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Matrix metalloproteinase-9 in bronchiolitis obliterans syndrome after lung transplantation

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ABSTRACT: Bronchiolitis obliterans syndrome (BOS) is a severe complication after lung transplantation (LTX).

In a retrospective cohort study 12 stable healthy recipients (non-BOS) and eight patients with BOS were enrolled after LTX and matrix metalloproteinases (MMP)-9, TIMP-1 and cell characteristics in bronchoalveolar lavage (BAL) samples (n=145) were analysed. BALs from patients with BOS were further divided according to whether they were obtained before (pre-BOS) or after manifestation of BOS (BOS group).

The MMP-9/TIMP-1 ratio was significantly increased in the BOS group compared with non-BOS or pre-BOS; furthermore, the ratio was negatively correlated with forced expiratory volume in one second. In zymography, the active form of MMP-9 was detected predominantly in the BOS group. In addition, zymography showed the banding pattern of neutrophil-derived MMP-9, indicating that polymorphonuclear neutrophils (PMNs) were the main source of MMP-9. According to that, MMP-9 was significantly correlated with the number of PMN. In immunocytochemistry, MMP-9 was also associated predominantly with PMN.

This is the first study to evaluate the expression of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinases-1 over time during manifestation of a fibroproliferative lung disease in patients. It demonstrates development of bronchiolitis obliterans syndrome after lung transplantation is associated with an imbalance of matrix metalloproteinases-9/tissue inhibitors of metalloproteinase-1 ratio.

KEYWORDS: Bronchiolitis obliterans syndrome, bronchoalveloar lavage, lung transplantation, matrix metalloproteinase, polymorphonuclear cells, tissue inhibitors of matrix metalloproteinases

Despite medical improvements, 5-yr survival after lung transplantation (LTX) remains <50% [1]. The major cause of mortality and morbidity is chronic allograft rejection, clinically manifested as bronchiolitis obliterans syndrome (BOS). Histopathological features of BOS suggest injury and inflammation of epithelial cells and subepithelial structures of the airways, which lead to excessive fibroproliferation due to ineffective epithelial regeneration and aberrant tissue repair [2].

The matrix metalloproteinases (MMPs) represent a family of (currently) 23 zinc-containing enzymes. Some of the MMP family members are thought to be responsible for the turnover of extracellular matrix (ECM). MMP-9 belongs to the gelatinases with a relative molecular weight of 92 kD [3, 4]. It contributes to the migration of inflammatory cells through the ECM, basement membrane and endothelial layer in several lung diseases, which are characterised by recruitment of polymorphonuclear neutrophils (PMN) and parenchymal destruction [5–10, 11]. It is suggested that PMN play a major role in the pathogenesis of BOS [12, 13]; however, the role of MMP-9 to PMN in BOS remains unclear.

The major specific inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs). An imbalance in the MMP/TIMP ratio may be critical in bronchial tissue destruction and repair in patients with asthma, lung fibrosis, cystic fibrosis or chronic obstructive pulmonary disease [14–19]. Recently, it was suggested that an imbalance of MMP-9 and TIMP-1 may be involved in the development of BOS after LTX [20].

The current study investigated the hypothesis that the airway remodelling in BOS might be

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influenced by the balance of the MMP-9/TIMP-1 ratio. The kinetics of MMP-9 and TIMP-1 in bronchoalveolar lavage (BAL) samples during the development of BOS was investigated and it was hypothesised that PMN was the main source of MMP-9 in BOS.

METHODS

Patients

In a retrospective cohort study, patients who underwent a LTX from January 1995 up to January 2001 and regularly attended the post-operative follow-ups for $\geqslant$1 yr after LTX, were investigated. According to these criteria, 29 recipients were enrolled in this study. Informed consent was obtained from all patients. This study conforms to the Declaration of Helsinki. Patients received a standard triple immunosuppressive regimen consisting of cyclosporine A, adjusted to maintain whole blood levels 200–250 ng·mL^{-1} or tacrolimus (0.3–0.6 mg·kg^{-1}·day^{-1}), adjusted to maintain blood levels 5–15 ng·mL^{-1}, azathioprine (0.5–1 mg·kg^{-1}·day^{-1}) or mycophenolat-mofetil (2–3 g·day^{-1}), both adjusted to a white blood cell count >5.000·mm^{3}, and steroids, which were tapered over the time following transplantation. Episodes of acute cellular allograft rejection were treated with *i.v.* methylprednisolone pulse therapy (usually 10 mg·kg^{-1}·day^{-1} on three consecutive days). In case of resistance to therapy, rabbit-antithymocyte-globulins (Pasteur Merieux, Lyon, France) treatment was administered. Diagnosis of BOS was confirmed by histological findings if available, and/or by decline in lung function over time, as proposed by the International Society for Heart and Lung Transplantation [21]. The following groups were defined. 1) Non-BOS included clinically stable patients without signs of BOS; they did not show any irreversible alteration in lung function, defined by forced expiratory volume in one second (FEV_1) >80% of baseline (derived from the two best consecutive FEV_1 after LTX). 2) Pre-BOS/BOS included patients with BOS; they showed an irreversible decline in lung function, defined by an irreversible >20% decrease in FEV_1 of baseline during the course of the study, and/or confirmation by histopathological findings. BALs were divided according to whether they were obtained before diagnosis of BOS (pre-BOS, defined by FEV_1 >80% of baseline value at time point of bronchoscopy) or after the diagnosis of BOS was established (BOS group, defined by FEV_1 <80% at time point of bronchoscopy).

BALs were excluded from the study pool during episodes of acute rejection and pulmonary infections. The diagnosis of acute rejection was primarily based on pathological findings in transbronchial biopsies and/or typical clinical and radiological findings to support the diagnosis. BALs were only recruited in clinically stable status $\geqslant$3 months after the acute episode. The diagnosis of infection was primarily made by clinical findings (fever, chills, sore throat, coughing, roentgenological findings, *etc.*). When recipients were clinically asymptomatic, but had a positive BAL culture for bacteria, fungi or virus, BALs were included in the study pool.

Seven patients had to be excluded from study due to recurrent histories of infections and/or acute rejections, and one patient was excluded because of diagnosis of lymphoma in the lung 1 yr after LTX. Subsequently, a total of n=20 patients (n=12 non-BOS, n=8 BOS) and 145 BALs (n=79 non-BOS, n=28 pre-BOS, n=38 BOS group) were recruited for this study. For detailed patient characterisation, see table 1.

Post-operative follow-up

Patients were evaluated in $\leqslant$3-month intervals, whenever patients showed signs of clinical deterioration. Pulmonary function tests were performed at each presentation using a body plethysmograph (Masterscreen Body; Jager Toennis, Höchberg, Germany). A fiberoptic bronchoscopy follow-up with concurrent BAL was performed in a 3-month interval in the first year post-operatively, then later every 6 months, or whenever clinical parameters indicated a deterioration in patient status as previously described [22]. The BALs were taken from patients through a flexible bronchoscope wedged into a subsegmental bronchus of either the lingual or the right middle lobe. BALs were obtained by instilling a total volume of 200 mL of isotonic saline solution in sequential 20-mL aliquots. The total of the recovered fluid was pooled, mixed, and the volume was measured. Samples of fluid were processed immediately for cytological, microbiological and immunological analyses. Transbronchial biopsies were obtained when diagnosis remained unclear.

TABLE 1 Clinical characteristics of eight transplant recipients with and 12 without bronchiolitis obliterans syndrome (BOS)

	BOS	Non-BOS
Patients n	8	12
Sex F:M	3:5	6:6
Type of transplantation		
Single-lung	1	4
Double-lung	7	8
Heart-lung	0	0
Underlying disease		
COPD	2	2
α_1-Antitrypsin deficency	1	3
Primary pulmonary hypertension	1	1
Idiopathic lung fibrosis	1	3
Cystic fibrosis	3	1
Cor pulmonale	0	1
Hämosiderosis	0	1
Age yrs	45.0 (34.3–49.5)	37.5 (25.0–54.2)$^{\#}$
Days of follow-up	1288 (1116–1634)	886 (480–2303)¶
FEV_1 after follow-up % baseline value	46.5 (42.3–49.2)	85.5 (80–93.3)$^{+}$
BOS grade 0	0	12
BOS grade I	1	0
BOS grade II	1	0
BOS grade III	6	0
Histopathological confirmation	5	0

Results expressed as median (25–75th percentile) or n. F: female; M: male; COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in one second. $^{\#}$: p=0.652; ¶: p=0.894; $^{+}$: p<0.0001.

Immunocytochemistry

For immunocytochemistry, BAL cytospins were processed using an alkaline phosphatase-antialkaline phosphatase technique as previously described [23]. The monoclonal antibody used was anti-MMP-9, recognising the latent (92 kD) and active (83 kD) forms of MMP-9 (Merckbioscience, Darmstadt, Germany).

Concentration of BAL, protein quantification

According to the diluting effect of instillation fluid and the expected low concentration of MMP-9 and TIMP-1, the supernatants of BAL were concentrated ~3–5-fold by centrifugation at 4℃ using ultrafiltrators (Centriprep 10; Amicon, Redford, MA, USA) according to the manufacturer's recommendations. The total protein concentration of lavage was determined by the bicinchoninic acid protein assay (Sigma-Aldrich, St Louis, MO, USA), where a protein concentration of 0.7–2.1 $mg \cdot mL^{-1}$ was measured. Each sample of concentrated BAL was adjusted with distilled water to an equal protein concentration of 0.5 $mg \cdot mL^{-1}$.

MMP-9, TIMP-1 assay

MMP-9 and TIMP-1 in concentrated BAL fluid were determined by commercially available ELISA (Biotrak; Amersham Pharmacia, Braunschweig, Germany) according to the manufacturer's instructions. The total amount of free and complexed enzyme was measured, not the active MMP-9. The detection limits of the assays were 4–128 $ng \cdot mL^{-1}$ for MMP-9, and 3.1–100 $ng \cdot mL^{-1}$ for TIMP-1.

Zymography

MMPs were detected in the BAL by using their capacity to degrade gelatine, as previously described [6]. Molecular sizes of bands were identified by comparison with pre-stained standard protein, purified active MMP-9 and active MMP-2 (both BIOSCIENCE, La Jolla, Canada). The molecular weight of the gelatinolytic bands were estimated in relation to pre-stained low-range molecular weight markers (Bio-Rad laboratories, Hercules, CA, USA).

To confirm enzymatic activity was caused by MMPs and not by other gelatin degrading enzymes (such as serine proteases), selected gels were incubated with 10 mM ethylenediaminetetraacetic acid. MMPs are completely inhibited under these conditions (data not shown).

To confirm identity of MMPs, selected samples were activated with 2 mM p-amino-phenylmercuric acetate (APMA) and incubated for 2 h at 37℃ before zymography was performed. APMA activates latent MMPs by cleavage of the N-terminal domain. Each of the activated samples underwent characteristic band shift to a lower molecular-weight form with APMA (data not shown).

Statistical analysis

The data set consists of an unequal number of sporadically taken repeated measurements from each patient. The results for each patient are illustrated by averaged values. Monte Carlo methods were used to estimate the underlying distribution of the measurement values (MMP-9, TIMP-1, ratio of TIMP-1 to MMP-9 and cell differential) of the groups of patients (BOS, pre-BOS, non-BOS) by randomly choosing one observation for each patient in the group over 10,000 iterations.

Group values are presented by median values with 25th and 75th percentile for continuous variables, and proportions with 95% confidence interval (CI) for binary response. In paired observations (BOS group *versus* pre-BOS), the groups were compared using the Wilcoxon signed rank test, and for independent observations (BOS group *versus* non-BOS, pre-BOS *versus* non-BOS) using the Wilcoxon rank sum test or Fishers exact test (patient characteristics). Correlations were obtained by Spearmans rank correlation coefficient with 95% CI. Thereby, the percentile at zero of the simulated distribution corresponded to a p-value for a test for dependence. All tests were two-sided and the significance level was set to 5%.

RESULTS

Patients' characteristics

Patients were similar according to age, sex and time of follow-up after LTX. The values for FEV1 after follow-up were significantly lower in patients with BOS than patients without. Both groups differed in some underlying diseases, due to of the limited number of patients in this study (table 1). Median time of follow-up was 1,244 days (25–75th percentiles: 891–1667) during which a total of 145 BALs (median six tests per patient (5.75–8.5)) were obtained from 20 patients.

Differential cell count

The differential cell count of BALs showed a significantly elevated percentage of PMN in patients after manifestation of BOS compared with patients without BOS (BOS group *versus* non-BOS: 31% (11.5–54.5) *versus* 2% (1–5); p=0.005; fig. 1). The percentage of PMN was higher in patients with BOS compared with patients with pre-clinical stages of BOS (BOS group *versus* pre-BOS: 31% (11.5–54.5) *versus* 3% (1.5–9); p=0.047; fig. 2). There was a corresponding lower percentage of alveolar macrophages in BOS group compared with the other two groups, but no significant differences in percentages of eosinophils or lymphocytes (data not shown).

MMP-9 and TIMP-1

MMP-9 was significantly increased in patients after manifestation of BOS compared with patients without BOS (BOS *versus* non-BOS group: 53.35 $ng \cdot mL^{-1}$ (32.5–92.2) *versus* 18.83 $ng \cdot mL^{-1}$ (5.8–83.2); p=0.037). TIMP-1 was significantly decreased in patients after manifestation of BOS compared with patients without BOS (BOS *versus* non-BOS group: 21.48 (10.9–68.9) *versus* 95.01 $ng \cdot mL^{-1}$ (19.8–126.8); p=0.031). The ratio of MMP-9 to TIMP-1 was significantly higher in patients after manifestation of BOS than in patients without BOS (BOS *versus* non-BOS group: 2.14 (1.2–3.8) *versus* 0.26 (0.2–1.5); p=0.0003; figs 1 and 3).

In patients with BOS, there was a trend towards increased expression of MMP-9 in BALs obtained after manifestation of BOS, compared with BALs obtained during pre-clinical stages of BOS, (pre-BOS *versus* BOS group: 26.60 $ng \cdot mL^{-1}$ (5.0–43.7) *versus* 53.35 $ng \cdot mL^{-1}$ (32.5–92.2); p=0.28). A trend towards a decrease of TIMP-1 after manifestation of BOS was found

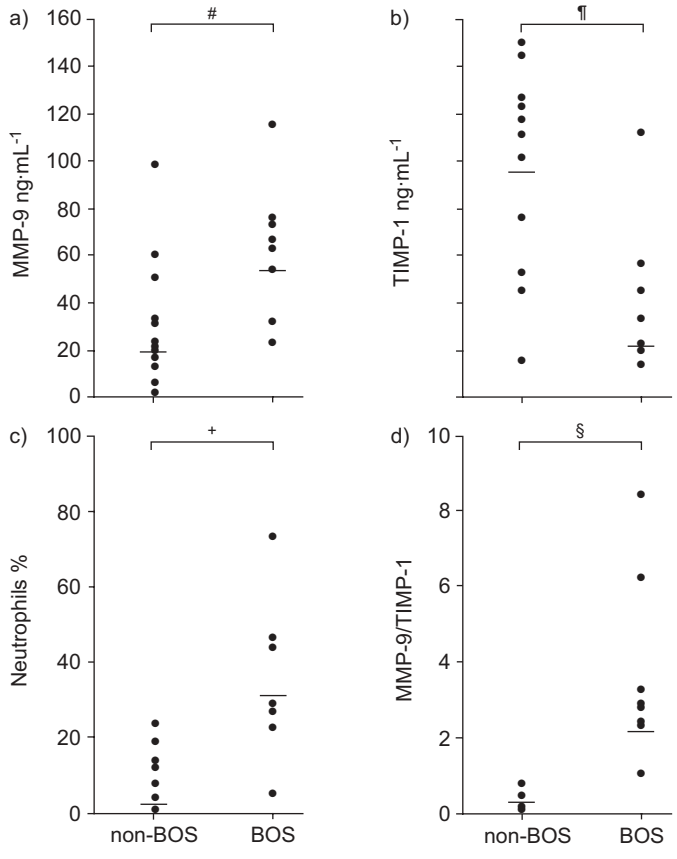

FIGURE 1. Mean concentration of a) matrix metalloproteinase (MMP)-9, b) tissue inhibitors of metalloproteinase (TIMP)-1, c) neutrophil count (percentage to the total cell count) and d) the ratio between MMP-9 and TIMP-1 in bronchoalveolar lavage samples from patients without bronchiolitis obliterans syndrome (BOS; non-BOS, n=12 patients) and patients with BOS (n=8 patients). ●: mean of all values of one patient; –: median of one group. #: p=0.037; ¶: p=0.031; +: p=0.005; §: p=0.0003.

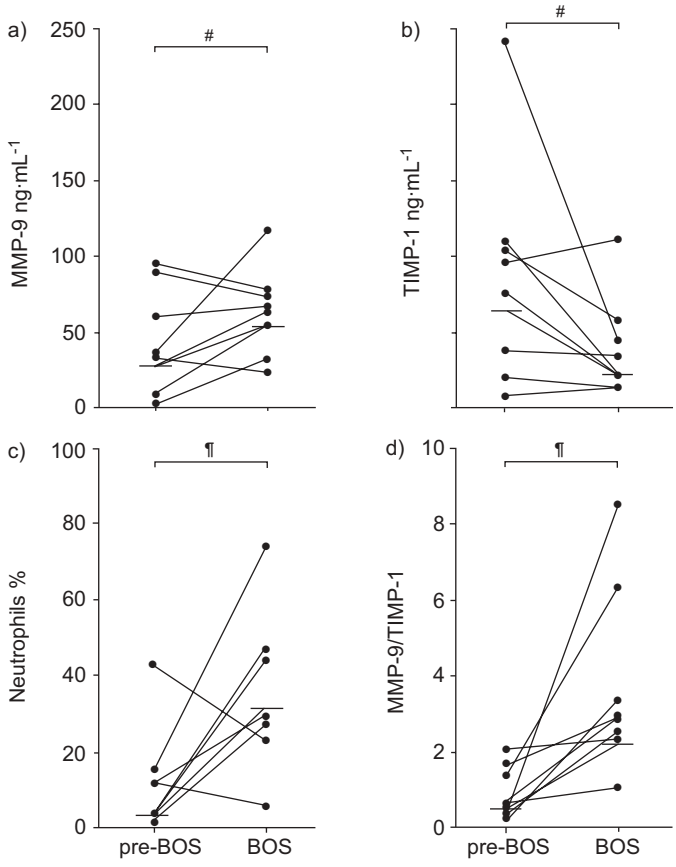

FIGURE 2. Mean concentrations of a) matrix metalloproteinase (MMP)-9, b) tissue inhibitors of metalloproteinase (TIMP)-1, c) neutrophil count (percentage to the total cell count) and d) ratio of MMP-9 to TIMP-1 in bronchoalveolar lavage samples from patients with pre-bronchiolitis obliterans syndrome (pre-BOS) or BOS, n=8 patients. ●: mean of all values of one patient; –: median of one group. #: p=nonsignificant difference; ¶: p=0.047.

(pre-BOS *versus* BOS group: 63.58 ng·mL^{-1} (62.9–126.4) *versus* 21.48 ng·mL^{-1} (10.9–68.9) p=0.17). However, there was a significant increase of the ratio of MMP-9 and TIMP-1 in BALs obtained after manifestation of BOS compared with pre-clinical stages of BOS (pre-BOS *versus* BOS group: 0.45 (0.1–0.5) *versus* 2.14 (1.2–3.8); p=0.047) (fig. 2). Ratio of MMP-9 and TIMP-1 during pre-BOS tended to be higher in comparison to non-BOS, but this observation was not significant (pre-BOS *versus* non-BOS) 0.45 (0.1–0.5) *versus* 0.26 (0.2–1.5); p=0.25).

Gelatinolytic activity

Zymographic analyses revealed an increased activity of MMP-9 in all patients with BOS compared with patients without BOS. Figure 4 demonstrates a representative zymographic analysis of a clinically stable patient without signs of BOS and a patient with BOS grade II. The zymographies of all patients with BOS presented predominately increased lytic bands of 92, 130, and 220 kD in most samples compared with patients without BOS. Furthermore, an 85-kD band corresponding to the activated form of MMP-9 was detected in 83.3% (62.5–87.5) of patients with BOS. There was a significantly lower prevalence of the 85 kD form in patients without BOS (18.2% (8.3–36.4; p=0.019)).

Correlation of MMP-9 to BAL cells

MMP-9 immunoreactivity in patients with BOS appeared to be primarily associated with PMN and not with macrophages or lymphocytes (fig. 5). MMP-9 was slightly, but significantly positively correlated with percentage of PMN in BAL cell count (r=0.470; p=0.016; fig. 6). A negative correlation of MMP-9 and percentage of lymphocytes (r=-0.333; p=0.014) was found. No correlation was seen between MMP-9 and macrophages (p=0.07), in addition, TIMP-1 was not correlated with any cellular compounds of BAL (p>0.05) and with MMP-9 (p=0.07).

Correlation to lung function

A mild, but significantly negative correlation between the ratio of MMP-9 to TIMP and relative FEV1 values was observed (r=-0.447, p=0.003; fig. 7). No significant correlation was found between lung function and either MMP-9 (p=0.31), TIMP-1 (p=0.17), relative PMN (p=0.09), macrophages (p=0.20), or lymphocyte count (p=0.26) of BAL cells.

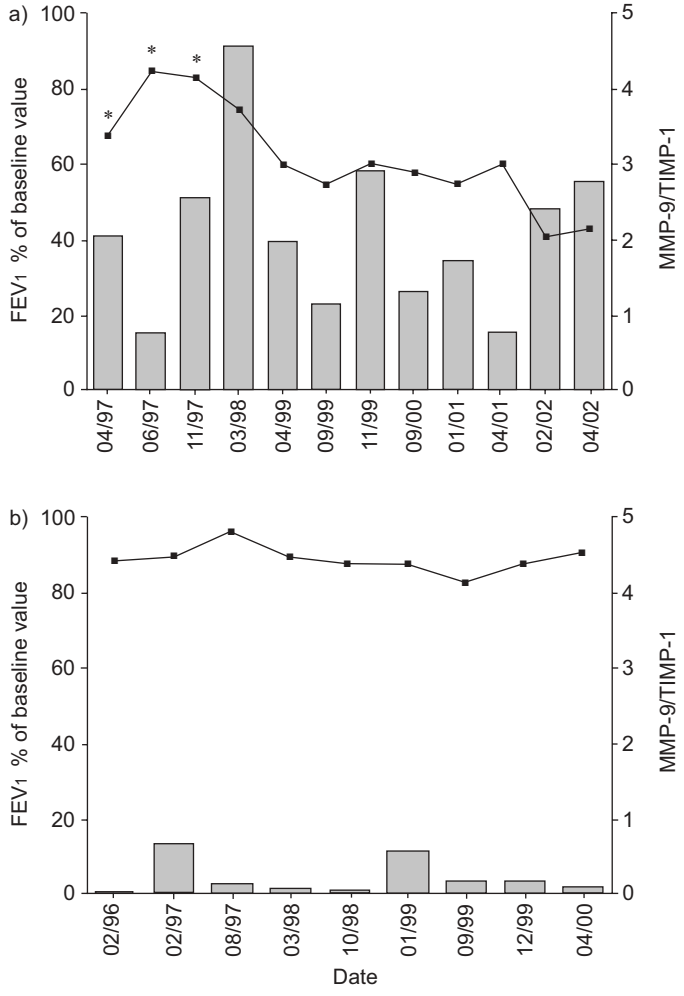

FIGURE 3. Representative diagram of a patient with bronchiolitis obliterans syndrome (BOS) grade III (a) and a clinical stable patient without BOS (b). The line indicates forced expiratory volume in one second (FEV_1) in percentage of baseline value during the follow-up. The columns depict the ratios of matrix metalloproteinase-9 (MMP-9) to tissue inhibitors of metalloproteinase (TIMP)-1. *: bronchoalveolar lavages at pre-BOS.

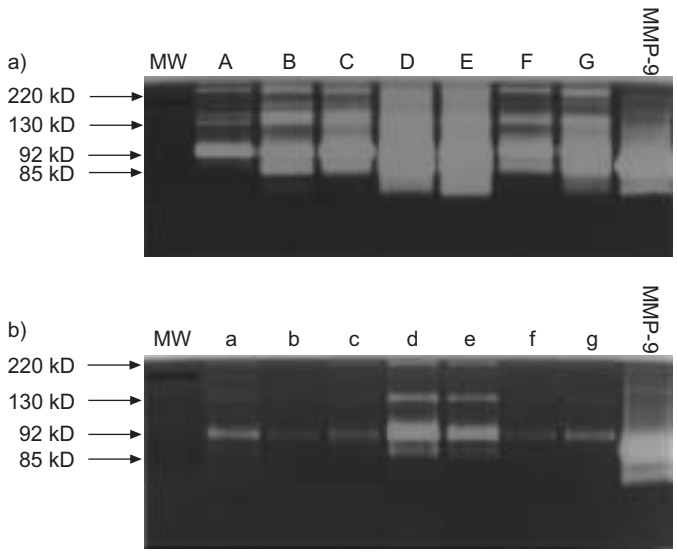

FIGURE 4. Representative gelatin zymographies of bronchoalveloar lavage (BAL) of a patient with bronchiolitis obliterans syndrome (BOS) grade II (a) and a clinical stable patient without BOS (b). a) Lanes A–G: BALs of a patient with BOS during follow-up after lung transplantation. Lanes A–C: pre-clinical stages of BOS (pre-BOS). Lanes D–G: stages after manifestation of BOS. b) Lanes a–g: BAL samples of a patient without BOS during follow-up after lung transplantation (non-BOS). MMP-9: positive control of active matrix metalloproteinase-9 (5 $\mu g \cdot lane^{-1}$).

DISCUSSION

This study found a significant increase of MMP-9 in BALs of patients with BOS compared with clinical stable recipients without BOS. The excess of MMP-9 was not counterbalanced by a simultaneous increase in TIMP-1. After onset of BOS, a significant increase in MMP-9/TIMP-1 ratio was seen, which was significantly negative correlated to lung function. There was a trend to a slightly increased ratio in pre-clinical stages of BOS compared with the clinically stable patients without BOS (not statistically significant). These results implicate a local imbalance between MMP-9 and TIMP-1 with an excess of MMP in the development of BOS compared with patients without BOS.

In this study, zymography analyses demonstrated the 85 kD active form of MMP-9 in the vast majority of patients with manifestation of BOS in contrast to patients without BOS. This finding reveals that proteases are overexpressed in BOS, leading to free activity in the alveolar compartment. Furthermore, zymographic analysis of patients with BOS revealed increased lytic bands at 95 kD, 130 kD and 220 kD, in contrast with clinical stable patients, where only faint bands were expressed. The overwhelming activity of MMP-9 in patients with BOS suggests that MMPs are not sufficiently inhibited. TIMP-1 is a 28.5 kD glycoprotein and known as a specific inhibitor of MMP-9. It inhibits the MMP-9 activity by forming a complex of 1:1 stoichiometry with MMP-9 [3]. Imbalances between MMP-9 and TIMP-1 may cause a proliferation of ECM, a well known crucial feature of several lung diseases [5, 9, 16–18].

These findings support data by TRELLO *et al*. [25], who found an increased gelatinolytic activity in BALs in stable lung transplant recipients. However, patients with BOS were excluded from this study. BEEH *et al*. [20] suggest the imbalance of MMP-9 and TIMP-1 is involved in the pathogenesis of BOS, although MMPs were detected only once at a random time point and in sputum samples, which is suggested not to reflect airway subepithelial proteinase activity [3].

The persistence of recruitment and activation of PMN in the alveolar space seems to play a major role in the pathogenesis of BOS [12, 13, 26]. In agreement with these observations, the current study has shown that an increased number of PMN in BALs is closely linked to the development and manifestation of BOS. However, a significant correlation between the number of PMN and lung function has been marginally missed. This may be due to the low number of patients in the current study. Some authors have proposed neutrophils as early and sensitive markers for the prediction of BOS [26, 27]. However, whether PMN qualify as early markers for BOS or whether they only reflect ongoing infections remains unclear [2]. Interestingly, a

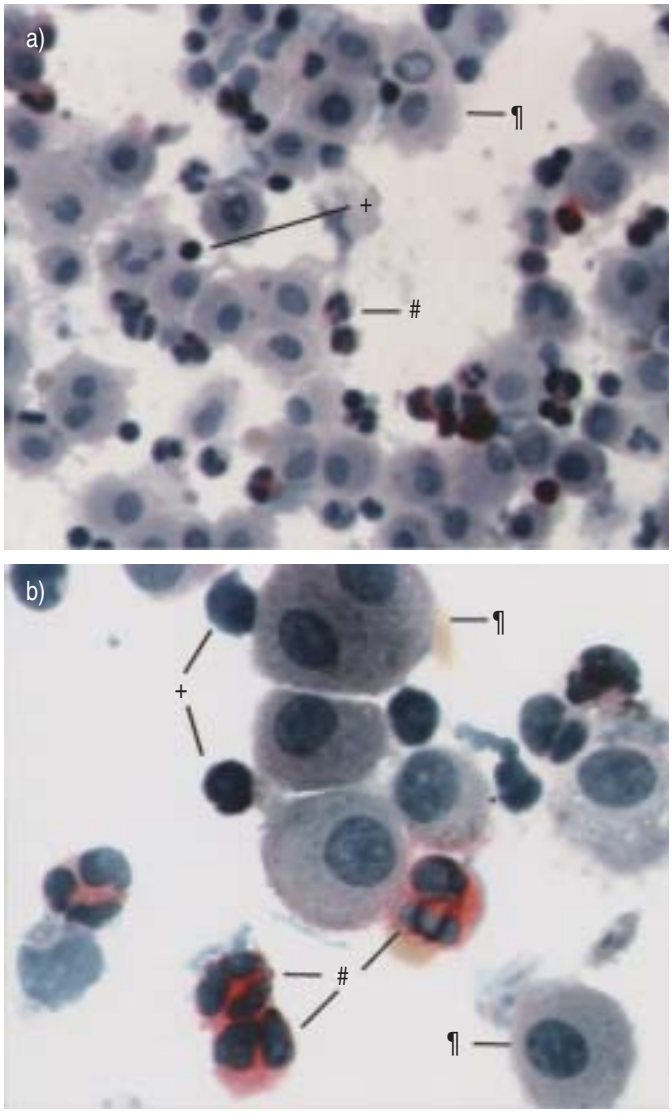

FIGURE 5. Representative immunocytochemical stainings of bronchoalveloar lavage cells of two patients after lung transplantation with bronchiolitis obliterans syndrome (stained with matrix metalloproteinase-9 antibody). #: positive stained polymorphonuclear neutrophils; ¶: macrophage; +: lymphocyte.

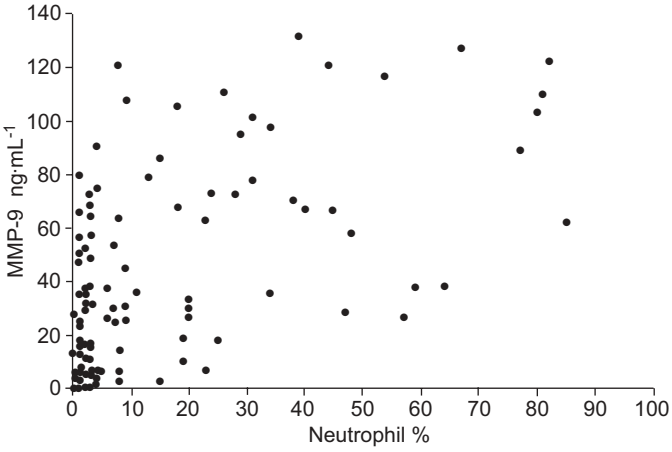

FIGURE 6. Correlation of matrix metalloproteinase (MMP)-9 and neutrophil count (percentage to the total cell count) in bronchoalveloar lavage of patients after lung transplantation (r=0.470; p=0.016).

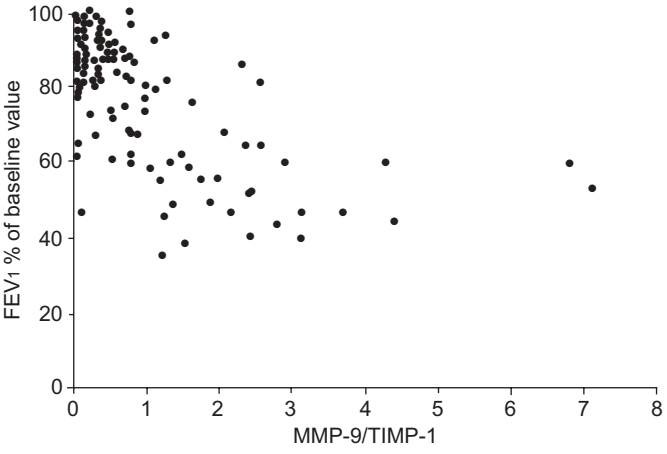

FIGURE 7. Correlation of ratio of matrix metalloproteinase (MMP)-9 to tissue inhibitors of metalloproteinase (TIMP)-1 in bronchoalveloar lavage after lung transplantation and lung function (values of forced expiratory volume in one second (FEV_1) in % of baseline value; r=-0.477; p=0.0032).

normal percentage of PMN in pre-BOS was found, which is in contrast to other authors [26, 27]. Further studies are needed to clarify this issue.

The origin of MMP-9 is controversial [3]. However, the results of the current study revealed PMN as the main source of MMP-9 in patients after LTX. First, a significant correlation between the percentage of PMN and the concentration of MMP-9 in BALs was found, with no positive correlation between MMP-9 and other cell compounds of BAL. These results are in accordance to recently published data [5, 6, 28]. Secondly, it was demonstrated in most zymographic analyses that the characteristic typical banding pattern of neutrophil-derived-MMP-9 consisted of the homodimeric form of MMP-9 (220 kD), the human neutrophil lipocalin-pro-MMP-9 complex (130 kD) and the pro-MMP-9 (92 kD). SUGA *et al*. [6] revealed in zymographic analyses with purified MMP-9 from neutrophils a similar characteristic banding pattern. Thirdly, the current study found MMP-9 immunoreactivity to be primarily associated with neutrophils and not with other compounds of BAL cells. However, it could be possible that a lack of immunostaining was based on a too prompt release of MMP-9 from macrophages, eosinophils or lymphocytes, so MMP-9 were not detected in association with these cells. More studies are necessary to evaluate MMP-9 expression by resident airway cells, such as epithelial cells, endothelial cells and fibroblasts, which may also contribute to MMP-9 expression in the airways. Nonetheless, data from the current study strongly suggest that airway neutrophils are a primary source of MMP-9 in these patients.

Despite solid statistical analysis, data from the current study need careful interpretation. It is possible that the development of BOS is the result of another mediator released by PMNs that

was not measured in this study. One limitation represents the retrospective study design, so a selection bias cannot be definitely excluded and only data were examined that were recorded in the patients charts. Although patient data are carried by a large number of BALs, total number of patients was limited. The current authors propose a large controlled prospective study for evaluation of MMP-9/TIMP as a predictive marker for the development of BOS.

In summary, an increased concentration of matrix metalloproteinase-9 without a counterbalancing increase of tissue inhibitors of metalloproteinase-1 in bronchiolitis obliterans syndrome after lung transplantation was found. It seems that the ratio of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinase-1 may play a role as a diagnostic tool of bronchiolitis obliterans syndrome. Furthermore, matrix metalloproteinase-9 expression was shown to be strongly associated to polymorphonuclear neutrophils. It seems that a cycle of release of proinflammatory cytokines stimulates polymorphonuclear neutrophils in the alveolar/bronchial compartment and that matrix metalloproteinase-9 expression leads to aggravation of the inflammatory response, both resulting in extracellular matrix proliferation.

REFERENCES

1 Trulock EP, Edwards LB, Taylor DO, *et al.* The Registry of the International Society for Heart and Lung Transplantation: Twentieth official adult lung and heart-lung transplant report 2003. *J Heart Lung Transplant* 2003; 22: 625–635.

2 Boehler A, Estenne M. Post-transplant bronchiolitis obliterans. *Eur Respir J* 2003; 22: 1007–1018.

3 Atkinson JJ, Senior RM. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol* 2003; 28: 12–24.

4 Parks WC. Matrix metalloproteinases in lung repair. *Eur Respir J* 2003; 22: Suppl. 44, 36s–38s.

5 Boulay ME, Prince P, Deschesnes F, Chakir J, Boulet LP. Metalloproteinase-9 in induced sputum correlates with the severity of the late allergen-induced asthmatic response. *Respiration* 2004; 71: 216–224.

6 Suga M, Iyonaga K, Okamoto T, *et al.* Characteristic elevation of matrix metalloproteinase activity in idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2000; 162: 1949–1956.

7 Cataldo DD, Gueders MM, Rocks N, *et al.* Pathogenic role of matrix metalloproteases and their inhibitors in asthma and chronic obstructive pulmonary disease and therapeutic relevance of matrix metalloproteases inhibitors. *Cell Mol Biol (Noisy-le-grand)* 2003; 49: 875–884.

8 Torii K, Iida K, Miyazaki Y, *et al.* Higher concentrations of matrix metalloproteinases in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. *Am J Respir Crit Care Med* 1997; 155: 43–46.

9 Hartog CM, Wermelt JA, Sommerfeld CO, Eichler W, Dalhoff K, Braun J. Pulmonary matrix metalloproteinase excess in hospital-acquired pneumonia. *Am J Respir Crit Care Med* 2003; 167: 593–598.

10 Delacourt C, Le Bourgeois M, D'Ortho MP, *et al.* Imbalance between 95 kDa type IV collagenase and tissue inhibitor of metalloproteinases in sputum of patients with cystic fibrosis. *Am J Respir Crit Care Med* 1995; 152: 765–774.

11 Pugin J, Widmer MC, Kossodo S, Liang CM, Preas HLN, Suffredini AF. Human neutrophils secrete gelatinase B *in vitro* and *in vivo* in response to endotoxin and proinflammatory mediators. *Am J Respir Cell Mol Biol* 1999; 20: 458–464.

12 Riise GC, Williams A, Kjellstrom C, Schersten H, Andersson BA, Kelly FJ. Bronchiolitis obliterans syndrome in lung transplant recipients is associated with increased neutrophil activity and decreased antioxidant status in the lung. *Eur Respir J* 1998; 12: 82–88.

13 DiGiovine B, Lynch JP. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. *J Immunol* 1996; 157: 4194–4202.

14 Mao JT, Tashkin DP, Belloni PN, Baileyhealy I, Baratelli F, Roth MD. All-trans retinoic acid modulates the balance of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with emphysema. *Chest* 2003; 124: 1724–1732.

15 Ruiz V, Ordonez RM, Berumen J, *et al.* Unbalanced collagenases/TIMP-1 expression and epithelial apoptosis in experimental lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2003; 285: L1026–L1036.

16 Choi KH, Lee HB, Jeong MY, *et al.* The role of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in cryptogenic organizing pneumonia. *Chest* 2002; 121: 1478–1485.

17 Ratjen F, Hartog CM, Paul K, Wermelt J, Braun J. Matrix metalloproteases in BAL fluid of patients with cystic fibrosis and their modulation by treatment with dornase alpha. *Thorax* 2002; 57: 930–934.

18 Ekekezie II, Thibeault DW, Simon SD, *et al.* Low levels of tissue inhibitors of metalloproteinases with a high matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio are present in tracheal aspirate fluids of infants who develop chronic lung disease. *Pediatrics* 2004; 113: 1709–1714.

19 Tanaka H, Miyazaki N, Oashi K, Tanaka S, Ohmichi M, Abe S. Sputum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio in acute asthma. *J Allergy Clin Immunol* 2000; 105: 900–905.

20 Beeh KM, Beier J, Kornmann O, Micke P, Buhl R. Sputum levels of metalloproteinase-9 and tissue inhibitor of metalloproteinase-1, and their ratio correlate with airway obstruction in lung transplant recipients: relation to tumor necrosis factor-alpha and interleukin-10. *J Heart Lung Transplant* 2001; 20: 1144–1151.

21 Cooper JD, Billingham M, Egan T, *et al.* A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 1993; 12: 713–716.

22 Tiroke AH, Bewig B, Haverich A. Bronchoalveolar lavage in lung transplantation. State of the art. *Clin Transplant* 1999; 13: 131–157.

23 Kelly EA, Busse WW, Jarjour NN. Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med* 2000; 162: 1157–1161.

24 Yano M, Omoto Y, Yamakawa Y, *et al*. Increased matrix metalloproteinase 9 activity and mRNA expression in lung ischemia-reperfusion injury. *J Heart Lung Transplant* 2001; 20: 679–686.
25 Trello CA, Williams DA, Keller CA, Crim C, Webster RO, Ohar JA. Increased gelatinolytic activity in bronchoalveolar lavage fluid in stable lung transplant recipients. *Am J Respir Crit Care Med* 1997; 156: 1978–1986.
26 Devouassoux G, Drouet C, Pin I, *et al*. Alveolar neutrophilia is a predictor for the bronchiolitis obliterans syndrome, and increases with degree of severity. *Transpl Immunol* 2002; 10: 303–310.
27 Reynaud-Gaubert M, Marin V, Thirion X, *et al*. Upregulation of chemokines in bronchoalveolar lavage fluid as a predictive marker of post-transplant airway obliteration. *J Heart Lung Transplant* 2002; 21: 721–730.
28 Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *J Allergy Clin Immunol* 2003; 112: 1064–1071.