that such an impact requires repeated exposure to tobacco smoke over time as the pulmonary disease progresses, possibly also affected by co-factors such as infectious agents.

It has previously been demonstrated that MMP-9 protein levels and activity are increased in the proximal airways of patients with asthma and that MMP-9 levels are further enhanced after allergen challenge [11, 20]. This previously reported, atopy-related increase in MMP-9 is likely to explain the increase in net gelatinase activity that was observed in the atopic-occasional smokers at baseline (prior to smoke exposure) in the present study.

The current study revealed no increased susceptibility to an acute exposure of tobacco smoke in terms of alterations in gelatinases and their inhibitors or the occurrence of inflammatory cells in the peripheral airways of atopic subjects. To the best of the current authors' knowledge, this is the first study to report on this matter, as most previous studies addressing atopy *versus* smoking have focused on the tobacco-induced increased risk of developing atopy and asthma [21, 22] and on the increased severity, inflammation and progress of atopic diseases, such as asthma in smokers [23–25].

Interestingly, the current study generated evidence that the BAL procedure *per se* can influence both cell counts and proteins in the lower airways, even though these iatrogenic effects seem modest. In comparison to what has been reported in a previous study on induced sputum [7], the percentage of eosinophils observed was increased in never-smokers and in atopic-occasional smokers in BAL2, harvested 14 days after BAL1. Moreover, a small increase in net gelatinase activity was found in the second BAL sample harvested from never-smokers. These observations encourage extra caution to be taken when designing studies involving repeated BAL procedures, and emphasise the need of including a longitudinal control for the procedure, in addition to the use of baseline controls.

In summary, the present study specifically assessed the impact of an acute and moderate dose of tobacco smoke on the concentrations of the quantitatively dominant gelatinases matrix metalloproteinase-2 and -9 and their inhibitors tissue inhibitor of matrix metalloproteinase-1 and -2, the gelatinase: gelatinase inhibitor ratio and the net gelatinase activity in bronchoalveolar lavage fluid from occasional smokers. Even though modest, transient alterations cannot be excluded, the main conclusion of the study is that an acute exposure to a moderate dose of tobacco smoke is not sufficient to cause any substantial, lasting impact on gelatinases or their inhibitors in the peripheral airways of humans. Naturally, the fact that a limited number of subjects were investigated means that the risk of type II errors cannot be totally disregarded. However, the three different means of assessing gelatinases in the study all generated results indicating a lack of substantial alterations in gelatinases after one acute exposure to smoke. Moreover, atopy seems to be of no major importance in this specific context. Indeed, the current findings are compatible with the fact that it takes many years of tobacco smoking to establish chronic obstructive pulmonary disease but, importantly, they should not be taken as evidence that occasional exposure to tobacco smoke is harmless. Rather, in view of the results of previous studies on gelatinases in patients with chronic obstructive pulmonary disease, the current results provide a rationale for hypothesising that repeated exposure to tobacco smoke is required for the mobilisation of gelatinases in naïve airways and for investigating proteases other than gelatinases in this context.

ACKNOWLEDGEMENTS

The authors would like to thank B. Balder (Lung Immunology Group, Dept of Internal Medicine/Respiratory Medicine and Allergology, Sahlgrenska Academy, University of Gothenburg, Sweden) for excellent technical assistance with the clinical logistics.

REFERENCES

1 Gueders MM, Foidart JM, Noel A, Cataldo DD. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases. *Eur J Pharmacol* 2006; 533: 133–144.
2 Chakrabarti S, Patel KD. Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 2005; 31: 599–621.
3 Finlay GA, Russell KJ, McMahon KJ, *et al.* Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. *Thorax* 1997; 52: 502–506.
4 Beeh KM, Beier J, Kornmann O, Buhl R. Sputum matrix metalloproteinase-9, tissue inhibitor of metalloprotinease-1, and their molar ratio in patients with chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and healthy subjects. *Respir Med* 2003; 97: 634–639.
5 Mercer PF, Shute JK, Bhowmik A, Donaldson GC, Wedzicha JA, Warner JA. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res* 2005; 6: 151.
6 van der Vaart H, Postma DS, Timens W, *et al.* Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. *Respir Res* 2005; 6: 22.
7 van der Vaart H, Postma DS, Timens W, Kauffman HF, Hylkema MN, Ten Hacken NH. Repeated sputum inductions induce a transient neutrophilic and eosinophilic response. *Chest* 2006; 130: 1157–1164.
8 Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from the central airways: confirmation using a radiolabeled aerosol bolus delivery technique. *Am J Respir Crit Care Med* 2001; 164: 1964–1970.
9 Pizzichini E, Pizzichini MM, Kidney JC, *et al.* Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: Inflammatory cells, lymphocyte subsets and soluble markers compared. *Eur Respir J* 1998; 11: 828–834.
10 Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH, Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. *Eur Respir J* 2000; 15: 109–115.
11 Mattos W, Lim S, Russell R, Jatakanon A, Chung KF, Barnes PJ. Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. *Chest* 2002; 122: 1543–1552.
12 Bozinovski S, Jones JE, Vlahos R, Hamilton JA, Anderson GP. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to

lipopolysaccharide through Akt/Erk activation of NF-κB and AP-1 *in vivo*. *J Biol Chem* 2002; 277: 42808–42814.
13 Churg A, Zay K, Shay S, *et al.* Acute cigarette smoke-induced connective tissue breakdown requires both neutrophils and macrophage metalloelastase in mice. *Am J Respir Cell Mol Biol* 2002; 27: 368–374.
14 Leclerc O, Lagente V, Planquois JM, *et al.* Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. *Eur Respir J* 2006; 27: 1102–1109.
15 Boschetto P, Quintavalle S, Zeni E, *et al.* Association between markers of emphysema and more severe chronic obstructive pulmonary disease. *Thorax* 2006; 61: 1037–1042.
16 Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, Selman M. Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest* 2000; 117: 684–694.
17 Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med* 2000; 162: 1355–1360.
18 Baraldo S, Bazzan E, Zanin ME, *et al.* Matrix metalloproteinase-2 protein in lung periphery is related to COPD progression. *Chest* 2007; 132: 1733–1740.
19 Sethi S, Maloney J, Grove L, Wrona C, Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; 173: 991–998.
20 Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidart JM, Louis R. Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. *Chest* 2002; 122: 1553–1559.
21 Gilliland FD, Islam T, Berhane K, *et al.* Regular smoking and asthma incidence in adolescents. *Am J Respir Crit Care Med* 2006; 174: 1094–1100.
22 Postma DS, Boezen HM. Rationale for the Dutch hypothesis. Allergy and airway hyperresponsiveness as genetic factors and their interaction with environment in the development of asthma and COPD. *Chest* 2004; 126: Suppl. 2, 96S–104S.
23 Chaudhuri R, Livingston E, McMahon AD, *et al.* Effects of smoking cessation on lung function and airway inflammation in smokers with asthma. *Am J Respir Crit Care Med* 2006; 174: 127–133.
24 Siroux V, Pin I, Oryszczyn MP, Le Moual N, Kauffmann F. Relationships of active smoking to asthma and asthma severity in the EGEA study. Epidemiological study on the genetics and environment of asthma. *Eur Respir J* 2000; 15: 470–477.
25 Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur Respir J* 2004; 24: 822–833.