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Z α_1-Antitrypsin Polymerizes in the Lung and Acts as a Neutrophil Chemoattractant *

Alan T. Mulgrew, Clifford C. Taggart, Matthew W. Lawless, Catherine M. Greene, Mark L. Brantly, Shane J. O'Neill and Noel G. McElvaney

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have shown a correlation between yoga breathing exercises and various physiologic improvements. These improvements include the following: increased exercise tolerance[4]; lowering of serum glucose and cholesterol levels[5]; decrease in urea levels[6]; as well as the slowing of the heart rate and the lowering of BP.[7]

Other studies on yoga breathing have demonstrated positive effects on patients with asthma. Two studies by Nagendra and Nagarathna[8,9] showed the beneficial effects of yoga breathing exercises for asthmatic patients. In the first study, peak expiratory flow rate values improved after yoga, and a majority of the patients were able to stop receiving or reduce their cortisone medications. The second study showed overall decrease in asthma attacks and medication usage in patients using yoga breathing. Singh et al[10] used a Pink City Lung Exerciser (Pulmotech; Jaipur, India) to mimic pranayama breathing. That study demonstrated an improvement in the measured breathing parameters of all patients over baseline. It also showed a statistically significant increase in the dose of histamine needed to cause a 20% reduction in FEV_1 during pranayama.

A literature search showed no other reports of spontaneous pneumothorax attributed to yoga breathing. Other case reports of unusual causes of pneumothorax include acupuncture,[11] cardiopulmonary resuscitation training,[12] molar extraction,[13] grass head (seed) aspiration,[14] paraquat intoxication,[15] cocaine use in pregnancy,[16] and a sewing needle injury.[17]

Conclusion

Yoga exercises have been practiced for hundreds of years. Now, an increasing number of Americans are turning to yoga and other alternative holistic methods to improve their health. Although further studies are needed to show their effect on health, many physicians may be asked to counsel their patients about the benefits and risks of these techniques. This case should illustrate that adverse side effects can occur when one pushes the body to physiologic extremes.

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Z α_1-Antitrypsin Polymerizes in the Lung and Acts as a Neutrophil Chemoattractant*

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Background: **α_1-antitrypsin (A1AT) is an abundant protein that is synthesized in the liver and is secreted into the plasma. From the plasma, A1AT diffuses into various body compartments, including the lung where it provides much of the antiprotease protection. The current understanding of the pathogenesis**

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of emphysema in A1AT-deficient individuals focuses on the polymerization of mutant protein within the liver, which results in a deficiency of circulating A1AT and a protease-antiprotease imbalance in the lungs.

Methods and results: **In this study, we evaluated BAL fluid samples from five healthy volunteers, five individuals with ZA1AT deficiency, and an individual with the PiZZ phenotype who had received a liver transplant. We show that the lung itself is a source of A1AT. In addition, the Z protein formed in the lung polymerizes, and these polymers are detectable in lung epithelial lining fluid by enzyme-linked immunosorbent assay and Western blot analysis. Finally, we show that polymeric ZA1AT is a potent neutrophil chemoattractant that is similar to polymerized MA1AT.**

Conclusions: **Our findings suggest that the polymerization of locally produced ZA1AT is a contributory factor to the lung inflammation experienced by those with A1AT deficiency and that standard antiprotease therapies may not address this problem.**

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Key words: chemoattractant; polymerization; Z α_1-antitrypsin

Abbreviations: A1AT = α_1-antitrypsin; DLCO = diffusing capacity of the lung for carbon monoxide; ELF = epithelial lining fluid; ELISA =enzyme-linked immunosorbent assay; IL = interleukin; NE =neutrophil elastase; OD = optical density; PAGE = poly-acrylamide gel electrophoresis

α_1-antitrypsin (A1AT) deficiency is a condition that is characterized by low serum levels of A1AT[1,2] and a substantially increased risk for the development of emphysema by the fourth or fifth decade.[3,4] There are also risks for the development of hepatic disease[5,6] and cutaneous panniculitis.[7] The A1AT gene is expressed in cells of several lineages, with the highest expression existing in hepatocytes. Serum A1AT is almost totally derived from hepatic production, but A1AT is also actively transcribed and secreted by other cells, including neutrophils, blood monocytes, macrophages, pulmonary alveolar cells, and intestinal epithelial cells.[8–10]

For editorial comment see page 1607

It is thought that the hepatic damage in A1AT deficiency results mainly from the retention and abnormal processing of polymerized ZA1AT in the liver.[11–13] In this context, efforts to increase secretion may have a therapeutic effect.[14] On the other hand, the lung disease associated with A1AT deficiency is thought to result from the markedly decreased antiprotease protection in the lung due to the inadequate secretion of polymerized ZA1AT from the liver with subsequent diminution of plasma and lung A1AT levels. Little is known about the local production and secretion of A1AT in the lung. This may be important because, although the secreted protein may be expected to have some antiprotease activity, albeit reduced, there is also evidence that A1AT, if polymerized, may be proinflammatory.[15] This fact has important consequences for therapy in patients with the condition. Presently, the lung disease associated with A1AT deficiency is treated with the standard therapies for COPD in addition to A1AT augmentation therapy. Augmentation therapy, by increasing the serum levels of A1AT, will in turn increase the A1AT levels on the epithelial and interstitial surfaces of the lung, thereby potentially preventing or hindering the progression of emphysema.[16–18] IV augmentation therapy with plasma-purified A1AT has been approved by the US Food and Drug Administration for the treatment of lung disease associated with A1AT deficiency since 1987.

In this study, we evaluate whether the local secretion of A1AT onto the surface of the lung is significant, whether Z protein in the lungs of individuals with A1AT deficiency is polymerized, and finally whether the secreted protein has a proinflammatory effect that may undermine the antiprotease effect of exogenously administered A1AT. To do this, we evaluated individuals with severe A1AT deficiency (Z homozygotes), nondeficient individuals (M homozygotes), and one individual with the Z deficiency phenotype who underwent liver transplantation. In this individual, the phenotype of the A1AT in his serum should reflect that of his transplanted liver, while any ZA1AT protein detected in his lungs would reflect local production and secretion.

Materials and Methods

Study Population

Five patients with A1AT deficiency (PiZZ phenotype), five PiMM control subjects, and one patient with the PiZZ phenotype who had received a liver transplant were evaluated as part of this study. All patients were clinically stable at the time of the study and had been free of infection for the previous 6 weeks. All patients in the study had been phenotyped previously by standard isoelectric focusing techniques. The patient with the liver transplant had undergone transplantation 10 years previously for A1AT-related liver disease. All patients in the study attended the respiratory outpatient clinic in Beaumont Hospital, Dublin, Ireland. All patients underwent blood sampling, as well as bronchoscopy and BAL. All patients gave fully informed consent, and study approval was obtained from the hospital ethics committee.

Determination of A1AT Levels

A1AT levels in serum and BAL fluid were determined using a double-sandwich enzyme-linked immunosorbent assay (ELISA) technique, as previously described.[19] This was performed using a polyclonal antibody that was capable of detecting all forms of A1AT (1:1000 dilution of serum) as well as with a monoclonal antibody (1:100 dilution of serum) developed by Wallmark et al.[20] This antibody was raised against PiZZ hepatocytic A1AT and was initially thought to be specific to the Z protein. However, some studies[21] have shown that this antibody detects polymerized A1AT as well as A1AT complexed with neutrophil elastase (NE) with no apparent affinity for native, latent, or cleaved forms of A1AT. In addition, this antibody has been used routinely to identify ZA1AT-deficient serum samples, and false-positive results have never been detected.[21] Fluid was derived from BAL

performed according to standardized guidelines set out by Klech and Pohl.[22] In brief, during fiberoptic bronchoscopy aliquots of 3×60 mL were injected into a division of the right middle lobe of the lung and reaspirated immediately. BAL fluid was filtered through sterile gauze and centrifuged at 1,100 revolutions per minute for 10 min. The supernatant was divided into aliquots and stored at −80°C.

Western Blot Analysis of A1AT Protein

BAL specimens were separated by 8% nondenaturing polyacrylamide gel electrophoresis (PAGE) and were transferred onto a nitrocellulose membrane. After probing with monoclonal antibody to ZA1AT diluted 1:500, antimouse IgG-alkaline phosphatase conjugate (I-Block; Promega; Madison, WI) was added (1:7500), and the blot developed with a chemiluminescent reagent (CDP-Star; Applied Biosystems; Foster City, CA).

Generation of Polymerized ZA1AT

The purification of ZA1AT was accomplished using a modification of the method described by Sugiura et al.[23] Briefly, 950 mL plasma obtained from a single PIZZ individual, and verified by isoelectric focusing,[24] was fractionated using ammonium sulfate. The fraction, which was obtained with an ammonium sulfate saturation of between 50% and 80%, was passed through a cibacron blue column (Sepharose F3GA-CL-4B column; Amersham Biosciences; Little Chalfont, UK). The fractions containing A1AT were then further purified by chromatography on a diethylaminoethanol column (Sephacel; Amersham Biosciences) by eluting from this column using a linear gradient from 0 to 250 mmol/L NaCl. The final purification steps involved chromatography on a 50×100 column (Sephacryl S 200 HP XK; Amersham Biosciences), and passage through antihuman albumin and antihuman α_1-acid glycoprotein columns. The identity and purity of the ZA1AT protein was confirmed by sodium dodecyl sulfate gel electrophoresis, isoelectric focusing at pH 4 to 5, nephelometry and amino acid analysis, and protein sequencing (Harvard University Microchemistry Facility; Cambridge, MA). The Z protein was polymerized by incubation at a final concentration of 0.1 to 0.5 mg/mL in 50 mmol/L Tris (pH 7.4) and 50 mmol/L KCl at 37°C over a 10-day time-course. Polymerization was confirmed using 8% nondenaturing PAGE. Polymerized Z protein was separated into aliquots and stored at −20°C prior to its use in further experiments.

Neutrophil Isolation

Neutrophils were isolated from heparinized (10 U/mL; Sarstedt; Wexford, Ireland) venous blood, as previously described.[25]

Chemotaxis Assays

Neutrophil migration in response to polymerized A1AT was evaluated using wells (Costar Transwells; Corning; Rochester, NY) with polycarbonate filters and a 3-μm pore size. Briefly, stimuli were placed into the lower chamber of the well, and neutrophils were placed in the top chamber. Following chemotaxis, the wells were removed and washed in phosphate-buffered saline solution, and the nonadherent neutrophils were removed with a sterile swab. The wells then were stained (Diff-Quik; Clin-Tech; Essex, UK) and rinsed in sterile water, and the cells were counted by microscopy. The data were presented as the mean ± SE, and *t* tests were performed using specific software (Prism 3.0; GraphPad Software; San Diego, CA).

Results

Patient Demographics

The study population included five PiZZ patients, whose conditions were confirmed by A1AT levels and isoelectric focusing. Four of the patients were ex-smokers with established lung disease (*ie*, FEV_1, 43 ± 14% predicted; FEV_1/FVC ratio, 38 ± 2% predicted; diffusing capacity of the lung for carbon monoxide [DLCO], 34± 12% predicted). One patient was a newly diagnosed nonsmoker (*ie*, FEV_1, 99% predicted; FEV_1/FVC ratio, 72% predicted; DLCO, 96% predicted). All patients were men, with an age range of 24 of 67 years. BAL fluid analysis revealed total cell counts of 1×10^7 to 4.2×10^7 cells, with 9 to 19% neutrophils present.

Five PiMM individuals were also studied. All were healthy nonsmokers (*ie*, FEV_1, 100.5 ± 3% predicted; FEV_1/FVC ratio, 81 ± 15% predicted; DLCO, 77 ± 2% predicted). All patients were men, with an age range of 30 to 60 years. BAL fluid analysis revealed total cell counts of 4×10^6 to 2×10^7 cells, with 0 to 2% neutrophils present.

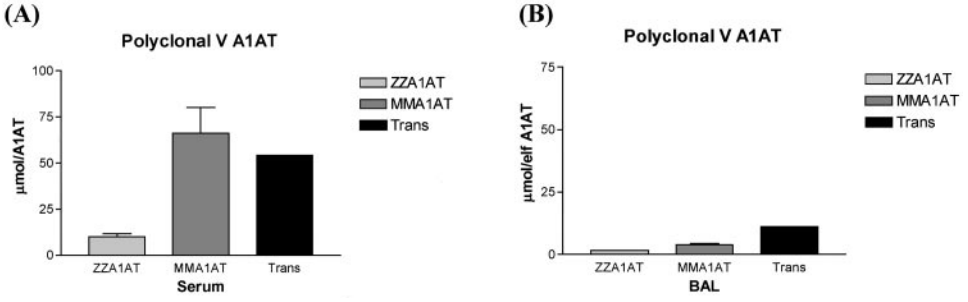

FIGURE 1. The determination of A1AT levels with polyclonal antibody. A1AT levels in serum and BAL fluid were determined by ELISA using a polyclonal antibody for A1AT. *Left*, *A*: serum levels of A1AT were low in PiZZ patients, and were in the normal range for PiMM patients and the transplanted patient. *Right*, *B*: BAL fluid levels of A1AT were low in PiZZ patients, and were in the normal range for PiMM patients and the transplanted patient.

One male patient was PiZZ (age, 64 years) and had received a liver transplant 9 years previously. He had no history of smoking and no history of significant lung disease. Spirometry revealed the following: FEV_1, 83% predicted; FEV_1/FVC ratio, 73% predicted; DLCO, 67% predicted. BAL fluid analysis revealed 3×10^7 cells, with 21% neutrophils present.

A1AT Levels and Western Blot Analysis of A1AT Protein

Serum levels of A1AT were obtained using a polyclonal antibody against A1AT (Fig 1) and a monoclonal antibody, which is specific for polymerized/elastase-complexed A1AT (Fig 2, *top*, *A*, and *middle*, *B*). As MA1AT protein does not polymerize at body temperature, it can be inferred that the A1AT detected by this antibody is either polymerized Z protein or ZA1AT or MA1AT complexed with NE. The serum levels of A1AT (Fig 1, *left*, *A*: PiZZ patients, 9.98 ± 1.72 μmol/L; PiMM patients, 66.08 ± 13.86 μmol/L; transplant patient, 54 μmol/L), which were measured using the polyclonal antibody, were as expected.[23] Using the polyclonal antibody, BAL levels of A1AT were as follows: PiZZ patients, 1.412 ± 0.05 μmol/L/epithelial lining fluid (ELF); PiMM patients, 3.712 ± 0.45 μmol/L/ELF; transplant patient, 10.9 μmol/L/ELF (Fig 1, *right*, *B*). The monoclonal antibody did not detect A1AT protein in the serum of nondeficient individuals or in the transplanted individual, but did detect A1AT protein in the serum of Z individuals, suggesting that the ZA1AT protein in these individuals was either polymerized or complexed with NE (Fig 2, *top*, *A*; optical density [OD], 0.496 ± 0.007). ZA1AT was also detectable in the BAL fluid from Z individuals (OD, 0.4424 ± 0.0123) and the transplant patient (OD, 0.4672 ± 0.0137) using the monoclonal antibody (Fig 2, *middle*, *B*). However, it was not clear whether the ZA1AT protein detected by ELISA using the monoclonal antibody was polymerized or complexed with NE. Therefore, the BAL samples were separated by PAGE, and Western blot analysis of these BAL fluids was performed using the monoclonal antibody. The results show that the majority of the A1AT protein in the Z-deficient individuals (Fig 2, *bottom*, *C*, lane 2) was polymerized, not complexed to NE, due to the slow mobility of the ZA1AT on the gel (A1AT-NE complexes migrate with a similar mobility to that of monomeric A1AT).[21] Likewise, there were also large amounts of polymerized A1AT in the transplant patient BAL fluid (Fig 2, *bottom*, *C*, lane 3) but not in the BAL fluid from the PiMM patient (Fig 2, *bottom*, *C*, lane 4). The presence of ZA1AT polymers in the BAL fluid from A1AT-deficient individuals has been demonstrated previously by transverse urea gradient gel methodology, and we have now confirmed this finding by ELISA and Western blot analysis.[26]

Neutrophil Chemotaxis Assay

Native M and Z proteins had no appreciable effect on neutrophil migration, whereas polymerized Z protein had an effect analogous to the potent neutrophil chemoattractant interleukin (IL)-8 (Fig 3). Although polymerized MA1AT protein has been shown previously to be a neutrophil chemoattractant,[15] this is the first demonstration of polymerized ZA1AT as a neutrophil chemoattractant, which is more physiologically relevant in the context of ZA1AT deficiency.

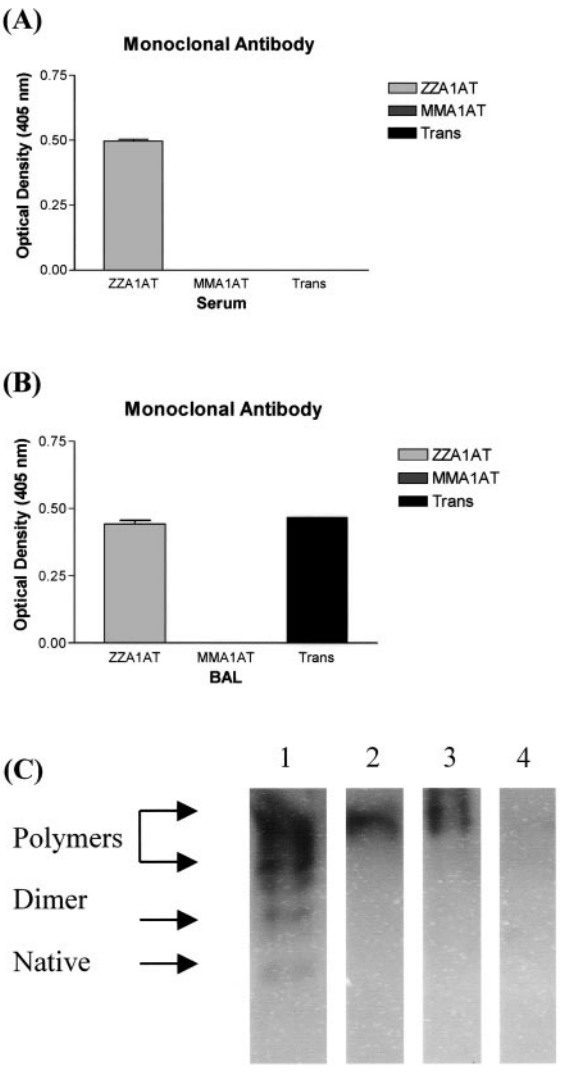

FIGURE 2. ELISA and Western blot analysis of A1AT protein using monoclonal antibody. *Top*, *A*: the monoclonal antibody detected Z protein only in the serum of PiZZ patients, indicating its specificity. *Middle*, *B*: Z protein was detected in PiZZ patients and the transplanted patient only. *Bottom*, *C*: BAL fluid samples were run on 8% nondenaturing PAGE. Ten microliters of the sample were added to each well followed by Western blotting using the monoclonal antibody to ZA1AT. Lane 1, polymerized ZA1AT standard; lane 2, PiZZ patient BAL fluid; lane 3, transplant patient BAL fluid; lane 4, PiMM patient BAL fluid.

DISCUSSION

This study shows for the first time that A1AT is locally produced on the epithelial surface of the lung. The source

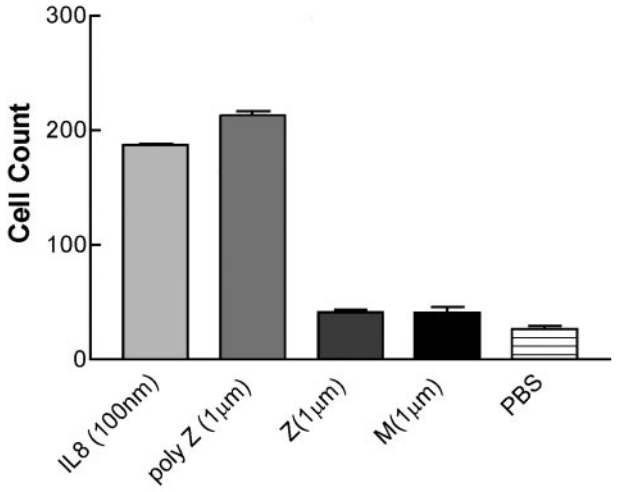

FIGURE 3. Chemotaxis assays. The data are shown as the mean of three separate experiments. Cell counts are represented on the vertical axis, and the chemoattractants used in the lower well are represented on the horizontal axis. IL-8 was used as a positive control, and phosphate-buffered saline solution was used as a negative control. Polymeric ZA1AT (poly Z) was significantly more chemotactic than were the nonpolymeric forms (Z and M).

of this A1AT could be respiratory epithelial cells, macrophages, or, less likely, neutrophils.[9,10] In addition, unlike MA1AT protein, this ZA1AT protein polymerizes at body temperature, and, in addition to being an ineffective antiprotease inhibitor, may become a strong neutrophil chemoattractant, thus representing an ongoing source of inflammation in the lungs of individuals with A1AT deficiency.

How important is the presence of airway polymerized Z protein chemoattraction *in vivo*? Some of the Z protein in serum is polymerized. This possibly represents an opposing chemoattraction to that of the polymerized ZA1AT protein on the lung epithelial surface.[21] However, although the net effect of these opposing forces is difficult to quantify, it should be noted that even PiZZ patients with near-normal lung function have high neutrophil concentrations on their respiratory epithelial surfaces.[27] Previously, this increased neutrophil burden was attributed to leukotriene B_4 or IL-8 release from neutrophils or epithelial cells.[28,29] Our findings emphasize that neutrophil accumulation in the lungs of A1AT-deficient individuals is multifactorial and that chemoattraction due to polymerized Z protein is another potential cause of this neutrophil-dominated inflammation.

The findings in this study have implications for the therapy of this disorder. In the past, it was assumed that all of the A1AT present on the epithelial or interstitial surfaces of the lung was derived from serum. It was therefore rationalized that in order to treat A1AT deficiency in the lung one had to increase serum levels of A1AT above a putative therapeutic threshold, and that this A1AT then would diffuse into the various lung compartments, protecting them against NE-mediated damage. This study shows that A1AT is also produced locally in the lung and that this production is quite significant. The Z protein, although less competent than the M protein, is an antiprotease in its native state,[30] raising the possibility that this local production could be augmented to therapeutic effect. This has been attempted in the past, although with the intent of increasing the liver secretion of A1AT using typhoid vaccine, estrogen-progesterone combinations, tamoxifen, or danazol with varying degrees of success.[31–33] However, we have shown in this study that the ZA1AT present in the BAL fluid of the transplant patient and in that of deficient patients is not only polymerized, and thus inactive, but also could act as a potent neutrophil chemoattractant.

A more rational approach to therapy would be to attempt to inhibit the polymerization of the Z protein both intracellularly and extracellularly. This would increase the secretion of active nonpolymerized Z protein from the liver, potentially ameliorating the liver disease and, from the respiratory epithelial surface, providing anti-NE protection and avoiding the proinflammatory effects of polymerized ZA1AT. This approach is feasible as has been shown by a 2002 study,[34] where a 6-mer peptide was selectively annealed to ZA1AT, preventing its polymerization without interfering with the function of other similar proteins. However, ZA1AT, even in its unpolymerized form, exhibits diminished anti-NE capacity, and such a therapeutic approach may have to be accompanied by standard augmentation therapy.

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