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Immune-modulating effects of alpha-1 antitrypsin

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Abstract

Alpha-1 antitrypsin (AAT) is a circulating serine protease inhibitor (serpin) that inhibits neutrophil elastase in the lung, and AAT deficiency is associated with early-onset emphysema. AAT is also a liver-derived acute phase protein that, in vitro and in vivo, reduces production of pro-inflammatory cytokines, inhibits apoptosis, blocks leukocyte degranulation and migration, and modulates local and systemic inflammatory responses. In monocytes, AAT has been shown to increase intracellular cAMP, regulate expression of CD14, and suppress NF κ B nuclear translocation. These effects may be mediated by AAT's serpin activity or by other protein-binding activities. In preclinical models of autoimmunity and transplantation, AAT therapy prevents or reverses autoimmune disease and graft loss, and these effects are accompanied by tolerogenic changes in cytokine and transcriptional profiles and T cell subsets. This review highlights advances in our understanding of the immune-modulating effects of AAT and their potential therapeutic utility.

Keywords

apoptosis; autoimmunity; diabetes; inflammation; protease; transplantation

Introduction

Alpha-1 antitrypsin (AAT) is a major liver-derived circulating protein that functions as a natural inhibitor of various serine proteases and is a component of the acute-phase response. For many decades, the protein's function as a serine protease inhibitor ("serpin") was its best-known and best-understood role. An extensive body of work established the importance of AAT in inhibiting excessive neutrophil elastase activity in the lung, and over 75 polymorphisms in the AAT gene have been found, some of which are associated with AAT deficiency and are linked to diseases of the lung and liver (reviewed in Crystal, 1990 and Janciauskiene et al., 2011). This work formed the basis for the development of augmentation therapy with AAT for the treatment of early-onset emphysema associated with AAT deficiency (Crystal, 1990).

Less well understood is AAT's role in the hepatic acute-phase response. During inflammation the liver synthesizes acute phase proteins (APPs), including AAT, which are

thought to contribute to the inflammatory response but which also play a central role in limiting local and systemic inflammation (Tilg et al., 1993). Thus, APPs play a dual role, and AAT has been shown to induce the production of both pro-inflammatory interleukin 1 (IL-1) and anti-inflammatory IL-1 receptor antagonist (IL-1Ra) in peripheral blood mononuclear cells (PBMCs) (Tilg et al., 1993). Other studies have shown that AAT inhibits neutrophil superoxide production (Bucurenci et al., 1992), prevents hepatocyte apoptosis (Van Molle et al., 1997), inhibits lipopolysaccharide (LPS)-induced monocyte activation (Janciauskiene et al., 2004a), and functions as an endogenous inhibitor of proinflammatory cytokine production in whole blood (Pott et al., 2009).

These studies have led to an evolution in our understanding of the biological roles of AAT, from a protein that functions primarily as an antiprotease to one that also has subtle and nuanced roles as an APP with broad anti-inflammatory and anti-apoptotic properties. In this emerging view, AAT is a protein with diverse immune-modulating effects and there is an interest in determining whether AAT may contribute in some way to various inflammatory conditions and whether it may have future therapeutic utility in indications beyond augmentation therapy for AAT deficiency.

Structure and function

AAT is 52-kDa, 394-amino acid glycoprotein that is a typical member of the serpin superfamily (Huber and Carrell, 1989). Members of this family have a common molecular structure with a 5-stranded β -sheet-A and a mobile reactive center loop that acts as a pseudosubstrate for the cognate protease leading to a covalently bound complex (Elliott et al., 1998). The principal serine protease targets of AAT are neutrophil elastase and proteinase 3, but the protein also inhibits trypsin, kallikreins 7 and 14, and matriptase (Janciauskiene et al., 2011). There is also some evidence that AAT can inhibit non-serine proteases, such as the cysteine protease caspase-3 (Petrache et al., 2006; Zhang et al., 2007) and the metalloprotease TACE (tumor necrosis factor- α -converting enzyme) (Bergin et al., 2010), although the molecular mechanisms involved are unclear. The molecular basis for the anti-inflammatory and anti-apoptotic effects of AAT is also unclear: while in some cases this may involve the reactive center loop (Tilg et al., 1993), in other instances these effects are observed with forms of AAT lacking protease inhibitory activity (Janciauskiene et al., 2007). Some of these effects may be mediated by non-serpin binding activities of AAT, the structural basis of which is only partly understood. The thyroxine and corticosteroid binding proteins in plasma are members of the serpin family and AAT shares with these proteins a β -barrel motif that could serve as ligand-binding site (Huber and Carrell, 1989). Such a motif may account for the apparent association of AAT with cholesterol and localization within lipid rafts (reviewed by Lewis, 2012). Binding of AAT to IL-8 depends on one or more of the three glycosylation moieties on AAT (Bergin et al., 2010; Huber and Carrell, 1989). AAT also binds to the EspB protein from enteropathogenic *E. coli* and to the metalloprotease aggrecanase-1, but the binding mechanisms and functional significance of these interactions are unknown (Knappstein et al., 2004; Yoshida et al., 2005). In addition, as reviewed by Lewis (2012), AAT binds heat shock proteins, which may play a role in diabetes; the C-terminal end of AAT blocks human immunodeficiency virus entry; and AAT

induces vascular endothelial growth factor production, which may reduce endothelial cell apoptosis.

AAT is the most abundant serpin in human plasma and is synthesized primarily in the liver, although neutrophils, mononuclear phagocytes, islets, and intestinal epithelial cells may also produce the protein. AAT generally circulates at a concentration of 1.0-1.5 g/L but during the acute-phase response the plasma concentration increases rapidly into the 2.0-3.0 g/L range (Crystal, 1990; Janciauskiene et al., 2011). Over 75 alleles of the AAT gene have been described, some of which lead to AAT deficiency, in some cases to < 10% of normal levels (Crystal, 1990). Even when AAT plasma levels are in the normal range, AAT can be modified at sites of inflammation by oxidation or polymerization, which may produce local or systemic functional deficiency (reviewed in Janciauskiene et al., 2004a).

Anti-inflammatory effects

AAT is an APP and as such is involved in modulation of local and systemic inflammatory responses. For example, AAT attenuates bleomycin-induced pulmonary fibrosis in hamsters (Nagai et al., 1992) and inhibits the lethal response to TNF in mice (Libert et al., 1996). In vitro, AAT inhibits neutrophil superoxide production (Bucurenci et al., 1992), protects cultured lung endothelial cells from LPS injury (Tunen et al., 1988), and inhibits LPS-mediated monocyte activation (Janciauskiene et al., 2004a). Production of the inflammatory cytokines IL-6 and IL-8 after stimulation with *S. epidermidis* or LPS is greater in whole blood from AAT-deficient subjects compared to healthy controls (Pott et al., 2009). Although the molecular basis for these effects is unclear, AAT has been shown to inhibit NF κ B activation (Pott et al., 2009) and increase cAMP synthesis (Janciauskiene et al., 2007), both of which have been shown to modulate cytokine production in mononuclear cells.

It is plausible that the anti-inflammatory effects may result in part from AAT's serpin activity, since proteases released at sites of injury are important drivers of inflammation. In isolated human PBMCs, AAT preferentially induces IL-1Ra, an effect that appears to be mediated by the serpin-enzyme complex (SEC) receptor, suggesting that the serpin activity of AAT is indeed important (Tilg et al., 1993). It has also been suggested that the anti-inflammatory effects of AAT may be mediated by inhibition of serine proteases that signal via protease-activated receptors (PARs) (Lewis, 2012). PARs are broadly involved in diverse inflammatory responses and proteases known to activate PARs include neutrophil elastase, proteinase 3, and trypsin (Shpacovitch et al., 2008), all targets of AAT. Although this is a plausible mechanism, direct involvement of AAT in modulating PAR signaling has yet to be demonstrated. On the other hand, more recent data suggest that other mechanisms may also be involved. Suppression of silica-induced acute inflammation in mice by AAT was not abrogated by oxidative inactivation of the protein, suggesting that the anti-inflammatory effect was not mediated by classic antiproteolysis (Churg et al., 2001). Similarly, both native and modified (inactive) forms of AAT inhibit the release of TNF α and IL-1 β , and enhance the release of the anti-inflammatory cytokine IL-10, from LPS-stimulated monocytes, again suggesting that the protective effects of AAT are independent of serine protease inhibition (Janciauskiene et al., 2004a).

In contrast to studies that have consistently shown anti-inflammatory activities of the full-length AAT protein, some studies have suggested that a C-terminal cleavage product – a 36-residue peptide (C-36 peptide) generated after formation of the serpin-enzyme complex – is pro-inflammatory, stimulating neutrophil activation and inducing monocyte cytokine release (Janciauskiene et al., 2004b; Subramaniam et al., 2006), although the *in vivo* relevance of this observation is uncertain. Interestingly, on monocytes the C-36 peptide appears to act as a weak LPS-like agonist: in the absence of LPS, C-36 produces modest stimulation of TNF α and IL-1 β release, while in the presence of LPS, C-36 partly antagonizes the robust cytokine release stimulated by LPS alone, suggesting that C-36 acts as either a pro- or anti-inflammatory immune modulator depending on the context (Subramaniam et al., 2006).

Anti-apoptotic effects

AAT has been shown to inhibit the lethal response to TNF and galactosamine (GalN) in mice (Libert et al., 1996). Further study revealed that this protective effect is mediated by inhibition of TNF-induced apoptosis of hepatocytes in mice, an effect that appears to be indirect (i.e., involving additional factors *in vivo*) because AAT does inhibit TNF-induced apoptosis in hepatoma cells in culture (Van Molle et al., 1997). AAT also reduces renal ischemia/reperfusion injury in a murine model by preventing apoptosis and inflammation. In this model, the anti-apoptotic effect may have been mediated by reduced caspase-1-like and caspase-3-like activity observed in renal homogenates, although direct inhibitory effects by AAT on caspases could not be demonstrated *in vitro* (Daemen et al., 2000).

In cell culture, AAT inhibits apoptosis of vascular smooth muscle cells, apparently by inhibition of extracellular matrix degradation by cell-derived proteases and possibly by inhibition of caspase activation; these data have led to the suggestion that AAT and related serpins are natural survival factors in serum (Ikari et al., 2000). AAT has also been shown to inhibit staurosporine-induced apoptosis of primary lung endothelial cells, and AAT interacted directly with and inhibited caspase-3 in cell-free systems, albeit with lower potency than the inhibition of elastase (Petrahe et al., 2006). Importantly, the anti-caspase-3 activity required active AAT, because heat- and oxidation-inactivated AAT showed significantly diminished caspase inhibition, as did AAT conformers with cleavage of the reactive center loop. Intriguing also was the finding that AAT colocalized intracellularly with caspase-3 in lung endothelial cells, suggesting that the anti-apoptotic effect of AAT in this model is fundamentally different from the classical serpin activity on extracellular elastase (Petrahe et al., 2006).

Consistent with the findings in primary lung endothelial cells, AAT has been shown to inhibit TNF α - or streptozotocin (STZ)-induced apoptosis of insulin-producing β -cells, an effect that appeared to be due to direct inhibition of caspase-3 activity (Zhang et al., 2007). Moreover, as shown for lung endothelial cells, AAT was found to localize intracellularly in β -cells, and this was the presumed site of caspase-3 inhibition (Zhang et al., 2007). Further, therapeutic administration of AAT prevented STZ-induced diabetes in mice (Zhang et al., 2007). These findings were supported by similar data in the rat insulinoma cell line INS-1E, in which AAT prevented TNF α -induced apoptosis, and by data showing that AAT increased insulin production in isolated rat islets (Kalis et al., 2010).

Effects on innate immunity

Innate immunity comprises all cellular and humeral components of the immune system that are not antigen-specific, including mononuclear and polymorphonuclear phagocytic cells, dendritic cells, and natural killer cells, and the cytokines and chemokines produced by these cells. The inflammatory response is part of innate immunity and, as already discussed, numerous studies have documented effects of AAT on production of pro- and anti-inflammatory cytokines by PBMCs *in vitro*, as well as effects of AAT on local and systemic inflammation *in vivo*. Indeed, a consistent theme that has emerged from *in vitro* and *in vivo* studies of the immune-modulating effects of AAT is that these activities are mediated predominantly by effects on cells of the innate immune system, including monocytes, neutrophils, and dendritic cells.

Numerous studies have documented AAT-mediated inhibition of pro-inflammatory cytokine release by human monocytes or PBMCs (Tilg et al., 1993; Janciauskiene et al., 2004a, 2007; Nita et al., 2005, 2007; Pott et al., 2009). The mechanism involves suppression of NF κ B nuclear translocation (Churg et al., 2001; Nita et al., 2007) as well as regulation of expression and release of membrane-bound CD14 (Nita et al., 2007); the effects on CD14, in turn, may be a consequence of AAT-stimulated increases in intracellular cAMP in monocytes (Janciauskiene et al., 2007). Interestingly, at early time points AAT transiently augments LPS-stimulated cytokine release before inhibiting cytokine production at later time points, suggesting that AAT modulates inflammatory responses in a context-dependent manner (Nita et al., 2007).

Several independent reports have also documented suppression by AAT of neutrophil activation and migration (Bucurenci et al., 1992; Churg et al., 2001; Nita et al., 2005; Bergin et al., 2010, 2014). Bergin and colleagues have shown that AAT inhibits neutrophil migration by binding the chemoattractant IL-8 and by controlling the membrane expression of the Fc receptor Fc γ RIIIb, which is required for the chemotactic response to soluble immune complexes (Bergin et al., 2010). This group also showed that AAT regulates membrane TNF α expression and degranulation in neutrophils (Bergin et al., 2014).

Finally, several studies have provided evidence that AAT alters the maturation and promotes tolerogenic responses of dendritic cells (DCs), including IL-10 production (Lewis et al., 2008; Tawara et al., 2012; Ozeri et al., 2012). Since DCs are a key link between innate and adaptive immunity, this effect may be important in explaining tolerogenic responses to AAT therapy in models of autoimmune disease (see below).

Effects on adaptive immunity

Adaptive immunity comprises all cellular and humeral components of the immune system that are antigen-specific, principally B and T lymphocytes and antibodies. Several studies have shown that AAT has no direct effects on T cells *in vitro*, and yet there are effects on T cell subsets *in vivo*, which are therefore presumed to be indirect. In a variety of models of autoimmunity or allotransplantation, AAT therapy reduces lymphocytic infiltrates, alters T cell receptor repertoire, expands regulatory T cells (Tregs), and favorably alters the ratio of Tregs to T effector (Teff) cells, all without evidence of direct T cell effects (Song et al.,

2004; Lu et al., 2006; Lewis et al., 2008; Koulmanda et al., 2008; Grimstein et al., 2011; Subramanian et al., 2011; Tawara et al., 2012). The presumption is that these effects on adaptive immunity result from changes in the cytokine milieu (e.g., suppression of TNF α , IL-1 β , and IL-6, and increases in IL-10) together with induction of tolerogenic DCs, which affect T cell lineage commitment and favorably alter the Treg/Teff balance. These changes may be further augmented by AAT-mediated reductions in proliferation and activation of B cells, which like DCs are important antigen-presenting cells (Mizrahi et al., 2013). Suppression of B cell activity is consistent with reductions in autoantibodies that have been observed in models of autoimmunity after AAT therapy (Song et al., 2004; Grimstein et al., 2011).

AAT therapy in models of autoimmunity and transplantation

AAT therapy has been evaluated in a variety of animal models of autoimmunity and allotransplantation. Most of the studies have involved type 1 diabetes (T1D) and islet allografts, but models of arthritis, multiple sclerosis, and skin and bone-marrow transplantation have also been studied. The nonobese diabetic (NOD) mouse is a standard model for autoimmune T1D. In this model, recombinant adeno-associated virus (rAAV)-mediated AAT gene therapy prevents the onset of disease, reduces insulinitis, and alters the TCR repertoire (Song et al., 2004; Lu et al., 2006). Further, injection of human AAT in the NOD mouse prevents development of disease and reverses new-onset diabetes and is associated with increased β -cell mass, tolerance to syngeneic islet allografts, induction of tolerogenic transcriptional profiles in pancreatic lymph nodes, and ablation of insulin resistance (Koulmanda et al., 2008; Ma et al., 2010). Injection of human AAT has also been shown to prolong islet allograft survival in the mouse, an effect that was associated with reduced cellular infiltration and pro-inflammatory responses in islets, expansion of tolerogenic DCs and Tregs, and downregulation of key inflammatory pathways including the TNF α -TLR4-NF κ B axis (Lewis et al., 2005, 2008; Koulmanda et al., 2012).

In the collagen-induced arthritis (CIA) mouse model of rheumatoid arthritis, injection of human AAT or gene therapy with rAAV-AAT resulted in delayed disease onset, reduced autoantibody levels, and decreased serum levels of B cell activating factor (BAFF) but had no direct effects on T cells in vitro (Grimstein et al., 2011). As noted for the NOD and islet allograft studies, these results suggest that the effect on CIA disease progression is mediated by changes in cytokine production and effects on B cells. Sustained expression of human AAT in a transgenic mouse prevented the onset of experimental autoimmune encephalomyelitis (EAE) induced by injection of MOG-35-55 peptide, enhanced levels of Tregs, and decreased MOG peptide-induced IL-17, IL-1 β , and IL-6 production by splenic cells (Subramanian et al., 2011). While the disease protection in this model was impressive, it can be argued that this was mainly a model of impaired T cell responses to a highly immunogenic vaccination protocol and the relevance to AAT therapy in established autoimmunity is unclear. Finally, injection of human AAT reduced graft-versus-host disease (GvHD) in a mouse model of allogeneic bone marrow transplantation (BMT), which was associated with reduced expansion of alloreactive Teff, enhanced recovery of Tregs, and suppression of LPS-induced pro-inflammatory cytokine production, but there were no direct effects on T cells in vitro (Tawara et al., 2011). Concordant with these data, ex vivo addition

of AAT to mixed lymphocyte cultures from patients with chronic GvHD resulted in suppression of IL-32 and decreased lymphocyte proliferation (Marcondes et al., 2011).

Impressive as these results are of AAT therapy in mouse models of autoimmunity or transplantation, it must be remembered that in every case this involved injection of, or gene therapy with, human AAT. In the mouse there are at least five AAT genes and it is unclear whether AAT is a component of the acute phase response (reviewed in Lu et al., 2006). Human AAT appears to be immunogenic in the mouse and it can produce fatal anaphylaxis in the NOD strain (Ma et al., 2010). Therefore, we should be cautious in our interpretation of the significance of the results. While it is possible that human AAT is exerting molecule-specific anti-inflammatory and anti-apoptotic effects in these mouse models, it is also possible that the effects are, at least in part, the result of nonspecific immunologic responses to a foreign protein. Nevertheless, based on the efficacy in preclinical models, several pilot clinical trials have been launched to evaluate the utility of AAT in preserving residual islet function in patients with recent-onset T1D (Lewis, 2012), including the Immune Tolerance Network's RETAIN trial (Ehlers and Nepom, 2012).

Opportunities and challenges

Accumulating evidence suggests that, in addition to its classic serpin activity, AAT exerts anti-inflammatory and cytoprotective effects. These data are enhancing our understanding of the roles AAT may play in the acute phase response and diverse inflammatory conditions, and may open the door to novel therapeutic applications (Janciauskiene et al., 2011; Lewis, 2012). However, significant challenges remain. A definitive mechanism for the anti-inflammatory and anti-apoptotic effects at the molecular level is lacking. This, in turn, leads to uncertainty about the true nature of the 'active pharmaceutical ingredient' (API) that mediates these anti-inflammatory/cytoprotective effects. All marketed AAT products are prepared from pooled human plasma and are somewhat heterogeneous in terms of protein composition and chemical structures, such as modifications affecting protein charge, C-terminal amino acid truncations, and the presence of unrelated plasma proteins (FDA, 2013). There is no evidence that these structural variations or modifications affect the elastase-inhibitory activity, safety, or efficacy of AAT products for the approved indication (AAT deficiency with emphysema) (FDA, 2013), but their effect on the anti-inflammatory/cytoprotective activities in novel indications is unknown. Recombinant alternatives would be desirable, but would require strict control of glycosylation patterns and other potential modifications whose importance for biological activity remains unclear. AAT has a short half-life (3-5 days), requiring weekly intravenous infusions. If new indications require long-term treatment (months or years) this could be a significant burden. Finally, while AAT monotherapy has shown success in murine models of disease this is unlikely to be sufficient in human disease. For example, there are now many instances in which a monotherapy appeared to be curative in the NOD mouse but showed limited or no efficacy in human T1D. The more likely scenario in the clinic will be the use of AAT as an anti-inflammatory/cytoprotective drug in combination with one or more additional drugs, which could include a direct T cell modulator (e.g., anti-CD3 or anti-CD2), an anti-cytokine (e.g., anti-IL-6 or anti-TNF α), and/or an auto-antigen (e.g., GAD65 or proinsulin in the case of T1D) (Ehlers and Nepom, 2012). Among potential disease targets, T1D is compelling because it is

characterized by localized inflammation and killing of β -cells in islets and thus may benefit from an agent that has combined anti-inflammatory and anti-apoptotic effects (Strom, 2005). Moreover, definitive trials in new-onset T1D may benefit from initial pilot studies in islet transplantation, where residual islet cell mass can be estimated, thereby strengthening the mechanistic rationale for the use of AAT in this disease. A great deal of translational research still needs to be done to resolve these questions, but AAT presents an interesting prospect as a potential immune modulator that may have value in the treatment of autoimmunity and in improving graft survival after transplantation.

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