

TUESDAY, SEPTEMBER 5TH 2006

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### 309. Usefulness of bronchoalveolar lavage (BAL) in interstitial lung diseases

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**Contribution of BAL proteomic analysis in sarcoidosis (S) and in idiopathic pulmonary fibrosis (IPF/UIP)**

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In previous studies we analysed BAL protein composition of BAL from sarcoidosis, idiopathic pulmonary fibrosis and systemic sclerosis patients finding different protein profiles in each disease.(1,2) The aim of this study was to analyse the expression of groups of proteins involved in specific functions (such as immune response, protease/antiprotease or oxidant/antioxidant or coagulation systems). BAL from 9 patients with sarcoidosis, 11 with idiopathic pulmonary fibrosis (IPF/UIP) and 5 healthy controls was studied by 2D electrophoresis and MALDI-TOF-MS or immunoblotting to identify protein composition. The proteomic approach to the analysis of BAL enables us to identify groups of proteins potentially involved in the pathogenesis of these diseases, some of these proteins have a known function and role, as prothrombin significantly increased in IPF compared to controls ( $p < 0.05$ ) or alpha1B glycoprotein, significantly increased in S compared to controls ( $p < 0.01$ ), other proteins have not yet been particularly studied, such as calgranulin B and galectin, two low molecular weight proteins significantly increased in IPF compared to controls. ( $p < 0.05$ ). In S a possible pathogenetic role for antiproteases, in particular AAT, was indicated by increased levels of dimers (immunefunction?) significantly increased compared to controls and presence of fragments of this protein that resulted also an oxidation target. In conclusion proteomic approach to the study of ILD can be a valid aid to further characterize alveolar microenvironment

1) Rottoli P, Magi B, et al. *Proteomics* 2005, 5: 1423-1430.

2) Rottoli, Magi B, *Proteomics* 2005, 5: 2612-2618.

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**MMPs in BAL from patients with scleroderma fibrosing alveolitis**

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Extracellular matrix (ECM) proteins are degraded by matrix metalloproteinases (MMPs). Concentrations of various MMPs and their major inhibitor TIMP-1 have been shown to be elevated in serum from patients with fibrosing alveolitis due to systemic sclerosis (FASSc) and in BAL from other interstitial lung diseases. BAL fluid stored at  $-70^{\circ}\text{C}$  from 34 FASSc patients (lifetime non-smokers, 6 male, mean age 47y, mean disease duration 3.6y) who underwent BAL during routine clinical assessment were thawed and analysed by ELISA for MMP-2, -8, -9 and TIMP-1. 10 normal controls were used for comparison. Concentrations of MMP-8 and TIMP-1 were significantly elevated in FASSc BAL ( $3.0 \pm 13.5$  vs  $0.1 \pm 0.1$  ng/ml; and  $4.8 \pm 4.2$  vs  $1.8 \pm 0.8$  ng/ml [mean  $\pm$  SD,  $p < 0.01$  and  $< 0.05$  respectively]). No significant difference was observed for BAL MMP-2 and MMP-9. MMP-8 was notable by its negative correlation with all pulmonary function parameters in FASSc: % FEV<sub>1</sub> ( $r_s = -0.49$ ,  $p < 0.05$ ); % FVC ( $r_s = -0.38$ ,  $p < 0.05$ ); and % TLCO ( $r_s = -0.34$ ,  $p < 0.05$ ). These findings suggest an association between elevated BAL MMP-8 levels and reduced pulmonary function. The elevation in TIMP-1 suggests that this mechanism is important in attempting to attenuate MMP-8 induced ECM disruption. The results concur with the finding by others of increased MMP-8 in BAL from other fibrotic diseases such as idiopathic pulmonary fibrosis (IPF) and sarcoidosis<sup>1</sup>.

(1) Henry, M. T.; McMahon, K.; Mackarel, A. J.; Prikk, K.; Sorsa, T.; Maisi, P.; Sepper, R.; Fitzgerald, M. X.; O'Connor, C. M. Matrix Metalloproteinases and Tissue Inhibitor of Metalloproteinase-1 in Sarcoidosis and IPF. *Eur. Respir. J.* 2002, 20, 1220-1227.

Raynaud's and Scleroderma Association.

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**Increased expression of tumor necrosis factor receptors by bronchoalveolar cells in cryptogenic organising pneumonia**Qiao Ye<sup>1</sup>, Ulrich Costabel<sup>1</sup>, Josune Guzman<sup>2</sup>. <sup>1</sup>Department of Pneumology and Allergy, Ruhrlandklinik, Essen, Germany; <sup>2</sup>General and Experimental Pathology, Ruhr University, Bochum, Germany

TNF receptors (TNFR1 and TNFR2) and Fas are associated with apoptosis signalling and have been implicated in the pathogenesis of interstitial lung disease. Clinically, patients with cryptogenic organising pneumonia (COP) usually achieve a good response to corticosteroid therapy and subsequently have a much better prognosis compared to idiopathic pulmonary fibrosis (IPF). This may be due to the different pathogenesis. This study aimed to investigate the expression of TNFR1, TNFR2 and Fas by bronchoalveolar lavage (BAL) cells in patients with COP compared with IPF and controls.

Using immunocytochemistry, the expression of TNFR1, TNFR2 and Fas on BAL macrophages and lymphocytes was analysed in 9 patients with COP, 10 patients with IPF and 12 controls.

TNFR1 and Fas expression by alveolar macrophages (AM) was significantly higher in COP than in controls and IPF (see table, mean±SEM). The expression of TNFR2 by AM was increased in COP compared to controls. The expression of TNFR2 and Fas by lymphocytes was also significantly higher in COP than in controls and in IPF. There were significant correlations between the expression of TNFR1, TNFR2 and Fas by BAL macrophages. In addition, all three receptors positively correlated with the BAL lymphocyte percentages.

	COP	IPF	Controls
TNFR1 <sup>+</sup> AM, %	42 ± 8*** <sup>#</sup>	19 ± 4	12 ± 3
TNFR2 <sup>+</sup> AM, %	29 ± 7*	22 ± 4	12 ± 4
Fas <sup>+</sup> AM, %	46 ± 8*** <sup>#</sup>	25 ± 3	20 ± 4
TNFR2 <sup>+</sup> lym, %	49 ± 9*** <sup>#</sup>	29 ± 4	20 ± 5
Fas <sup>+</sup> lym, %	49 ± 7*** <sup>#</sup>	33 ± 3	27 ± 3

\*: p<0.05, \*\*: p<0.01 (COP vs controls);<sup>#</sup>: p<0.05, <sup>##</sup>: p<0.01 (COP vs IPF).

This study indicates upregulation of TNF receptors and Fas by BAL cells in patients with COP.

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**Long term follow up of the whole lung lavage programme in patients with pulmonary alveolar proteinosis: an update**

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Pulmonary alveolar proteinosis (PAP) is a rare lung disease characterized by respiratory insufficiency due to pulmonary surfactant accumulation, and is currently treated by whole lung lavage (WLL). We have previously reported on the long term benefit of WLL in a large cohort of PAP patients followed in Pavia, Italy, from 1990 to 2001 (Eur. Resp. J. 2004; 23: 526-31). We now report the longitudinal follow up of these and additional patients through 2005. Forty eight patients seen during this period received a diagnosis of PAP ((47 primary or idiopathic (37 M, 10 F); 1 secondary)). Thirty seven patients with primary PAP (40 ± 11 years of age at diagnosis) were entered into the longitudinal study and comprise the study group. Thirteen patients (35%; 7 M, 6 F) had mild symptoms not requiring therapy while 24 (65%; 20 M, 4 F) received WLL therapy. In 18 (51%; 15 M, 3 F), one WLL treatment was sufficient for disease resolution or sustained improvement. Six patients (16%; 5 M, 1 F) required repeated WLL treatments (4 in three patients, 2 in four patients). One subject with a poor clinical response to repeated (n=4) WLL treatments is currently undergoing plasmapheresis therapy. While useful in distinguishing primary from secondary PAP, baseline serum anti-GM-CSF antibody titers were similar in the untreated (296±237 µg/ml) and treated (396±269 µg/ml) patients, and did not predict the need for WLL therapy. Overall, long term follow up shows that WLL was effective in producing a lasting symptom-free period in 70% of patients with primary PAP and that most patients experienced benefit, as determined by spirometry, in the period immediately following WLL.

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**Lysozyme level and activity are decreased in the lungs of patients with idiopathic pulmonary fibrosis (IPF) and potentially contribute to increased susceptibility to pulmonary infection**

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Patients with end-stage Idiopathic Pulmonary Fibrosis (IPF) are prone to recurrent

and life-threatening pulmonary infections. In the present study we investigated potential mechanisms underlying this increased susceptibility, with special emphasis on the components of the innate host defense system, in particular on antimicrobial proteins of the lung.

We analyzed the expression pattern of different antimicrobial proteins (lysozyme, lactoferrin, lactoperoxidase, β-defensins) in the bronchoalveolar lavage fluid and in lung tissue of IPF patients on the RNA (Real-time PCR) as well as on the protein level (western blotting, ELISA, immunohistochemistry, activity assays). We found significantly decreased lysozyme levels in the bronchoalveolar lavage (BAL) fluid of IPF patients as compared to healthy controls. This was associated with a reduced lysozyme enzyme (muramidase) activity and an impaired bactericidal activity of these BAL fluids against different clinically relevant gram-positive and gram-negative pathogens in vitro. In contrast, levels of other antimicrobial proteins were not significantly altered in IPF lungs as compared to healthy controls. We conclude that decreased lysozyme levels and activity in the lung might be an important underlying mechanism for the increased susceptibility of IPF patients to pulmonary infections.

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**Identification of four proteins from a "sarcoidosis-associated protein profile": SELDI-TOF MS analysis of bronchoalveolar lavage fluid (BALF)**

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Investigating the sarcoid proteome, we have explored the differential protein profile (PP) of BALF from sarcoidosis patients (S, n=65) and healthy controls (C, n=23) using Surface Enhanced Laser Desorption/Ionization-Time Of Flight Mass Spectroscopy (SELDI-TOF MS). From 40 protein entities (2.75-185.62 kDa) differentially expressed in S vs. C, we have already reported identification of human serum albumin, alpha-1-antitrypsin and protocadherin-2 precursor (1). Aiming at identifying further proteins from the "sarcoidosis-associated PP" we have, in the current study, fractionated BALF samples by reverse-phase chromatography; the fractions were analysed by SDS-PAGE. The identity of 4 proteins, belonging to the cytokine, chemokine and anti-protease groups, was revealed by peptide mapping and confirmed by immunodepletion analysis. The protein with m/z (mass/charge ratio) 7.85 kDa was found upregulated in patients with parenchymal involvement (CXR-stage III); two proteins (m/z 11.73 and 79.37 kDa) were upregulated in S as a whole and the last protein with m/z 97.34 kDa was found downregulated in patients with CXR-stages I,II. These data support the concept of differential expression of proteins in distinct clinical subtypes of sarcoidosis.

Support: GA CR 310/05/2614, IGA MZ CR NR/9037, BMBF.

(1) Kriegova E, Melle C, Kolek V et al. AJRCCM in press; doi:10.1164/rccm.200507-1126OC.

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**The role of apoptosis in idiopathic pulmonary alveolar proteinosis**

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**Purpose:** Idiopathic pulmonary alveolar proteinosis (iPAP) is regarded as an autoimmune disease. Previous studies indicated that bronchoalveolar lavage fluid (BALF) levels of chemokines, surfactant protein D (SP-D) and KL-6 were significantly higher in patients with iPAP than in those with interstitial lung diseases, including idiopathic pulmonary fibrosis (IPF), interstitial pneumonitis associated with collagen vascular diseases (CVD), and sarcoidosis. However, lung inflammation has not yet been demonstrated in iPAP. Accordingly, it is plausible that apoptosis of pulmonary macrophages may play a role in iPAP.

**Design:** A prospective study

**Patients and methods:** Tumor necrosis factor receptor (TNFR)-I, TNFR-II, soluble Fas (sFas) receptor, sFas ligand, transforming growth factor (TGF)-β1 and TGF-β2 were measured using enzyme-linked immunosorbent assays in the BALF of 13 iPAP patients. To serve as disease controls, BALF was obtained from 20 patients with interstitial pneumonitis associated with CVD, 13 with IPF and 15 with sarcoidosis. To serve as lung controls, 17 patients without lung diseases were included.

**Results:** Compared with those of disease controls and lung controls, iPAP patients had significantly higher BALF levels of TNFR-I, TNFR-II, sFas receptor, sFas ligand, TGF-β1 and TGF-β2. In iPAP patients, positive correlations were found among BALF levels of TNFR-I, TNFR-II, sFas receptor, sFas ligand and TGF-β2. In addition, the values of alveolar-arterial PO2 difference were highly correlated with all variables measured in BALF.

**Conclusions:** Apoptosis may be involved in decreasing lung inflammation in iPAP.

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**Changes of BAL findings as no progression marker in idiopathic pulmonary fibrosis (IPF) patients treated with IFN-g**

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As no efficacious drugs are currently available for IPF, IFN-g-based regimens are more recently under investigation. Eight non-smoker male patients (mean age  $53\text{yrs} \pm 4$ ) affected by steroid not-responsive IPF, attending our Division, were treated with IFN-g 1b, 200 mcg three times/week s.c. Lung function testing and BAL collection were performed before (T0) and after 6 months (T1) of therapy. BAL cell differentials and T cell-subsets were assessed by flow cytometry. Mean FVC, TLC and  $\text{TLC}_{\text{CO-sb}}$  values at T0 were  $75 \pm 9$ ,  $66 \pm 9$ , and  $42 \pm 15$  (% of predicted). Mean  $\text{PaO}_2$  was  $70 \pm 12$  mmHg at rest (21%  $\text{FiO}_2$ ) with a  $\text{D(A-a)O}_2$  of  $29 \pm 14$ . Lung function tests and gases exchanges at T1 showed no significant variations. Mean BAL total cells were  $2.7 \pm 1.2 \times 10^5/\text{ml}$  at T0. Percentages of neutrophils and eosinophils were  $18 \pm 21$  and  $3.6 \pm 6$ . Lymphocytes were  $11 \pm 6$  ( $\text{CD4/CD8}$  ratio =  $0.8 \pm 0.6$ ). Mean BAL total cells were decreased at T1 ( $1.9 \pm 0.6 \times 10^5/\text{ml}$ ,  $p = \text{ns}$ ). Neutrophils and eosinophils were  $9.8 \pm 8$  and  $4 \pm 6$  ( $p = \text{ns}$ ). Lymphocytes were  $7 \pm 6$  ( $p < 0.05$ ) with  $\text{CD4/CD8} = 1 \pm 0.3$  ( $p = \text{ns}$ ). Neutrophils and eosinophils were inversely correlated with TLC and FVC at T0 ( $R = -0.7$  in all instances). A negative correlation was also found between neutrophils with  $\text{TLC}_{\text{CO-sb}}$  at T0 ( $R = -0.7$ ) and between T0-neutrophils and eosinophils with T1-TLC ( $R = -0.67$  and  $-0.69$ ). Neutrophils correlated with  $\text{D(A-a)O}_2$  ( $R = 0.8$ ) at T1. We confirm that mixed neutrophilic and eosinophilic alveolitis is related with a poor lung function in IPF patients. Although further studies are needed, our findings of no deterioration of lung function in association with the reduction trend of BAL total cells and neutrophils are suggestive of a quite good response to IFN-g.