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Concentration-dependent effects of native and polymerised α 1-antitrypsin on primary human monocytes, *in vitro*

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Summary

Background: α 1-antitrypsin (AAT) is one of the major serine proteinase inhibitors controlling proteinases in many biological pathways. There is increasing evidence that AAT is able to exert other than antiproteolytic effects. To further examine this question we compared how various doses of the native (inhibitory) and the polymerised (non-inhibitory) molecular form of AAT affect pro-inflammatory responses in human monocytes, *in vitro*. Human monocytes isolated from different donors were exposed to the native or polymerised form of AAT at concentrations of 0.01, 0.02, 0.05, 0.1, 0.5 and 1 mg/ml for 18 h, and analysed to determine the release of cytokines and to detect the activity of NF- κ B.

Results: We found that native and polymerised AAT at lower concentrations, such as 0.1 mg/ml, enhance expression of TNF α (10.9- and 4.8-fold, $p < 0.001$), IL-6 (22.8- and 23.4-fold, $p < 0.001$), IL-8 (2.4- and 5.5-fold, $p < 0.001$) and MCP-1 (8.3- and 7.7-fold, $p < 0.001$), respectively, compared to buffer exposed cells or cells treated with higher doses of AAT (0.5 and 1 mg/ml). In parallel to increased cytokine levels, low concentrations of either conformation of AAT (0.02–0.1 mg/ml) induced NF- κ B p50 activation, while 1 mg/ml of either conformation of AAT suppressed the activity of NF- κ B, compared to controls.

Conclusions: The observations reported here provide further support for a central role of AAT in inflammation, both as a regulator of proteinase activity, and as a signalling molecule for the expression of pro-inflammatory molecules. This latter role is dependent on the concentration of AAT, rather than on its proteinase inhibitory activity.

Background

α 1-antitrypsin (AAT) is one of the major serine proteinase inhibitors in human plasma, synthesised primarily in the liver, but also in extra-hepatic tissues and cells, including neutrophils, monocytes and alveolar macrophages [1–4]. Under normal conditions the daily production of AAT is 34 mg per kg body weight. The average concentration of AAT in plasma in healthy individuals is estimated to be

1.3 mg/ml, with a half-life of 3 to 5 days [5]. The concentration of AAT during acute phase processes rises by three- to fourfold above normal while, for example, local levels of AAT are shown to increase up to 11-fold [4]. The concentration of AAT in plasma also increases during oral contraceptive therapy and pregnancy [6]. A large number of studies has proven the importance of this rapid and high magnitude increase in AAT concentrations for the

local regulation of serine proteinase activity and tissue protection against proteolytic destruction [7,8]. AAT/proteinase complex formation and inhibitor inactivation presumably act as signals for inhibitor production and turnover in the acute phase state [9]. Human neutrophils, monocytes and alveolar macrophages can increase the expression of AAT in response to inflammatory mediators, such as IL-6 and endotoxins [1,7].

The distribution of AAT in tissue is not uniform, for example, it is reduced to approximately 10% of the plasma levels in the fluid of the lower respiratory tract [10], which explains the extremely low levels of the protein in alveolar fluid of individuals with inherited AAT deficiency. AAT also diffuses through endothelial and epithelial cell walls and is shown to be present in the epithelial lining fluid at 10 to 15% of total serum AAT concentration [11]. Moreover, by using a monoclonal antibody that specifically recognizes AAT polymers, we demonstrated that vascular, endothelium-bound AAT is in a polymeric form [12].

A change of a single amino acid in certain domains of the AAT molecule can block changes in the structure necessary for the protein folding and normal inhibitory activity, and can lead to the polymerization of mutant serpin [13]. Severe AAT deficiency of the homozygous PiZ phenotype, which differs from the normal M variant in the substitution of Glu-342 by Lys, is recognized as a hereditary condition predisposing to disease on the basis of low plasma levels (10% of normal) of the protein arising not from the lack of AAT synthesis, but from a blockage of its secretion [14]. Polymerisation of AAT is known to be involved in AAT deficiency-related diseases, such as liver cirrhosis, neonatal hepatitis and hepatocellular carcinoma, which are characterised by the formation of intracellular inclusions of polymerised AAT [15-17]. Recent studies provide evidence that AAT polymers are also present in the lungs of emphysema patients with Z-AAT deficiency and in the circulation of PiZ AAT carriers [12,18]. The normal M variant of AAT also forms polymers *in vitro*, and the process of polymer formation is shown to be dependent on temperature, pH and protein concentration [19].

Individuals with homozygous PiZ AAT deficiency have not only low baseline serum levels of AAT, but also an attenuated acute-phase response [20]. They are particularly susceptible to lung damage during bacterial exacerbations when there is a significant inflammatory cell influx and a release of various proteases. Normally, all of these proteolytic enzymes are counteracted by anti-proteinases, such as AAT, in amounts sufficient to prevent lung injury. Therefore, it is widely accepted that inherited AAT deficiency results in proteinase-antiproteinase imbalance and that this is the proximal cause of the observed pathologies. On the other hand, while low levels of native AAT

may result in uncontrolled proteolytic activity, dysfunctional forms of AAT, such as the polymerised, may have biological activities that differ from those of native AAT at physiological concentrations.

Various observations indicate that the native inhibitory form of AAT exerts its effects not only by inhibiting neutrophil elastase and other leukocyte derived proteinases. AAT was found to stimulate fibroblast proliferation and procollagen synthesis [21], up-regulate human B cell differentiation into IgE- and IgG4-secreting cells [22], interact with the proteolytic cascade of enzymes involved in apoptosis [23] and to express contrasting effects on the post-transcriptional regulation of iron between erythroid and monocytic cells [24]. In addition, AAT can inhibit neutrophil superoxide production [25], induce macrophage-derived interleukin-1 receptor antagonist release [26], and reduce TNF α -induced lethality [27].

Together these findings lead to an idea that the development of certain diseases might not only be ascribed to deficiency of AAT inhibitory activity, but also other properties of this protein, realised under inflammatory conditions. To address this possibility, we have tested whether native and polymerised forms of AAT differ *in vitro* in their effects on primary human monocytes, dependent on the protein concentration used. We find that both the native (inhibitory) and polymerised (non-inhibitory) form of AAT have similar effects as monocyte activators, with pro-inflammatory stimulation in low doses, and attenuation of pro-inflammatory activities at physiologically normal doses.

Results and discussion

AAT has been reported to influence cell function and behaviour via both direct and indirect mechanisms dependent on its concentration [28,29]. For example, native AAT has been shown to stimulate and inhibit cell proliferation, depending on cell type and the AAT concentration examined [29]. Similarly, earlier studies by Aoshiba and colleagues have shown that AAT has stimulatory and inhibitory effects on blood polymorphonuclear cells migration depending on its concentration [30]. AAT impaired chemotactic responsiveness at 2 and 10 mg/ml, but induced chemokinesis and chemotaxis with maximum potency at 0.2 mg/ml [30]. In addition, conformationally modified molecular forms of AAT, such as proteolytically cleaved, complexed with elastase or polymerised, were shown to act as potent activators and chemoattractants for neutrophils [31-33] and were therefore suggested to play a role in sustaining inflammatory reactions at the site of tissue injury.

In order to further investigate if AAT can express pro- and/or anti-inflammatory effects in a dose- and conformation-

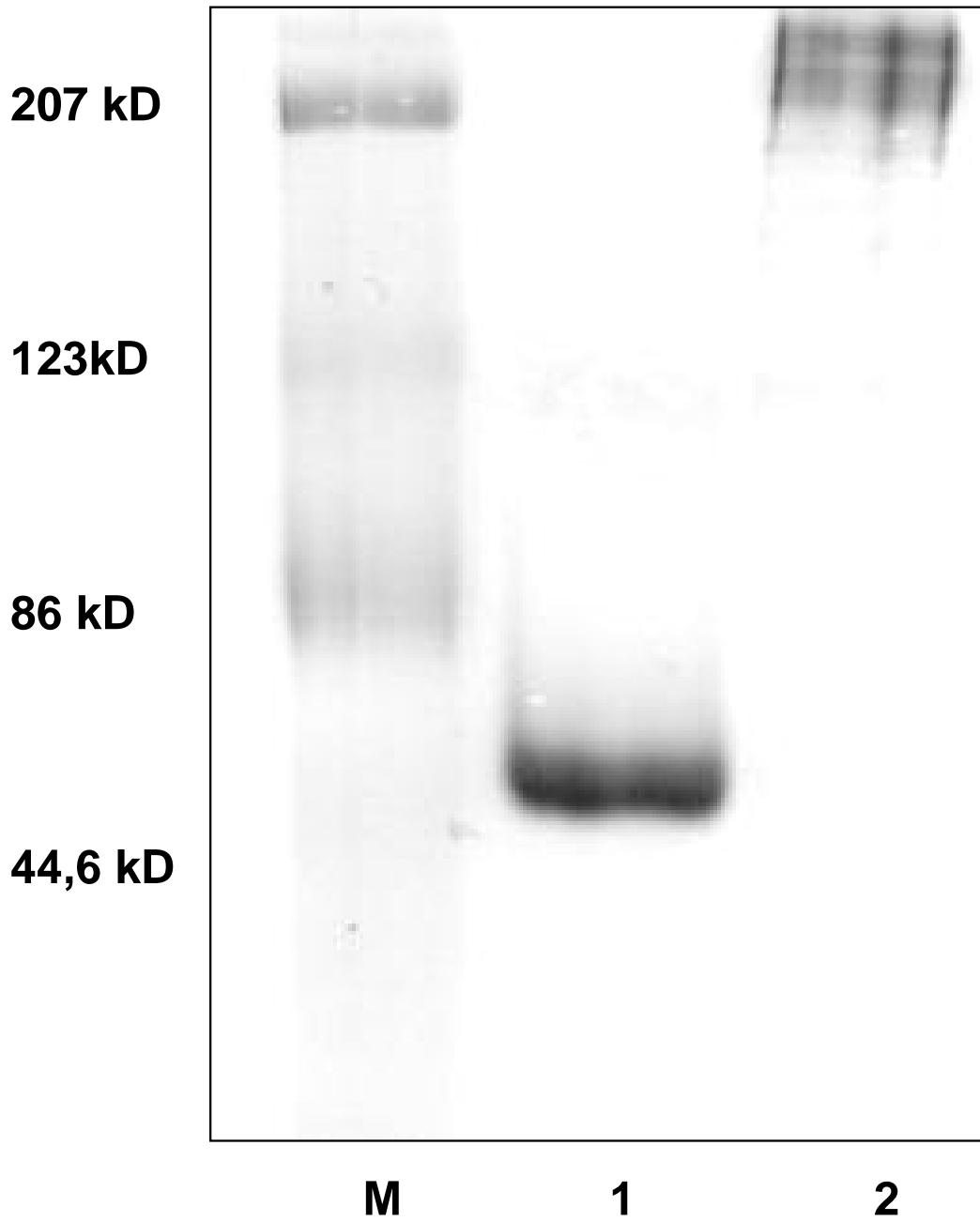


Figure 1

Native and polymerised AAT (10 µg/ml) analysed by 7.5% native PAGE. Lane M, molecular size markers, lane 1, native AAT and lane 2, polymerised AAT (incubated at 60°C, 3 h).

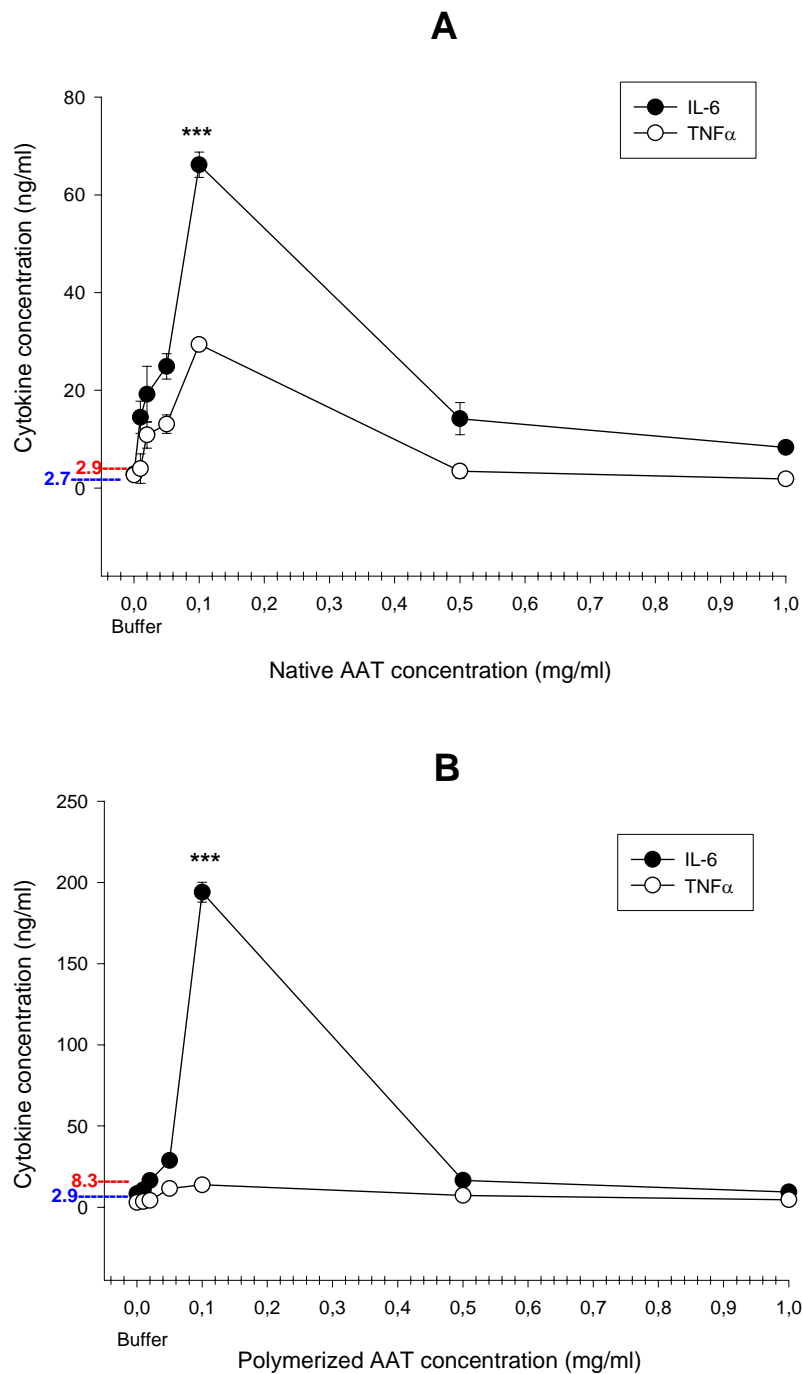


Figure 2
 Cytokines released from monocytes stimulated with various concentrations of native (A) or polymerized (B) form of AAT. Each point represents the mean \pm SD of six separate experiments. Significantly high up-regulation of the pro-inflammatory cytokines (TNF- α and IL-6) was found when cells were treated with low doses (0.05 and 0.1 mg/ml) of native or polymerized AAT, respectively, compared to control treated with equivalent amounts of buffer alone. The buffer control levels are indicated on the graphs (TNF- α in blue and IL-6 in red).

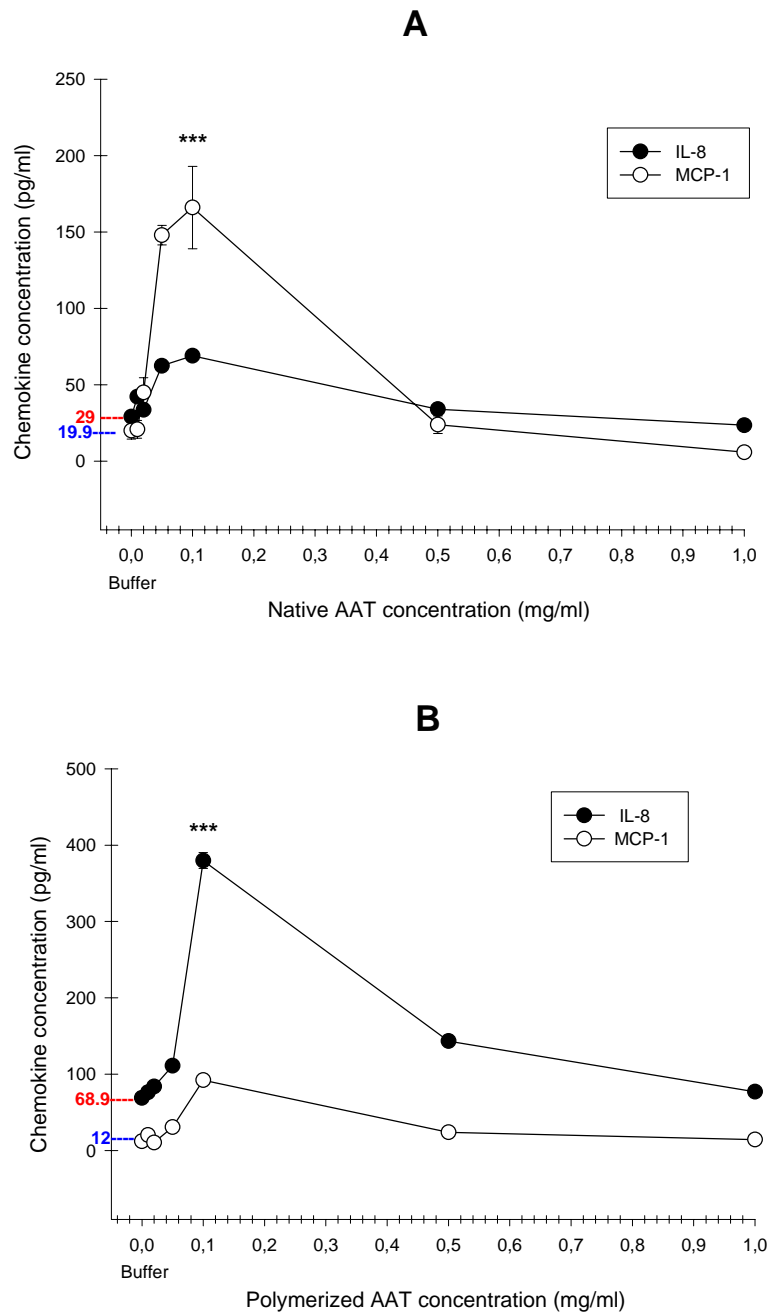


Figure 3

Chemokines released from monocytes stimulated with various concentrations of native (A) or polymerized (B) form of AAT. Each point represents the mean \pm SD of six separate experiments. Significantly high up-regulation of the pro-inflammatory chemokines (IL-8 and MCP-1) was found when cells were treated with low doses (0.05 and 0.1 mg/ml) of native or polymerized AAT, respectively, compared to controls treated with equivalent amounts of buffer alone. The buffer control levels are indicated on the graphs (MCP-1 in blue and IL-8 in red).

Table 1: A comparison between native (nAAT) and polymerised (pAAT) α 1-antitrypsin (0.1 mg/ml) effects on cytokine and chemokine release.

	IL-6 (pg/ml)		TNF α (pg/ml)	
	nAAT	pAAT	nAAT	pAAT
Mean	66.14	193.8	29.4	13.8
SD	2.6	6.04	1.2	1.8
t-value		33.7		12.5
Df		2.7		3.5
p-value		<0.001		<0.001
nAAT/pAAT				2.1
pAAT/nAAT		2.9		

	IL-8 (pg/ml)		MCP-1 (pg/ml)	
	nAAT	pAAT	nAAT	pAAT
Mean	68.9	379.8	166.0	92.2
SD	0.40	10.4	27.0	3.6
t-value		51.7		4.7
Df		2.0		2.1
p-value		<0.001		<0.01
nAAT/pAAT				1.8
pAAT/nAAT		5.5		

Mean-values of six separate experiments SD-standard deviation, Df-degree of freedom, t-value, calculated using Walsh t test [52].

dependent fashion we designed a model in which primary human monocytes were exposed to various concentrations of the native or the polymerised form of AAT for 18 h. Samples of native and polymerised AAT were subjected to native 7.5% PAGE analysis. Fig. 1, lane 1, shows a single molecular weight band corresponding to native AAT. Heating of native AAT for 3 h at 60°C resulted in the generation of different size polymers of AAT (Fig. 1, lane 2). As illustrated in Figures 2 and 3, monocytes exposed to native or polymerised preparation of the AAT at lower concentration (0.1 mg/ml) increase release of TNF α (10.9- and 4.8-fold, $p < 0.001$), IL-6 (22.8- and 23.4-fold, $p < 0.001$), IL-8 (2.4- and 5.5-fold, $p < 0.001$) and MCP-1 (8.3- and 7.7-fold, $p < 0.001$), respectively, compared to buffer exposed cells or cells treated with higher doses of either conformation of AAT (0.5 and 1 mg/ml). Thus, our data provide evidence that native and polymerised AAT at lower concentrations, i.e. 0.1 mg/ml and below, are sufficient to elicit an increase in pro-inflammatory cytokine (IL-6 and TNF- α) and chemokine (IL-8 and MCP-1) release from monocytes *in vitro*, whereas the cells treated with higher doses of either AAT manifest no activation at all. Although low-dose AAT induced cytokine and chemokine release is less pronounced relative to LPS (10 ng/ml) by approximately 10- to 100-fold (data not shown), it is still important to point out that the observed AAT effects are reproducible and statistically significant. In addition, statistical comparison between relative effects of native versus polymerised AAT (0.1 mg/ml) showed that native

AAT is significantly more potent stimulator of TNF α and MCP-1 release, compared to polymerised AAT whereas polymerised AAT expresses stronger effects on IL-6 and IL-8 release (Table 1). These findings may have biological importance and need to be further investigated.

It is known that nuclear transcription factor- κ B (NF- κ B) regulates host inflammatory and immune responses and cellular growth properties by increasing the expression of specific cellular genes [34]. Several studies have demonstrated that in monocytes, NF- κ B activation results in the transcription of immediate early genes that encode IL-6, TNF α , MCP-1, as well as several other molecules [35]. Numerous stimuli are known to activate NF- κ B, including the bacterial component LPS and other pro-inflammatory factors such as cytokines [36,37].

We have also studied the effects of native and polymerised AAT on the expression of transcription factor NF- κ B. Monocytes exposed to native or polymerised form of AAT at lower concentrations, such as 0.05 and 0.1 mg/ml activate NF- κ B expression, compared to control, buffer-stimulated cells (Figure 4), while the serum physiological level of either AAT (1 mg/ml) down-regulates NF- κ B expression to almost undetectable level. These findings correlate with the stimulatory effects of low dose of AAT on pro-inflammatory cytokine and chemokine release in monocytes described above (Figures 2 and 3) confirming the interrelationship between transcription factor, NF- κ B, activation

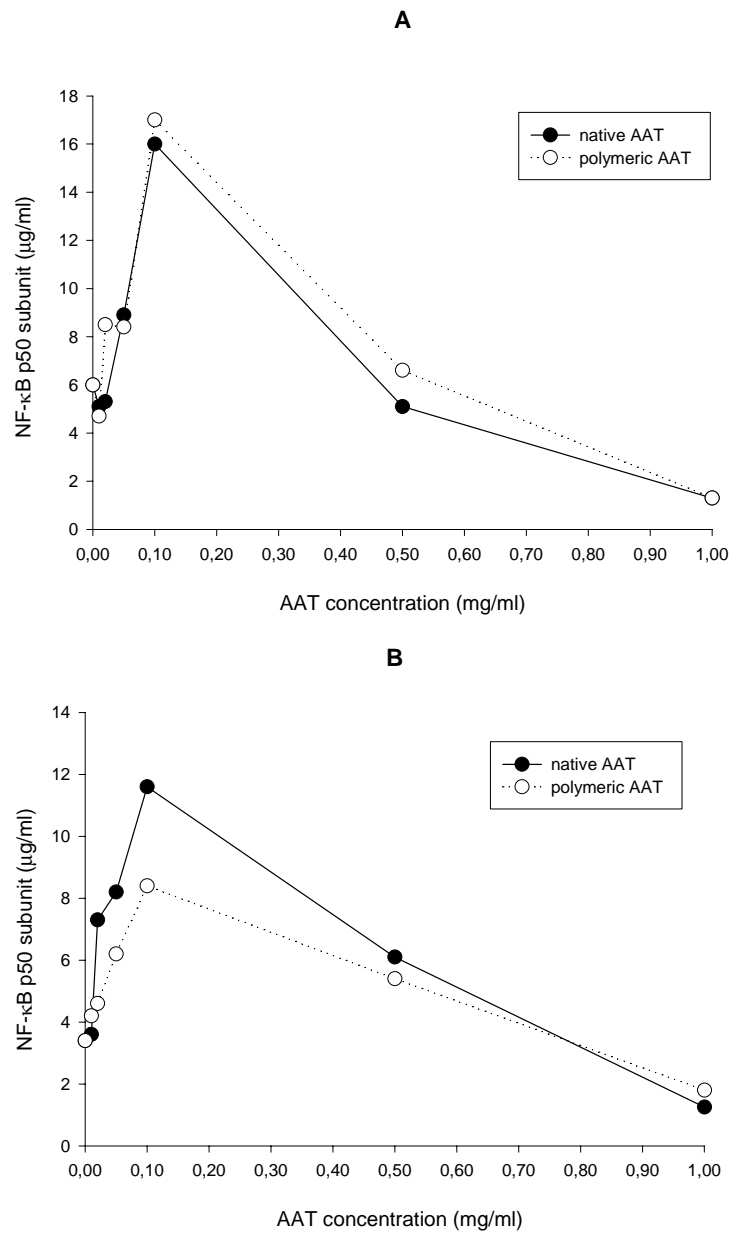


Figure 4

A and B. Monitoring of NFκB p50 activation in monocytes after 18 h of exposure to different concentrations of native or polymerized AAT by ELISA-based TransAM kit^{AM}. Cell nuclear extracts were prepared from monocytes alone or treated with different concentrations of AAT. NFκB activity assay was performed in 96-well plates with 10 μg of cell extract per well. Absorbance was measured by spectrophotometry at 450 nm. Specificity controls were performed by adding a molar excess (20 pmol/well) of mutant NFκB oligonucleotide (the positive signal remained unaffected) and wild-type NFκB oligonucleotide (a signal was abolished). A and B represent results from two independent experiments. Each point represents the mean of two experiments.

and pro-inflammatory cytokine release in response to native and polymerised AAT.

Surprisingly, the effects of both native and polymerised AAT on monocyte activity were parallel for all parameters measured and at all doses, although relative effects on specific cytokine or chemokine secretion were found to be different (Table 1). Equally important was the observation that the cellular responses showed a marked inverse dependence on dose, and were not dependent on the conformational form of AAT. Low AAT concentrations *in vivo* appear to be related to a state of general AAT deficiency, but may also reflect local depletion at different stages of inflammation. This latter may be the evolutionary basis for the inverse dependence on AAT concentration of the expression of pro-inflammatory molecules. The production of cytokines, chemokines, cell surface receptor/adhesion proteins, and other molecules by activated monocytes, macrophages and neutrophils can serve to orchestrate the immune response and is essential to host defence. The increased expression of a defined set of cytokines, such as IL-6 and TNF α , is believed to be one of the major pathological mediators of inflammatory diseases. Chemotactic cytokines (chemokines) are also generated locally, and this stimulates monocytes to migrate into the site of inflammation. In particular, IL-8 and MCP-1 have been implicated in diseases characterised by monocyte-rich infiltrates because of its ability to induce monocyte trafficking and activation [38]. IL-6, TNF α , IL-8 and MCP-1 are all hallmarks of inflammation and sustain the multiple inflammation-associated processes of lymphocyte attraction, differentiation, immunoglobulin secretion, primary monocyte recruitment, neutrophil attraction and up-regulation of extracellular matrix-degrading properties [39]. They are all most likely regulated by genes controlled from the NF- κ B promoter, since their expression correlates strongly with NF- κ B activation in our monocyte stimulation model. Based on our results, it is very likely that at low concentrations native as well as polymerised AAT may contribute to inflammatory events *in vivo*.

The inverse dependence on AAT concentration observed for the expression of pro-inflammatory reactance suggests a possible receptor-dependent feedback loop in which AAT is the key ligand. The cellular receptor for AAT has not been identified to date, although the cell surface receptors, termed serpin-enzyme complex (SEC) receptor [9], low density lipoprotein related (LRP) receptor [40], low density lipoprotein (LDL) receptor [41] and very low density lipoprotein (VLDL) receptor [42] which bind AAT- and other serpin-enzyme complexes and are involved in its internalisation and degradation, have been described. It is also known that AAT biosynthesis is responsive to levels of pro-inflammatory cytokines and to levels of inactivated

forms of AAT through their uptake at the SEC receptor [7,9,43]. A parallel feedback loop may exist for inhibitory-active AAT, and as we show here for the polymerised form of AAT, in which rising levels of these molecular forms are signals for a plateau in inflammation. By saturating a receptor(s), these forms of AAT at increasing concentrations may signal down-regulation of the expression of pro-inflammatory reactants, i.e. a pro-inflammatory role of AAT at low concentrations can be linked to the phenomenon of partial agonism [44].

The observations reported here provide further support for a central role of AAT in inflammation, both as a regulator of protease activity and as a signalling molecule. The fact that polymerised AAT has parallel effects to native AAT suggests a putative common receptor acting as a sensor for rising levels of un-reacted, non-degraded molecular forms of AAT. This idea is partially supported by our earlier studies showing that low doses of oxidised (non-inhibitory) form of AAT induce pro-inflammatory monocyte activation *in vitro* [45].

Conclusions

Here we show that AAT may play multiple roles at sites of inflammation, which may be determined not only by the conformational modifications of AAT, but also directly by the reduction in its concentration. It is well established that native AAT is the most abundant circulating proteinase inhibitor, which is responsible for the regulation of proteolytic enzymes and the maintenance of homeostasis in humans. A substantial number of pathological problems are suggested to result from any mechanism causing the reduction in the levels of the inhibitory active form of AAT, including inactivation by non-target proteinases, oxidative inactivation, polymerisation, or genetic aberrations in structure [46-48]. Our data show that at low concentrations (at approximately 10% of normal) AAT exerts direct pro-inflammatory effects in human monocytes, leading to the hypothesis that inherited and/or acquired AAT deficiency in itself may be a consequence of the development of chronic inflammatory processes. It also explains the greater susceptibility of individuals with severe Z-AAT deficiency to develop chronic inflammatory processes. Therefore, a marked increase in AAT concentration (from 4 to 5-fold and locally up to 11-fold) during acute inflammatory reactions appears to be an anti-inflammatory condition, and the further investigation of it may provide a new target for the development of therapeutic agents to regulate inflammation.

Methods

Specific reagents

Human plasma AAT (purity > 95%, and inhibitory activity > 75%) was obtained from Calbiochem, USA and from Clinical chemistry laboratory, University Hospital

Malmö, Sweden. Native AAT was diluted in PBS, pH 7.4, and, to ensure the complete removal of endotoxins, was decontaminated using endotoxin removing gel (Detoxi-Gel columns, Pierce, USA). AAT polymers were prepared as described previously [49] by incubating native AAT (final concentration 1 mg/ml) at 60°C for 3 h with gentle shaking. Polymer formation was confirmed by non-denaturing 7.5% PAGE and a concomitant loss of inhibitory activity against pancreatic elastase. The AAT-polymer mixture was reheated as necessary to ensure that the polymers were not contaminated with monomeric AAT. Protein preparations used in all assays were assayed for endotoxins using the LAL, Lumulus Amebocyte Lysate, Endochrome™ test (Sigma Chemical company, USA). In all samples of native and polymerized AAT used in the subsequent experiments endotoxin levels were less than 0.3 EU/mg of protein.

Isolation and culture of monocytes

Human monocytes were isolated from buffy coats from different donors by the Ficoll-Hypaque procedure as previously detailed [50]. Cell purity was >97% as determined on an AC900EO AutoCounter (Swelab Instruments, AB); cell viability was analysed by staining with 0.4 % trypan blue. Monocytes were plated at a density of 4×10^6 cells/ml into plastic dishes. After removal of non-adhering cells, the remaining adherent monocytes were cultured in RPMI 1640 (Gibco, Life Technologies, Paisley, Scotland) supplemented with 2 mM N-acetyl-L-alanyl-L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acid, 2% sodium pyruvate and 20 mM Hepes (Fluka, Chemie AG) without serum at 37°C in an atmosphere of 5% CO₂. Monocytes were treated with native or polymerised AAT at concentrations of 0.01, 0.02, 0.05, 0.1, 0.5 and 1 mg/ml for 18 h. Control wells contained the same volumes of PBS or were incubated in medium alone. At the end of the incubations cell-free supernatants were collected and stored at -20°C until analysis. It must be pointed out that no difference was observed between control cells alone and treated with PBS therefore all presented data are calculated from the buffer controls. Each experiment was done at a minimum in triplicate for each condition.

Cytokine and chemokine assays

Cell culture supernatants from monocytes treated with various concentrations of native and polymerised AAT were analysed to determine TNF- α , IL-6, IL-8 and MCP-1 by using sandwich DuoSet ELISA kits according to the manufacturer's instructions (R&D Systems, England). The detection limits for the assays were as follows: IL-8, 15.6 pg/ml, MCP-1, 7.8 pg/ml, TNF- α , 7.8 pg/ml, IL-6, 2.3 pg/ml.

Nuclear extract preparation and NF- κ B activation monitoring assay

Nuclear extracts from monocytes were prepared exclusively as described previously [51]. Protein concentrations were determined spectrophotometrically at 260 nm and using the Comassie Plus protein assay reagent according to instructions from the manufacturer (Pierce, IL, USA).

NF- κ B in nuclear extracts were analysed using TransAm kits (Activemotif, CA, USA) specific for p50 subunit according to manufactures instructions. Detection limit <0.5 µg cell extract per well.

Statistical analysis

The differences in the means in experimental results were analysed for their statistical significance by one-way analysis of variance (ANOVA) combined with a multiple comparisons procedure (Scheffé multiple range test) with the overall significance level of $\alpha = 0.05$. The Statistical Package for Social Sciences (SPSS for Windows, release 11.0) was used for the calculations.

Abbreviations

AAT, alpha1-antitrypsin, PiZZ, homozygous type Z deficiency variant; PiMM, normal AAT gene variant; MCP-1, monocyte chemoattractant protein-1; TNF α , tumour necrosis factor- α ; IL, interleukin, PBS, phosphate buffered saline; NF- κ B, nuclear factor- κ B, Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; LPS, lipopolysaccharide; SEC, serpin-enzyme complex; LRP, low density lipoprotein related protein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

Authors' contributions

RA carried out the experimental work and wrote the preliminary draft of the manuscript. LJ participated in the design of the study. SJ designed and coordinated the study, performed statistical analysis of the results, made major revision of the manuscript.

All authors read and approved the final manuscript.

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