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Polymerized α1-antitrypsin is present on lung vascular endothelium. New insights into the biological significance of α1-antitrypsin polymerization

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Polymerized α1-antitrypsin is present on lung vascular endothelium. New insights into the biological significance of α1-antitrypsin polymerization

Aims: The damage to lung tissue in chronic obstructive pulmonary disease (COPD) may involve the progressive loss of pulmonary vascular endothelial cells. Endothelial binding of α1-antitrypsin (α1-AT) derived from plasma has been identified, and α1-AT deficiency is a known genetic risk factor associated with α1-AT polymerization and COPD development. Therefore, in the present study we aimed to investigate if α1-AT is present on the lung vascular endothelium, and if it is in a polymeric form.

Methods and results: Postmortem paraffin-embedded tissue specimens from 15 COPD (chronic bronchitis and emphysema) cases with and without Z α1-AT (Glu342Lys) deficiency and from 10 cases without signs of COPD were studied. Immunohistochemistry was performed using the streptavidin–biotin method with a monoclonal ATZ11 antibody specific for polymeric α1-AT, and polyclonal antibodies against human α1-AT and neutrophil elastase. Vascular endothelium showed intense staining for α1-AT with the ATZ11 antibody in all cases; however, intensity of staining in patients with α1-AT deficiency was greater. No endothelial staining was observed with the anti-elastase antibody.

Conclusions: This is the first demonstration that α1-AT bound to the vascular endothelium of lungs is in a polymeric form, which also suggests a possible previously unknown role for polymeric α1-AT in vivo.

Keywords: α1-antitrypsin, COPD, endothelial cells, polymers

Abbreviations: α1-AT, α1-antitrypsin; COPD, chronic obstructive pulmonary disease

Introduction
It is now well recognized that endothelial cell functions are altered in sites of acute and chronic inflammation and it has been proposed that the disappearance of lung tissue may involve the progressive loss of pulmonary vascular endothelial cells. Endothelial dysfunction and intimal thickening have also been observed in the pulmonary arteries of patients with chronic obstructive pulmonary disease (COPD). The surface structure and composition of the endothelium play a major role in determining the metabolic status of endothelial cells, endothelial permeability to water and solutes, leucocyte adhesion and emigration, and microvascular resistance to pressure. Proteins adsorbed from the blood plasma and directly bound to the plasma membrane of endothelial cells are known to affect the expression of multiple endothelial cell markers. Most of the proteins at the endothelial surface are glycoproteins and proteoglycans. However, the biological role of each endothelial-bound protein is poorly understood.
Endothelial binding of α1-antitrypsin (α1-AT) derived from plasma has been identified. Findings that neutrophil-derived proteinases are capable of damaging endothelial-bound α1-AT led to speculation that preventing depletion of endothelial-bound proteinase inhibitors may help to minimize vascular damage during inflammation. α1-AT is an archetypal member of the serine proteinase inhibitor system in humans, and is a potent inhibitor of neutrophil elastase. α1-AT is found in most tissues and body fluids; it is an acute-phase reactant whose plasma concentration can rise by three- to four-fold above normal (average 1.34 mg/ml) during inflammation, infection and malignant diseases. The local balance between proteinases and endogenous inhibitors, such as α1-AT, is an important factor in determining whether inflammation results in connective tissue damage. When the concentration of α1-AT in plasma falls below 0.7 mg/ml the individual is considered to have α1-AT deficiency. The lack of circulating α1-AT results in uncontrolled proteolytic attack and early-onset panacinar emphysema. A single amino acid change in certain domains of the α1-AT can lead to polymerization of the mutant α1-AT into intracellular aggregates. The retention of Z-α1-AT polymers in the endoplasmic reticulum of hepatocytes can cause liver damage. Recent studies provide evidence that the polymeric form of α1-AT is present in the lungs of COPD patients with Z-α1-AT deficiency, and we have found that plasma from patients with Z-α1-AT deficiency contains a significant amount of circulating α1-AT polymers. These may have important implications for the pathogenesis of the disease, since polymerization obscures the reactive centre loop of α1-AT, rendering the protein inactive as an inhibitor of proteolytic enzymes.

Because α1-AT deficiency is known genetic risk factor for the development of COPD and because it has been proposed that the damage to lung tissue may involve the progressive loss of pulmonary vascular endothelial cells, we aimed to determine whether α1-AT is present on lung microvascular endothelium and if it is in a polymeric form, and to compare endothelial-bound α1-AT between COPD and normal lung tissues with and without Z-α1-AT deficiency.

Materials and methods

Tissue specimens

Lung tissue samples for immunohistochemical examination were obtained from the tissue bank at the Department of Pathology and Cytology, Malmö University Hospital. The tissues were prepared at autopsies performed between 1980 and 1990, fixed in formalin, embedded in paraffin wax and stored as paraffin blocks. The specimens for this study were selected via the autopsy diagnosis registry and classified as COPD (chronic bronchitis and emphysema) (n = 10) and without signs of COPD (n = 10). A microscopic examination of the liver was performed in all autopsies in order to identify cases with α1-AT-inclusion bodies in liver cells. This examination enabled us to identify five cases with liver histology indicative of a PiZ gene carrier and also to exclude PiZ gene carriers from COPD and control cases. However, it did not allow us to distinguish between hetero- and homozygous PiZ gene carriers. The lung microscopy in five PiZ carriers exhibited changes compatible with COPD (chronic bronchitis and emphysema). The study protocol was approved by the Ethics Committee of Lund University.

Immunohistochemistry

Monospecific antisera against human α1-AT and neutrophil elastase, negative control mouse IgG2b, and secondary, peroxidase-labelled antibodies were obtained from Dako (Glostrup, Denmark). Monoclonal antibody ATZ11, specifically reacting with polymerized and elastase-complexed α1-AT, is raised against the Z-α1-AT isolated from liver tissue and is available in our laboratory. The embedded tissues were sectioned at 4 μm and dried at 60°C for 1 h. The sections were deparaffinized, rehydrated, and microwaved twice for 5 min in 10 ml citrate buffer at pH 6. After cooling, the sections were washed in distilled water for 20 min. Immunohistochemical analyses were performed by an indirect, streptavidin–biotin method with an automated TechMate 500 Plus apparatus (Dako A/S). Blocking antibody was introduced and left to react for 20 min at room temperature and the primary antibody, monoclonal ATZ11 (1 : 50), polyclonal anti-α1-AT (1 : 20 000) and polyclonal antineutrophil elastase (1 : 100) antibody were added and allowed to react for 90 min at room temperature. The specificity of the ATZ11 antibody was tested by ELISA methods. The antibody was diluted with phosphate-buffered saline (PBS). Controls were performed in which the primary antibody was omitted or replaced with non-immunized mouse IgG (1 : 1000). The biotinylated secondary antibody and solutions supplied in the ChemMate Detection kit (Dako) were added and incubated for 30 min at room temperature. In this method, a biotinylated secondary antibody is detected with horseradish peroxidase-conjugated streptavidin, and peroxidase activity is detected with 3,3-diaminobenzidine chromogen.
inobenzidine tetrahydrochloride (DAB). The tissues were counterstained with haematoxylin. The specimens were analysed by microscopy, using an Olympus BX41 (Olympus Optical Co., Hamburg, Germany). Images were taken with an Olympus camera DP50 (Olympus Optical Co.) at an original magnification of $\times 100$ and $\times 400$.

**Results**

Our results demonstrated the positive immunoreactivity of vascular endothelial-bound $\alpha_1$-AT in all 25 cases investigated. Examples of positively stained endothelial cells with ATZ11 antibody in cases with morphologically and histochemically normal lung tissue are shown in Figure 1A,B. Positive staining was seen only on the endothelial layer, and not in the background or neighbouring alveolar epithelium. The polyclonal anti-$\alpha_1$-AT antibody manifested a nearly identical staining pattern of endothelial cell layers compared with the monoclonal ATZ11 antibody, but in addition the polyclonal anti-$\alpha_1$-AT showed background immunoreactivity (data not shown). It is important to emphasize that the ATZ11 antibody has no affinity to native, oxidized, latent and cleaved forms of $\alpha_1$-AT, but is known to cover a neoeptiote on both $\alpha_1$-AT complexed with enzyme and polymeric $\alpha_1$-AT, which means that this antibody cannot discriminate between these two forms of $\alpha_1$-AT. To investigate whether endothelial-bound $\alpha_1$-AT occurs both in a polymeric and in complex with elastase, we also stained with a polyclonal antibody to human neutrophil elastase. No endothelial staining was seen in any specimens with this antibody. The only positive staining was intracellular (Figure 1C), arguing against any significant contribution of $\alpha_1$-AT–elastase complexes to the endothelial staining pattern. Tsuji and coworkers have also shown that circulating $\alpha_1$-AT–elastase complex can pass through the endothelial cells without inducing any degenerative changes, and they did not detect $\alpha_1$-AT–elastase complexes on the endothelial layer. These findings still do not eliminate the possibility that other endothelial-bound $\alpha_1$-AT–enzyme complexes can be recognized by ATZ11 antibody, although in a Western blot analysis ATZ11 antibody did not react with $\alpha_1$-AT–cathepsin G or $\alpha_1$-AT–proteinase 3 complexes (data not shown).

Figures 2 and 3 show lung tissue with histological features of COPD, including focal fibrosis (Figures 2A,B and 3A), squamous metaplasia of bronchial epithelium (Figure 2A,C) and inflammation (Figures 2 and 3A,C). Immunostaining with ATZ11 antibody resulted in positive endothelial immunoreactivity in all cases. However, it must be pointed out that the intensity of staining in patients with $\alpha_1$-AT deficiency was much greater (Figure 3A,B). The alveolar inflammatory cells...
were mainly negative, although faint immunoreactivity could be detected with the ATZ11 antibody (Figure 2C). Both anti-neutrophil elastase and ATZ11 antibody reacted more intensely with inflammatory cells in Z-α1-AT deficiency cases (Figure 3C), thereby making it impossible to discriminate between the molecular forms of α1-AT in these cells.

**Figure 2.** Immunohistochemical analysis of endothelial-bound α1-antitrypsin (α1-AT) in lung tissue autopsy sections from chronic obstructive pulmonary disease cases with wild-type α1-AT. A,B. Lung fibrosis and chronic inflammation. Squamous metaplasia of the bronchial epithelium is seen. Positive α1-AT endothelial staining with monoclonal ATZ11 antibody (1:50) is indicated by arrows. C. Only weak immunostaining with ATZ11 antibody of inflammatory cells in the bronchial lumen can be seen. Arrow indicates cell staining.

**Figure 3.** Immunohistochemical analysis of endothelial-bound α1-antitrypsin (α1-AT) in autopsy sections of lung tissue from PiZ gene carrier with chronic obstructive pulmonary disease (COPD)-compatible pulmonary changes. A. Lung tissue changes characteristic of COPD. Both the endothelial layer and the inflammatory cells are immunoreactive with ATZ11. B. Close-up view of the endothelial staining with ATZ11 (1:50) of a small blood vessel. Background staining is also visible. Arrow indicates endothelial immunoreactivity. C. Immunostaining with ATZ11 antibody (1:50) of inflammatory cells in the alveolar lumina (indicated by arrows). These cells are also positive for immunostaining with a polyclonal antihuman α1-AT and anti-neutrophil elastase antibody (data not shown).
Discussion

Multiple forms of α₁-AT have been identified in biological fluids, including native, inhibitory, and non-inhibitory forms, the latter including oxidized, cleaved and polymeric forms.18 Dependent on its molecular form, α₁-AT has been reported to influence cell function and behaviour via both direct and indirect mechanisms.13,18 The studies described here are an extension of our previous work, which showed that polymeric α₁-AT is bound to the endothelium of temporal arteries.12 This indicated to us that polymerization of endothelial-bound α₁-AT may be a general phenomenon, and we sought to test this hypothesis by examining whether endothelial-bound α₁-AT in the lungs is in a polymeric form. The current study is the first convincing demonstration of endothelial-bound polymeric α₁-AT in normal and diseased lung tissue. Moreover, the presence of polymeric α₁-AT was independent of Z-α₁-AT deficiency.

The flexibility of the reactive centre loop of α₁-AT allows insertion of the reactive centre loop of one molecule into the Aβ-sheet of a second α₁-AT molecule which then extends to form chains of polymers.28 It is this polymerization that occurs spontaneously in Z-α₁-AT, underlies the formation of hepatic inclusions, and is associated with plasma deficiency of α₁-AT.22,23 The propensity for the wild type α₁-AT to undergo loop-sheet polymerization is much less pronounced. To date, in vivo, wild-type α₁-AT polymers have been described only in bile, where the interaction of α₁-AT with a denaturing milieu is thought to enhance polymer formation.31 Other conditions that are known to induce α₁-AT polymerization in vitro include high protein concentration, low pH and increased temperature.28 The potential cytotoxic effect of Z-α₁-AT polymers on hepatocytes and their occurrence in the circulation have prompted investigators to discuss the potential biological role of these polymers in extrahepatic tissues. Since polymerization abolishes α₁-AT inhibitory activity, it is proposed that polymer formation will exacerbate the already reduced antiproteinase screen and increase the susceptibility of the tissues to proteolytic attack. It has even been suggested by Parmar and coworkers that polymers of α₁-AT are proinflammatory and chemotactic for human neutrophils in vitro.32 In our experimental models, however, neither native nor polymeric α₁-AT at concentrations up to 0.5 mg/ml stimulated neutrophil chemotaxis, adhesion, superoxide generation or proteinase release.33 Our findings that polymeric α₁-AT is present on the vascular endothelium in both normal and diseased lungs do not support a proinflammatory role for polymeric α₁-AT, in vivo.

We have demonstrated that polymeric form of α₁-AT is bound to lung vascular endothelium in both COPD and control cases independent of α₁-AT PiZ deficiency, which allows us to propose that the presence of polymerized α₁-AT on the vascular endothelium is a general phenomenon. The levels and quality of polymeric, endothelial-bound α₁-AT might be one of the factors determining the susceptibility of individuals to develop vascular damage and related diseases. Further studies are needed to elucidate a biological role of vascular endothelial-bound AAT, in vivo.

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References

12. Janclausiene S, Dominaite R, Sternby NH et al. Detection of circulating and endothelial cell polymers of Z and wild type alpha...


