

Liver injury in α_1 -antitrypsin deficiency: an aggregated protein induces mitochondrial injury

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α_1 -Antitrypsin (α_1 AT), the archetype of the Serpin supergene family, is the principal blood-borne inhibitor of destructive neutrophil proteases including elastase, cathepsin G, and proteinase 3 (reviewed in ref. 1). This glycoprotein, is secreted by liver cells and is considered an acute-phase reactant because its plasma levels increase during the host response to inflammation/tissue injury. The classical form of α_1 AT deficiency, which affects 1 in 1,800 live births in Northern European and North American populations, is associated with a mutant molecule termed α_1 ATZ, which is retained as a polymer in the endoplasmic reticulum (ER) of liver cells (reviewed in refs. 2, 3). Homozygotes are predisposed to premature development of pulmonary emphysema by a loss-of-function mechanism in which lack of α_1 AT in the lung permits uninhibited proteolytic damage to the connective tissue matrix (4, 5). Cigarette smoking markedly increases the risk and rate of development of emphysema (6). One mechanism for this environmental risk factor involves the functional inactivation of residual α_1 AT by phagocyte-derived active oxygen intermediates (4, 5). However, a growing body of evidence suggests that other environmental factors and genetic traits affect the incidence and severity of lung disease among α_1 AT-deficient individuals (7). It is still not entirely clear whether heterozygotes for α_1 ATZ are predisposed to lung disease.

Homozygotes for α_1 ATZ ("PIZZ" individuals) are also at risk for liver disease. In fact, α_1 AT deficiency is the most common genetic cause of liver disease in children (2, 3), and it predisposes adults to chronic liver disease and hepatocellular carcinoma (8). However, in contrast to the pathobiology of lung disease, liver injury in this deficiency appears to involve a gain-of-function mechanism whereby retention of the mutant α_1 ATZ molecule in the ER triggers a series of events that are eventually hepatotoxic. The strongest evidence for a

gain-of-function mechanism comes from studies in which mice transgenic for mutant human α_1 ATZ develop liver injury with many of the histopathologic hallmarks of the human condition (9, 10). Because there are normal levels of anti-elastases in these mice, as directed by endogenous genes, the liver injury cannot be attributed to a loss of function.

Landmark nationwide prospective screening studies done by Sveger in Sweden have documented an extraordinary variation in the phenotypic expression of liver disease among homozygotes. In these studies, only 10–15% of the PIZZ population developed clinically significant liver disease over the first 20 years of life (11, 12). These data indicate that other genetic traits and/or environmental factors predispose a subgroup of PIZZ individuals to liver injury. Because only a subgroup of homozygotes develop liver disease and because there is an inherent bias in ascertainment in other clinical studies of α_1 AT deficiency, it has been very difficult to determine whether heterozygous ("PIMZ") individuals are at increased risk for liver disease.

The mutant α_1 ATZ molecule

The α_1 ATZ molecule is characterized by a point mutation that results in the substitution of lysine for glutamate 342 and accounts for defective secretion. As described by Lomas and Mahadeva (this Perspective series, ref. 13), this substitution reduces the stability of the monomeric form of the molecule and increases the likelihood that it will form polymers in the ER by the "loop-sheet" insertion mechanism (14, 15). Indeed, polymers have been detected in the ER of hepatocytes by electron microscopic analysis of a liver biopsy from a PIZZ individual, and in vitro studies indicate that α_1 ATZ undergoes polymerization to a certain extent spontaneously and to a greater extent during relatively minor perturbations, such as a rise in temperature (15). These observations have led Lomas et al. to speculate that increases in body temperature during systemic inflammation might exacerbate this tendency in vivo and that differences in incidence of severity of febrile illness might account for the variation in expression of liver disease among α_1 AT-deficient hosts (15).

The strongest evidence that polymerization causes retention of α_1 ATZ in the ER comes from studies in which the fate of α_1 ATZ is examined after the introduc-

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Nonstandard abbreviations used: α_1 -antitrypsin (α_1 AT); endoplasmic reticulum (ER); cyclosporine A (CsA).

tion of additional mutations into the molecule. For instance, Kim et al. (16) introduced a mutation, F51L, into the α_1 AT molecule at amino acid 51. This mutation is remote from the Z mutation, E342K, but was predicted on the basis of structural characteristics to impede loop-sheet polymerization. Indeed, the F51L mutation makes the α_1 ATZ molecule less prone to polymerization and more efficient at folding in vitro, and it moderates the intracellular retention properties of α_1 ATZ in microinjected *Xenopus* oocytes (17) and in yeast (18). However, we have recently found that a novel, naturally occurring variant of α_1 AT, bearing both the same E342K substitution that is found in α_1 ATZ and a carboxyl-terminal truncation, is retained in the ER for at least as long as α_1 ATZ, even though it does not polymerize (19). These results could indicate that there are mechanisms other than polymerization which determine whether mutant α_1 AT molecules are retained in the ER. An alternative possibility is that polymerization of α_1 ATZ is not the cause of ER retention, but rather its result.

It is still not entirely clear what proportion of the newly synthesized mutant α_1 ATZ molecules is converted to the polymeric state in the ER. In one cell culture model system, we have found that $17.0\% \pm 1.9\%$ of α_1 ATZ is in the insoluble fraction at steady state (19), but comparable in vivo data are not yet available. It is also not known whether polymeric molecules are degraded in the ER less rapidly than their monomeric counterparts or whether polymeric molecules, when retained in the ER, are more hepatotoxic than their monomeric counterparts. Indeed, recent studies on the effect of temperature on the fate of α_1 ATZ have indicated the high degree of complexity involved in these issues. Although Lomas et al. showed that a rise in temperature to 42°C increases the polymerization of purified α_1 ATZ in vitro (15), Burrows et al. found that a rise in temperature to 42°C improves secretion of α_1 ATZ and decreases its intracellular degradation in a model cell culture system, whereas lowering the temperature to 27°C diminishes intracellular degradation of α_1 ATZ without any change in the small amount of α_1 ATZ secreted (20). Consistent with the well-established role that temperature plays in most biochemical processes, these results suggest that changes in temperature have the potential to affect multiple steps in the pathways by which α_1 ATZ is translocated through the secretory and degradative compartments, as well as affecting the relative proportions of α_1 ATZ in the monomeric and polymeric state. On the basis of these considerations, as well as long-standing clinical experience with α_1 AT-deficient children and other children with liver disease, and in the absence of clear epidemiological evidence, it seems unlikely that there is a simple relationship between febrile episodes and phenotypic expression of liver disease in α_1 AT-deficient patients.

The fate of mutant α_1 ATZ in the ER

Several studies have shown that α_1 ATZ is degraded in the ER and that the proteasome is a key component of the degradation pathway (21–23). Degradation of α_1 ATZ is markedly reduced by specific proteasome inhibitors in yeast and mammalian cells (22, 23). In a mammalian cell-

free system, degradation of α_1 ATZ is, at least in part, attributable to a pathway involving the transmembrane ER chaperone calnexin, polyubiquitination of calnexin, and targeting of the α_1 ATZ-polyubiquitinated calnexin complex by the proteasome (23). There is also evidence for the involvement of ubiquitin-independent proteosomal and nonproteosomal pathways in degradation of α_1 ATZ in the mammalian cell-free system (24).

As discussed below, autophagy may represent one non-proteosomal mechanism for degradation of α_1 ATZ (25). Because this finding is based on the effect of chemical inhibitors of autophagy, which have other effects on cellular metabolism, definitive evidence for the role of autophagy in degradation of α_1 ATZ will require more detailed, probably genetic studies. Cabral et al. have provided evidence for a nonproteosomal degradation pathway that is sensitive to tyrosine phosphatase inhibitors (26). In their studies, degradation of α_1 ATZ in a hepatoma cell line was not affected by proteasome inhibitors but was reduced by tyrosine phosphatase inhibitors. Although this finding was originally interpreted to suggest that there were cell type-specific differences in the role of proteosomal and nonproteosomal degradation mechanisms and that nonproteosomal degradation mechanisms were more important in hepatocytes, subsequent studies have shown that the proteasome still plays a major role in degradation of α_1 ATZ in hepatoma cell lines (27). We therefore conclude that non-proteosomal mechanisms, sensitive to tyrosine phosphatase inhibitors, are particularly important in specific cell lines rather than specific cell types. The relative contributions of proteosomal and nonproteosomal mechanisms to the disposal of α_1 ATZ in vivo are still unknown.

The mechanism by which the proteasome gains access from the cytoplasm to α_1 ATZ on the luminal side of the ER membrane is also uncertain. Although retrograde translocation from the ER to the cytoplasm has been demonstrated for some luminal substrates of the proteasome, there is very limited evidence for retrograde translocation of α_1 ATZ. Werner et al. detected α_1 ATZ free in the cytosolic fraction of yeast when the proteasome was inhibited (22), but only a small fraction of the total α_1 ATZ in the ER could be detected (22), and there has been no other evidence for retrotranslocation. Recent studies have provided evidence for extraction of substrates through the ER membrane by the proteasome (28). The AAA ATPase Cdc 48/p97 and its partners appear to play an important role in this process (29).

In order to determine whether the fate of α_1 ATZ is different in α_1 AT-deficient hosts susceptible to liver disease ("susceptible hosts") as compared with α_1 AT-deficient individuals who are protected from liver disease ("protected hosts"), Wu et al. transduced skin fibroblasts from PIZZ individuals, with or without liver disease, with amphotropic recombinant retroviral particles designed for constitutive expression of the mutant α_1 ATZ (30). The PIZZ individuals were carefully selected to ensure appropriate representation. Susceptible hosts were defined as having severe liver disease by clinical criteria. Protected hosts were discovered incidentally and had never had clinical or biochemical evidence of liver dis-

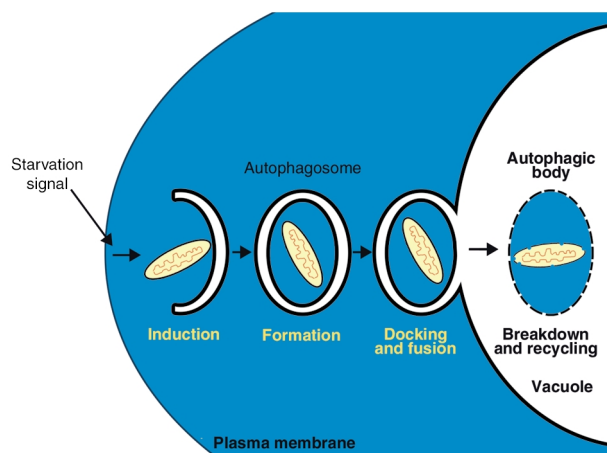


Figure 1
Schematic model of events that characterize the autophagic response. Adapted from ref. 50 with permission.

ease. Human skin fibroblasts were selected because they do not express the endogenous α_1 AT gene but, presumably, express other genes involved in the postsynthetic processing of secretory proteins. Each fibroblast cell line expressed the human α_1 AT transgene. Unlike wild-type α_1 AT, mutant α_1 ATZ protein was selectively retained intracellularly in every case. However, cells from susceptible and protected hosts differed in that the former showed a delay in degradation of α_1 ATZ after it accumulated in the ER. Thus, these data provide evidence that alterations in quality control mechanisms, such as the ER degradation pathway, can predispose α_1 AT-deficient hosts to liver injury. Further elucidation of the mechanisms by which the cell degrades α_1 ATZ or mounts protective cellular response pathways to it will be critical for further understanding the hepatotoxic effects of this aggregated protein.

Cellular responses to ER retention of α_1 ATZ

Several recent studies have shown that ER retention of mutant α_1 ATZ provokes a rather specific cellular response with autophagy as a major feature. Autophagy is thought to be a general mechanism whereby cytosol and intracellular organelles, such as ER, are first sequestered from the rest of the cytoplasm, allowing them to be degraded subsequently within lysosomes (Figure 1). This process has been observed in many cell types, especially in response to nutritional or other forms of stress or during the cellular remodeling that accompanies differentiation, morphogenesis, and aging. Our studies show that autophagosomes develop in several different model cell culture systems genetically engineered to express α_1 ATZ, including human fibroblasts, murine hepatoma, and rat hepatoma cell lines (Figure 2). Moreover, in a HeLa cell line engineered for inducible expression of α_1 ATZ, autophagosomes appear as a specific response to the expression of α_1 ATZ and its retention in the ER (25). There is a marked increase in autophagosomes in hepatocytes in transgenic mouse models of α_1 AT deficiency and a disease-specific increase in

autophagosomes in liver biopsies from patients with α_1 AT deficiency (Figure 3). Mutant α_1 ATZ molecules can be detected in autophagosomes by immune electron microscopy, often together with the ER molecular chaperone calnexin. Intracellular degradation of α_1 AT is partially reduced by chemical inhibitors of autophagy, suggesting that autophagy also contributes to the quality control mechanism for disposal of α_1 ATZ (25).

Taken together, these results have suggested that the autophagic response is induced to protect liver cells from the toxic effects of aggregated α_1 ATZ retained in the ER. We have also speculated about the role of autophagy in protecting liver cells from tumorigenesis. Several recent studies have shown that autophagic activity is decreased in tumors and that reconstitution of autophagic activity inhibits tumorigenesis *in vivo* (31, 32). In our studies, autophagosomes are predominantly found in liver cells with dilated ER in both human and transgenic mouse liver (25). Previous studies in transgenic mouse models of α_1 AT deficiency have shown that hepatocarcinogenesis evolves within nodular aggregates of hepatocytes that are negative for α_1 AT expression by immunofluorescent staining (33).

It is not yet clear whether autophagy is substrate-specific for α_1 ATZ or provides a more general response to aggregated proteins retained in the ER. No such cellular response has been described in studies of mutant proteins that aggregate in the cytoplasm or nucleus. Although there has been some mention of autophagic vacuoles around the aggresomes that form when CFTR $\Delta F508$ accumulates in the presence of proteasomal inhibitors (34, 35), the histologic pictures in cells expressing α_1 ATZ or CFTR $\Delta F508$ are quite distinct (25). Autophagy is not induced by tunicamycin or thapsigargin, agents that cause a generalized form of ER stress (N. Mitzushima, personal communication), but Russell bodies, which have been described in cells retaining certain mutant Ig molecules in the ER (36), share many characteristics with autophagosomes. Autophagy has also been implicated in the cellular response to the mutant form of the ER membrane protein peripheral myelin protein-22 that causes a gain-of-function disease affecting Schwann cells

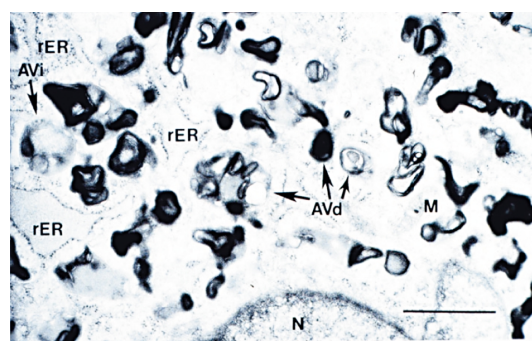


Figure 2
Autophagic vacuoles in fibroblasts engineered for expression of mutant α_1 ATZ. Transmission electron micrograph shows nascent autophagosomes (AVi) and degradative autophagosomes (AVd) intercalated within and around cisternae of rough ER (rER) that is dilated with granular material. N, nucleus; M, mitochondrion. Reproduced from ref. 25 with permission.

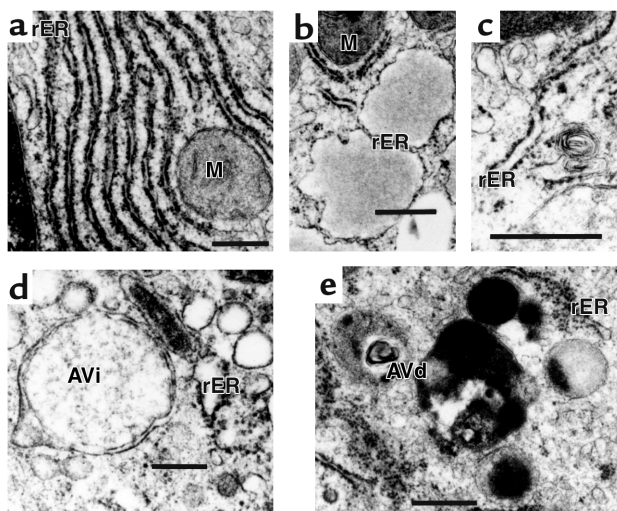


Figure 3
Autophagic vacuoles in human liver tissue. Transmission electron micrograph of a liver biopsy specimen from an individual homozygous for α_1 ATZ is shown. (a) One of the relatively normal hepatocytes that can be seen in such livers. (b) A hepatocyte with markedly dilated rough ER (rER). (c) An autophagic vacuole evolving adjacent to rER. (d) Nascent autophagosomes; (e) degradative autophagosomes. Bar, 100 nm. Taken from ref. 25 with permission.

in Charcot-Marie-Tooth disease and Dejerine-Sottas syndrome (37). Further studies of these structures may therefore shed additional light on whether the autophagic response is substrate-specific.

Recently, we examined the autophagic response to ER retention of α_1 ATZ *in vivo* by testing the effect of fasting on the liver of the PiZ mouse model of α_1 ATZ deficiency (38). Starvation is a well-defined physiologic stimulus of autophagy, as well as a known environmental stressor of liver disease in children. The results show that there is a marked increase in fat accumulation and in α_1 AT-containing, ER-derived globules in the liver of the PiZ mouse, induced by fasting. These changes were particularly exaggerated at 3–6 months of age. Three-month-old PiZ mice had a significantly decreased tolerance for fasting compared with nontransgenic C57 Black mice. Although fasting induced a marked autophagic response in wild-type mice, the autophagic response was already activated in PiZ mice to levels that were more than 50% higher than those in the liver of fasted wild-type mice, and they did not increase further during fasting. These results indicate that autophagy is constitutively activated in α_1 AT deficiency and that the liver is unable to mount an increased autophagic response to physiologic stressors. Based on our search of the literature, the only other condition in which there is accumulation of autophagic vacuoles under homeostatic conditions is Danon disease (39). In contrast to α_1 AT deficiency, however, autophagosomes accumulate in Danon disease because of a genetic defect in the terminal phases of autophagy, *i.e.*, the fusion of autophagic vacuoles with lysosomes and subsequent degradation within autolysosomes (39, 40).

In the course of our ultrastructural studies of the liver of the PiZ mouse and of patients with α_1 AT deficiency,

we have recently been struck by the degree of mitochondrial autophagy that is induced (Tekman, J.H., et al., manuscript submitted for publication). A comparison of the liver from four α_1 AT-deficient patients with livers from eight patients with other liver diseases and four normal livers showed a marked specific increase in mitochondrial autophagy associated with α_1 AT deficiency. Even more interesting is the observation that many mitochondria that are not surrounded by autophagic vacuolar membranes are nevertheless damaged or in various phases of degeneration in liver cells from α_1 AT-deficient hosts. This damage is characterized by the formation of multilamellar structures within the limiting membrane, condensation of the cristae and matrix, and, in some cases, dissolution of the internal structures, often leaving only electron-dense debris compressed into a thin rim at the periphery of the mitochondrion.

Mitochondrial autophagy and injury are also marked in the liver of the PiZ transgenic mouse model of α_1 AT deficiency. Immunofluorescence analysis shows the presence of activated caspase-3 in the PiZ mouse liver (Tekman, J.H., et al., manuscript submitted for publication). Because cyclosporine A (CsA) has been shown to reduce mitochondrial injury (41) and inhibit starvation-induced autophagy (42), we examined the effect of CsA on PiZ mice. We found that it significantly reduces hepatic mitochondrial injury, increases activation of caspase-3 and improves the animals' tolerance of starvation. These results provide evidence for the novel concept that mitochondrial damage and caspase activation play a role in the mechanism of liver cell injury in α_1 AT deficiency. Although this analysis suggests that there is mitochondrial injury that is separate from the autophagic process, the possibility that autophagy plays some role in mitochondrial damage cannot be completely excluded. One model of mitochondrial damage in this deficiency holds that accumulation of α_1 ATZ in the ER is in itself responsible for mitochondrial dysfunction, and, indeed, there is now ample evidence in the literature for functional interactions between mitochondria and closely apposed ER cisternae (43, 44). Recent studies show that specific signals are transmitted between these two intracellular compartments (45, 46) and that mitochondrial dysfunction, including release of cytochrome *c* and caspase-3 activation, is associated with the ER dilatation and stress induced by brefeldin A, tunicamycin, or thapsigargin (47, 48). It is not yet known, however, whether mitochondrial dysfunction in the latter cases is due to ER dilatation and/or ER stress or to independent effects on mitochondria by these experimental drugs. A second possible explanation, not necessarily incompatible with the first, envisages mitochondrial dysfunction as a result of the autophagic response to ER retention of α_1 ATZ. In this scenario, mitochondria are recognized nonspecifically by the autophagic response, which is constitutively activated to somehow remove and degrade areas of the ER that are distended by aggregated mutant protein. Although our data indicates that CsA inhibits hepatic mitochondrial injury *in vivo*, this benefit could reflect the drug's known effects on the mitochondrial permeability transition (41), on autophagy (42), or both.

The CsA findings are also noteworthy for their therapeutic implications. They indicate that CsA can prevent mitochondrial damage even under circumstances in which α_1 ATZ continues to accumulate in the ER. Thus, they provide a proof-in-principle for mechanism-based therapeutic approaches to liver disease in α_1 AT deficiency — pharmacological intervention directed at distal steps in the pathobiological pathway that leads to liver injury (the mitochondrial step, for instance), rather than at the primary defect or the early events in the pathway. Our previous studies have shown that chemical chaperones, phenylbutyric acid (20), and iminosugars (49) mediate a partial but significant increase in secretion of mutant α_1 ATZ. It is therefore now also possible to consider chemoprophylactic strategies involving combinations of drugs that affect sequential steps in α_1 AT hepatotoxicity.

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