ABSTRACT

The Z variant (Glu342Lys) of α₁-antitrypsin polymerises and accumulates in the hepatocyte endoplasmic reticulum predisposing to neonatal hepatitis and liver cirrhosis. The resultant secretory defect leaves the lungs vulnerable to elastolysis and early-onset emphysema. There is currently no cure for the liver or lung disease other than organ transplantation. This review discusses the evolving understanding of the molecular pathogenesis of the condition and how this has led to the emergence of novel treatment strategies for α₁-antitrypsin-related liver disease.

Key words: Alpha-1 antitrypsin, polymerisation, liver disease, emphysema.

INTRODUCTION

Alpha-1 antitrypsin (AT) is a member of the serine proteinase inhibitor superfamily. It is primarily synthesised in hepatocytes and secreted into plasma from where it enters into the lungs and protects the alveoli from unregulated neutrophil elastase activity. The normal variant is termed M-AT, according to its isoelectric point. Severe α₁-antitrypsin deficiency most commonly occurs due to a point mutation in the α₁-antitrypsin gene, resulting in a substitution of a glutamic acid for a lysine at position 342 (342GluLys); Z-AT. Despite being known about for 50 years, it remains under-recognised. It affects 1 in 2000 in northern Europe, 1 in 4500 in the US and 1 in 5000 in the UK. It is now well established that self-aggregation (polymerisation) of Z-AT leads to retention of Z-AT as periodic acid Schiff–positive (PAS-positive), diastase resistant inclusions within the hepatocyte endoplasmic reticulum (ER), which predisposes to neonatal hepatitis and liver cirrhosis. The resultant secretory defect (10–15% of the levels of those of the normal M allele) predisposes to early-onset emphysema. Retrospective studies have identified that up to 25% of those with Z-AT may suffer from liver cirrhosis or liver cancer in late adulthood. Other than organ transplantation, there is no effective treatment for Z-AT-related disease. Thus, reducing polymerisation and/or aggregation of Z-AT remains the major goal in the treatment of Z-AT-related liver disease.
hepatocytes, and the degree of plasma deficiency. Other polymerising mutants of α₁-antitrypsin resulting in hepatic inclusions and plasma deficiency include: Siiyama (Ser53Phe) and Mmalton (Phe deleted), S (GluVal) and I (Arg→Cys) variants of α₁-antitrypsin. These polymerising variants can also interact with the Z variant to form heteropolymers, inclusion bodies and liver cirrhosis. The process of polymerisation is a generalised mechanism by which serpin mutants are associated with disease: polymerisation of antithrombin, α₁-antichymotrypsin, C1-inhibitor and neuroserpin mutants is associated with thrombosis, emphysema, angioedema, and familial encephalopathy with neuronal inclusion bodies, respectively.

Figure 1. Polymerisation of Z-alpha-1 antitrypsin

Figure 1A. Mechanism of Z α₁-antitrypsin polymerisation
The structure of α₁-antitrypsin is centred on β-sheet A and the exposed mobile reactive centre loop. Polymer formation results from the Z-AT (E342K at P17; Z) or other mutations in the shutter domain, which destabilise β-sheet A to favour partial loop insertion and the formation of an unstable intermediate (M*). β-sheet A can accept the loop of another Z-AT molecule, to form a Z-AT dimer, which then extends into Z-AT polymers. The individual molecules of AT within the polymer are shown in different shades of grey.

Figure 1B. Western blot analysis of bronchoalveolar lavage fluid for Z-AT polymers in cigarette smoke-exposed mice models
Proteins were analysed on 7.5% non-denaturing PAGE

Top gel: a polyclonal anti-human antitrypsin antibody, detected α₁-antitrypsin polymers in transgenic mice for Z-AT and monomeric species in control (non-cigarette smoke exposed) Z-AT, and both cigarette-exposed and control transgenic mice for M-AT.

Bottom gel: a monoclonal anti-human oxidised α₁-antitrypsin antibody, detected oxidised polymeric Z-AT in cigarette smoke-exposed Z-AT mice, and monomeric oxidised α₁-antitrypsin in cigarette smoke-exposed M-AT mice. Oxidised conformations of α₁-antitrypsin were not detected in control Z-AT or M-AT mice. Results suggest that cigarette smoke induces the formation of Z-AT oxidized polymers.
Lung Disease in Z α₁-Antitrypsin Deficiency

Interestingly, despite the severe deficiency of such a major proteinase inhibitor, there is variability in the expression of pulmonary disease even in members of the same family.\textsuperscript{19-21} Several aspects of the pulmonary disease in PiZZ individuals are variable, for example age at the onset of lung disease, pulmonary function (spirometric measures of airflow obstruction and reduction in gas transfer factor), asthma-related phenotypes, extent of chronic bronchiitis and bronchiectasis, distribution of emphysema (basilar predominance vs. diffuse emphysema), disease-related morbidity and mortality.\textsuperscript{22} These findings suggest that modifier genes, environmental exposure, and gene-environment interactions also determine disease expression.\textsuperscript{21,23} Cigarette smoking is clearly associated with rapid progression of the lung disease in PiZZ homozygotes. However, this in itself does not fully explain the variability seen in the severity of emphysema in the PiZZ group. Occupational exposure to mineral dust and the use of kerosene heaters have been associated with reduced FEV1 in non-smokers, independent of smoking status.\textsuperscript{22,23}

PiZZ individuals with COPD have increased lung neutrophils and increased leukotriene B4 and interleukin-8.\textsuperscript{24-27} Z-AT polymers have been identified in bronchoalveolar lavage fluid and explanted lung tissue from Z-AT homozygotes.\textsuperscript{24,25} Interestingly, these extracellular Z-AT polymers are co-localised with and chemotactic to neutrophils.\textsuperscript{24,28}

Liver Disease in Z Homozygotes

Following screening of 200,000 PiZZ neonates, Sveger identified variability in the clinical presentation, suggesting involvement of genetic and environmental gene modifiers in the expression of liver disease.\textsuperscript{6} A more detailed description of the clinical aspects of the liver disease related to Z-AT is described elsewhere.\textsuperscript{7,9-13}

Figure 2. Proposed mechanism of liver disease in PiZZ individuals

Misfolded Z-AT protein is recognised by the quality control apparatus of the hepatocyte and degraded by the endoplasmic reticulum (ER) associated degradation (ERAD) proteasomal pathway. However, Z-AT proteins can escape this process and form Z-AT polymers which aggregate. These intracellular-inclusion bodies initiate the ER overload response. See main text for more details.
Activation of the ER-Overload Response in Severe α₁-Antitrypsin Deficiency

The pathophysiology of liver injury in PiZZ homozygotes is a consequence of the gradual accumulation of aggregated Z-AT in the ER of Z-AT cells. The relationship between the intracellular aggregation of misfolded Z-AT proteins and ER stress has been intensively studied. The aggregated Z-AT activates the ER-overload response (Figure 2). A key step in the regulation of the ER overload response involves transmembrane transducers for sensing ER stress such as transmembrane Protein Kinase RNA (PKR)-like ER Kinase PERK, regulator of G-protein signalling 16 (RGS16) and calnexin. Calnexin is a transmembrane ER chaperone in liver cells that binds to misfolded proteins suggesting the importance of proteasomal activity in disposal of misfolded proteins.

In particular, activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which causes a general inhibition of protein synthesis, and induction of the transcription factor ATF4 that binds to the amino acid response element to encode factors involved in the inflammatory response. We and others have shown that ER accumulation of Z-AT polymers is associated with activation of PERK, which when activated induces NF-κB activity, in keeping with activation of the ER overload response. Because IκB has a shorter half-life than NF-κB, PERK-mediated translational attenuation shifts the ratio of IκB to NF-κB, thereby freeing NF-κB to translocate to the nucleus, where it induces the transcription of genes involved in the inflammatory response, as demonstrated by significant secretion of inflammatory cytokines such as IL-6. Increased IL-6 activity could perpetuate the liver injury by increasing translation of Z-AT via its well characterised IL-6 promoter that would subsequently lead to further aggregation and accumulation of Z-AT, and so on. The level of RGS16 upregulation in the liver of PiZZ individuals is associated with the hepatic levels of Z-AT polymer inclusion bodies.

Autophagy in Severe α₁-Antitrypsin Deficiency

The importance of autophagy in PiZZ individuals was demonstrated by the identification of three gene products; ATG5, ATG6 and ATG16L1, which are necessary for digestion or degradation of aggregated Z-AT. Autophagy also determines how much aggregated Z-AT accumulates in the ER. The presence of aggregated Z-AT, rather than the soluble Z-AT, specifically activates autophagy. This was supported by the finding that accumulation of a mutant α₁-antitrypsin Saar (AT Saar) that does not aggregate, induced activation of unfolded protein response (UPR) and ER-associated degradation (ERAD) or the proteasomal degradation pathway that degrades soluble Z-AT.

Potential Treatment Options for Severe α₁-Antitrypsin Deficiency

To date, there is no effective specific treatment for Z-AT-related liver disease. A major distinction
Figure 4A. 4M dissociates intracellular Z-AT polymers
Z-AT cells were incubated for 24 hours, which allowed retention of Z-AT polymers as inclusion bodies before adding in 4M peptide (P<0.001). 4M dissociated over 90% of Z-AT polymers in inclusion bodies thereby resulting in the monomeric conformation of the Z-AT in the inclusion (P<0.001).

Figure 4B. 4M facilitates secretion of Z-AT monomers
The dissociation of inclusion Z-AT polymers with 4M resulted in the secretion of Z-AT monomers (P<0.001) in the supernatant (***).

Figure 4C. 4M prevents intracellular polymers and facilitates secretion of Z-AT monomer
**Top gel:** Analysis of metabolic labelling by Western blot demonstrates the distribution of Z-AT proteins in Z-AT cell lysate, supernatant and accumulation in inclusion bodies. Treatment with 4M completely prevented aggregation of intracellular Z-AT and subsequently increased secretion of α1-antitrypsin into the supernatant.

**Bottom graph:** 4M increased the concentration of monomeric Z-AT in the supernatant and reduced inclusion body α1-antitrypsin. Levels of α1-antitrypsin from pulse-chase for lysates (white bars), supernatants (black bars) and inclusion bodies (grey bars) are presented on histograms. Results are expressed as relative to percentage control, t=0 (100%), showing a time-dependent aggregation into inclusion bodies of Z-AT cells and secretion of AT from Z-AT+4M cells into supernatant (**).

Figure 4D. 4M reduces activation of PERK in Z-AT cells
RT-PCR analysis demonstrated that ER retention of Z-AT resulted in 3.04-fold upregulation of PERK mRNA in hepatic Z-AT cells when compared to vector control (P<0.001). Treatment with the inhibitor of polymerisation, 4M was able to significantly inhibit Z-AT-induced upregulation of PERK mRNA in Z-AT cells (**) (P<0.001). In contrast, the unrelated four-mer peptide (4U) had no effect on PERK mRNA.

Figure 4E. 4M inhibits NF-κB activation in Z-AT cells
Activation of ER-overload response in Z-AT cells induced by ER retention of Z-AT is further supported by upregulation of NF-κB, which could be inhibited by 4M; (**) (P<0.001).
between pathogenesis of liver and lung disease in severe $\alpha_1$-antitrypsin deficiency is gain of function and loss of function, respectively. The liver disease relates to the intracellular accumulation of Z-AT polymers in hepatocytes, rather than unopposed elastolysis in the lung due to lack of $\alpha_1$-antitrypsin. Therefore, antitrypsin augmentation therapy is used for the lung disease, but is not indicated for liver disease relating to severe $\alpha_1$-antitrypsin deficiency.

Several strategies are currently being studied for treatment of severe $\alpha_1$-antitrypsin deficiency-related liver disease including; gene therapy, the potential of combining human induced pluripotent stem cells (iPSCs) with genetic correction, enhancing autophagic clearance of aggregated Z-AT by drugs or transfer of a hepatocyte-directed master gene, and inhibitors of Z-AT polymerisation. Other strategies under evaluation also include gene therapy using small-interfering RNA, ribozymes and peptide nucleic acid.

Studies have suggested that enhancing the clearance of Z-AT polymers/aggregates by drugs; carbamezpine (CBZ), rapamycin or liver-directed transfer of transcription factor EB (TFEB) is of benefit in cell and animal models. CBZ promotes autophagic and proteasomal degradation of both soluble Z-AT and insoluble Z-AT polymers, and was found to decrease the hepatic load of Z-AT and hepatic fibrosis in a mouse model of Z-AT deficiency-associated liver disease. Although CBZ changes the rate of intracellular degradation of Z-AT, it does not enhance Z-AT secretion. Rapamycin activates autophagy by inhibiting rapamycin kinase. TFEB gene transfer enhances autophagy and reduces activation of NF-κB and IL-6.

Other studies have targeted secretion of $\alpha_1$-antitrypsin by interfering with Z-AT polymerisation in PiZZ individuals using 4-phenylbutyric acid and glycerol and imino sugar compounds. However, neither of these resulted in any change in plasma concentrations of $\alpha_1$-antitrypsin in Z-AT.

Another approach to treat Z-AT-related liver disease would be to inhibit the formation of Z-AT polymers. An allosteric cavity that is distinct from the interface involved in polymerisation was identified as a target for rational structure-based drug design to block polymer formation. From a library of 1.2 million commercial drug-like compounds/chaperones, only four compounds reduced the rate of Z-AT polymerisation in vitro. One compound; CG blocked Z-AT polymerisation, had no effect on $\alpha_1$-antichymotrypsin, antithrombin, wildtype and mutant Syracuse neuroserpin. Although CG blocks Z-AT polymerisation/reduces Z-AT aggregates in a cell model of the disease, it does not increase the secretion of Z $\alpha_1$-antitrypsin from the cells.

**Inhibition of Polymerisation by Targeting Strand 4a**

It was recognised that polymerisation could be prevented by synthetic peptides with homology to strand 4a. However, these peptides are large and also anneal to M-AT and other members of the serpin superfamily such as $\alpha_1$-antithrombin, and therefore were unsuitable as therapeutic agents. Understanding the distinct conformation adopted by the Z-AT protein facilitated the design of a shorter six-mer synthetic peptide (Ac-FLEAIG-NH$_2$ (6M)) (Figure 3A). This six-mer peptide preferentially bound to Z-AT rather than M-AT and other common circulating serpins. However, the specific binding of the six-mer to pathologic Z-AT renders the Z-AT as inactive, and therefore would not address the predisposition of PiZZ homozygotes to develop emphysema.

Recently, a shorter four-mer synthetic peptide, Ac-TTAI-NH$_2$ (4M) has been identified that specifically anneals to the pathologic Z-AT. Our cell studies showed that it entered the ER of the Z-AT cell without any cytotoxicity, and was able to abrogate Z-AT polymerisation in vivo (Figure 3B). It was also able to dissociate existing intracellular Z-AT polymers/aggregates (Figure 4A). In addition, metabolic labelling demonstrated that in a cell model it was able to significantly restore normal secretion of Z-AT (Figure 4B,C). Furthermore it reduced the ER overload response as demonstrated by inhibition of PERK-dependant NF-κB activity (Figure 4D,E). Our findings uniquely demonstrate the potential of this strategy for prevention of Z-AT polymer formation in the liver, with preservation of inhibitory function in the tissues.

**SUMMARY**

The scientific community has achieved great understanding of the molecular mechanisms for of Z-AT related liver and lung disease. The recognition of the underlying mechanism of Z-AT aggregation and its cellular consequences, and the understanding of degradation pathways and gene corrective approaches, signify an exciting time in the field of Z antitrypsin related liver disease. Ongoing work will define whether these strategies alone or in combination will come to fruition and provide amelioration from this devastating disease.
REFERENCES


