Abstract. Background: Dysregulation of matrix metalloproteinases (MMPs) has been implicated in lung injury associated with inflammatory disorders and several lung diseases such as pulmonary fibrosis.

Objective: We studied a murine model of lipopolysaccharide (LPS)-induced chronic inflammation in order to analyse the relationship between MMP activity in bronchoalveolar lavage fluid and collagen deposition in lung tissue. BP2 mice were exposed to repeated aerosols of LPS of E. coli for 8 months.

Results: The inflammatory reaction induced by LPS increased throughout the time of exposure and was associated after 10 weeks with collagen deposition in the alveolar walls. Meantime, we observed in BAL fluid from LPS-exposed mice an early induction of MMP-9 correlated with neutrophil recruitment. MMP-2 increased during the early inflammatory phase, and also during the development of the fibrotic phase.

Conclusion: Repeated exposure of mice to an aerosol of LPS can lead to pulmonary interstitial fibrosis and MMPs seem to be associated with this process.

Key words: Collagen deposition – LPS – Chronic inflammation – Lung – Metalloproteinase – Neutrophil

Introduction

Fibrosis is characterized by a qualitative and quantitative alteration of extracellular matrix (ECM) deposition with an accumulation of mesenchymal cells which replace normal tissue. However, the biochemical and cellular mechanisms involved in the development of pulmonary fibrosis remain poorly understood. It has been suggested that acute and chronic lung injury associated with pulmonary inflammation participate in the pathogenesis of fibrosis, either of known aetiology (asbestos inhalation, bleomycin toxicity, paraquat ingestion) or unknown causes (idiopathic pulmonary fibrosis [IPF]) [1].

Pulmonary inflammation is characterized by an enhanced activation of inflammatory cells, either recruited in the pulmonary tissue, such as polymorphonuclear neutrophils, or resident in the alveolar space, such as alveolar macrophages. The enhanced activation of the inflammatory cells is involved in the disruption of the tissue through the release of mediators, namely reactive oxygen species (H$_2$O$_2$, O$_2^–$, OH, …), cytokines (tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 … ) and proteases that injure cellular and extracellular components [2]. The recruitment of mesenchymal cells, such as fibroblasts is also induced by the release of cytokines which allow the migration and proliferation of these cells. Finally, the local production of ECM by these accumulating mesenchymal cells leads to the progressive deposition of collagens, elastin, fibronectin and proteoglycans [3].

Inflammatory cells may accomplish some of their critical functions by releasing degradative enzymes into the pericellular space [2]. Among the group of proteolytic enzymes, the matrix metalloproteinases (MMPs) constitute a family of Zn-dependent endopeptidases responsible for the degradation and turn over of the ECM (for review see 3). The MMPs can cleave most of the constituents of the ECM including collagen, proteoglycan, laminin, fibronectin and elastin. These enzymes which are secreted in a latent, pro-enzyme form, require activation to digest an ECM and are inhibited by tissue inhibitors of metalloproteinases [4]. MMPs are expressed at low levels in normal adult tissues and their upregulation appears to play an important role in the development of numerous pathological processes of the respiratory system [5]. Among the MMPs, gelatinase A, a 72-kDa type IV collagenase/MMP-2 and gelatinase B, a 92-kDa type IV colla-
genase/MMP-9, degrade denatured collagen [4, 5]. It has been suggested that MMP-2 and MMP-9 contribute to acute lung damage by facilitating the migration of inflammatory cells, as well as to the disruption of basement membrane components and extracellular matrix remodeling [5]. MMP-2 and MMP-9 also differ in their cellular distribution. MMP-9 is the form mainly found in polymorphonuclear leukocytes and is also the major MMP produced by macrophages. By comparison fibroblasts and other connective tissue cells preferentially produce MMP-2 [4]. An important role of the recruited polymorphonuclear neutrophils and MMP activity in the bronchoalveolar lavage (BAL) fluid in the development of damage to alveolar ECM has been suggested [6]. Moreover, in vivo administration of endotoxin, in various animal species induced a sustained inflammation characterized by a recruitment of neutrophils which was accompanied by an increase of MMP-2 and MMP-9 activities in the BAL fluid [7, 8, 9]. Finally, it has been reported that MMP levels were significantly higher in BAL fluid from patients with ARDS compared with healthy volunteer control subjects [10].

Therefore, it is suggested that pulmonary fibrosis may be initiated during the acute phase of lung injury which can evolve into a chronic pulmonary inflammation. We hypothesized that chronic pulmonary inflammation involving massive neutrophil recruitment and a dysregulation of the MMP activity could lead to pulmonary fibrosis. The purpose of this study was to analyze collagen deposition in lung sections and MMP activity in BAL fluid in a murine model in which chronic inflammation was induced by repeated bacterial endotoxin (lipopolysaccharide, LPS) aerosol administration.

Materials and methods

Reagents

Lipopolysaccharide (LPS) of *Escherichia coli* (0.55 BS), hydroxyproline, gelatin, ethylene diamine tetraacetic acid (EDTA), phenanthroline, N-ethylmaleimide (NEM), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). May-Grünewald and Giemsa stains were obtained from RAL (Paris, France). Sodium pentobarbital came from Sanofi santé animale (Libourne, France); RPMI 1640, penicillin, streptomycin, L-glutamine, sodium dodecyl sulfate (SDS) and TRIS solution were from Eurobio (Les Ulis, France). Reagents for electrophoresis and transfer were purchased from Sigma (St. Louis, MO, USA). SDS-PAGE (polyacrylamide gel electrophoresis) protein molecular weight markers (100–225 kDa) (Amersham, Little Chalfont, UK), conditioned media from 3T3 fibroblasts, which contain high level of MMP-2 (13), and recombinant human MMP-2 and MMP-9. Enzyme amount was quantified by measuring the intensity of the negative bands using a densitometer analyzer "Densylab" software package (Bioprobe Systems, Les Ulis, France). Results were expressed as a percentage of the intensity of the band of migration of a sample loaded onto each gel. A sample was used as internal standard in order to normalize the loading for the zymograms and to allow comparison between all the zymograms.

Zymography

Proteases were detected by their capacity to degrade specific substrates as previously described [12]. Aliquots of BAL fluid were loaded onto a 4.5% acrylamide stacking gel/7% separating acrylamide gel containing 1 mg/ml of gelatin, in the presence of sodium dodecyl sulfate (SDS) and subjected to electrophoresis. Gels were then washed twice with 2.5% triton X-100, rinsed with water and incubated at 37°C overnight in an activation buffer containing 50 mM Tris, 2 mM CaCl₂, 2 mM ZnCl₂, pH 8. After incubation, the gels were stained with Coomassie Brilliant Blue and destained in a solution of 25% ethanol and 10% acetic acid. Zones of enzymatic activity were revealed by negative staining: proteolysis areas appeared as clear bands against a dark background. To determine the inhibition profile of the enzyme activities, we also incubated gels in the presence of one of the following inhibitors in the activation buffer: 10 mM EDTA, 10 mM Phenanthroline, (two inhibitors of MMPs) and 1 mM PMSF, 2 mM NEM, (two inhibitors of serine protease). Molecular weights of gelatinolytic bands were estimated using recombinant protein molecular weight markers (100–225 kDa) (Amersham, Little Chalfont, UK), conditioned media from 3T3 fibroblasts, which contain high level of MMP-2 (13), and recombinant human MMP-2 and MMP-9.

Histological preparations and hydroxyproline measurement

Lungs from mice sacrificed throughout the study were removed after BAL. Two lobes were fixed in 10% formalin for histological study and the other lobes were frozen.

Paraffin embedded sections (5μm) were stained using Hematoxyline-eosine-safran (HES), May-Grünewald Giemsa (MGG), Perls and Picro-sirius hematoxyline dyes.

For immunohistochemistry, cryostat sections (5μm) from frozen lung were placed on gelatine slides and treated for 15 min with 4% paraformaldehyde in phosphate buffered saline (PBS). All incubations were realized at room temperature. Slices were incubated with 2% bovine serum albumin (BSA) in PBS for 1 h. Pro-III collagen or pro-IV collagen antibodies (diluted 1:100), were incubated for 1 h. Then sections were rinsed with PBS and incubated with the peroxo-cyclase-conjugated antibody (anti rabbit IgG, 1/100) for 1 h. Finally, collagen were revealed with a mixed (100:1; v/v) of diaminobenzidine (0.4 mg/ml) and 30% hydrogen peroxide solution (1/30). Slides were counterstained with Harris hematoxyline. Control experiments were carried out using rabbit IgG or the secondary antibody alone.

The extent of pulmonary fibrosis was inferred by estimating total lung collagen as reflected by measuring the hydroxyproline (OH-Pro)