

Effect of Recombinant α_1 -Antitrypsin Fc-Fused (AAT-Fc) Protein on the Inhibition of Inflammatory Cytokine Production and Streptozotocin-Induced Diabetes

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α_1 -Antitrypsin (AAT) is a member of the serine proteinase inhibitor family that impedes the enzymatic activity of serine proteinases, including human neutrophil elastase, cathepsin G and neutrophil proteinase 3. Here, we expressed recombinant AAT by fusing the intact AAT gene to the constant region of IgG1 to generate soluble recombinant AAT-Fc protein. The recombinant AAT-Fc protein was produced in Chinese hamster ovary (CHO) cells and purified using mini-protein A affinity chromatography. Recombinant AAT-Fc protein was tested for antiinflammatory function and AAT-Fc sufficiently suppressed tumor necrosis factor (TNF)- α -induced interleukin (IL)-6 in human peripheral blood mononuclear cells (PBMCs) and inhibited cytokine-induced TNF α by different cytokines in mouse macrophage Raw 264.7 cells. However, AAT-Fc failed to suppress lipopolysaccharide-induced cytokine production in both PBMCs and macrophages. In addition, our data showed that AAT-Fc blocks the development of hyperglycemia in a streptozotocin-induced mouse model of diabetes. Interestingly, we also found that plasma-derived AAT specifically inhibited the enzymatic activity of elastase but that AAT-Fc had no inhibitory effect on elastase activity.

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INTRODUCTION

α_1 -Antitrypsin (AAT) is a serine protease inhibitor and treatment with AAT prolongs islet allograft survival in mice (1). Moreover, AAT gene therapy modulates cellular immunity and efficiently prevents the development of type 1 diabetes in nonobese diabetic mice (2–4). AAT significantly reduces cytokine- and streptozotocin (STZ)-induced β -cell apoptosis by abolishing caspase-3 activity (5). AAT treatment also induces immune tolerance (6) and dampens inflammation,

resulting in the expansion of the functional mass of β -cells in nonobese diabetic (NOD) mice (7), whereas this effect is not observed in a nonautoimmune mouse strain (8).

The association of AAT with diabetes was initially investigated by analyzing the meconium of infants delivered via cesarean section to diabetic mothers (9). The highest AAT concentration was observed in the third trimester of the diabetic pregnancy. However, the increased concentration of AAT in the sera of preg-

nant diabetic women did not correlate with the glycemic control value (9). A study reported that the urine sample of diabetic patients had markedly increased AAT concentrations (10). The presence of increased AAT levels in patients with diabetes mellitus was suggested by the reduction of inhibitory capacity on serum proteinases (11–14); however, the precise molecular mechanism underlying the loss of AAT-mediated inhibitory functions is not clear.

The hereditary disorder of AAT deficiency is caused by mutations in the α_1 -antitrypsin (*SERPINA1*) gene (15,16), and the most common form of AAT deficiency occurs because of the Z mutation (17,18), resulting in aberrant folding and accumulation of the protein in the endoplasmic reticulum (ER). This step leads to ER stress and contributes significantly to liver disease. AAT is also synthesized by monocytes, neutrophils and epithelial cells (19,20). The unfolded AAT protein is activated in quiescent monocytes and

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contributes to an inflammatory phenotype, with Z mutation (ZZ) monocytes exhibiting enhanced cytokine production and activation of the nuclear factor (NF)- κ B pathway when compared with normal M variant (MM) monocytes (19). These findings changed the previous paradigm of lung inflammation in AAT deficiency due to defects in AAT-mediated inhibition of neutrophil proteinase.

AAT-mediated suppression of formyl-met-leu-phe (fMLP)-stimulated and non-stimulated neutrophil adhesion to fibronectin, as well as the inhibition of lipopolysaccharide (LPS)-induced interleukin (IL)-8 release and delayed neutrophil apoptosis, is independent of the inhibition of neutrophil proteinase activity (21). The effect of AAT on TNF α -induced self-expression is inhibited by the oxidation and modification of AAT, which abolishes the serine protease-inhibitor activity of AAT (22). These data further support the idea that the antiinflammatory function of AAT is independent of its inhibitory effect on elastase. In general, the patterns of gene expression regulated by native and oxidized AAT are similar, with neither of them stimulating proinflammatory genes or cytokine expression (23).

In the present study, we generated a recombinant form of AAT as a chimeric protein fused to Fc and successfully produced much recombinant AAT-Fc protein from stable clones of Chinese hamster ovary (CHO) cells. We evaluated the antiinflammatory properties of the purified AAT-Fc protein *in vitro* and *in vivo*. Our results demonstrate that the biological properties of AAT-Fc are very similar to those of plasma-derived AAT and that the antiinflammatory activity of AAT-Fc is more potent than that of plasma-derived AAT.

MATERIALS AND METHODS

Recombinant AAT-Fc Protein Expression

Human pCAGGs-IgG1/AAT plasmids were cotransfected with the pSV-dihydrofolate reductase (DHFR) vector (ATCC, Manassas, VA, USA) into the

DHFR-deficient CHO cell line (DG44). Stable clones were selected in medium containing G418 (500 μ /mL) and subsequently subjected to methotrexate selection for gene amplification as previously described (24). The CHO stable clones that secrete soluble recombinant AAT-Fc were grown in serum-free medium (CHO-SSFMII; Life Technologies, Carlsbad, CA, USA), as previously described (24).

Purification of AAT-Fc

The single cell clone selected was cultured in a 2-liter flask. The culture medium was first filtered through membranes with a pore size of 0.2 μ m to remove debris. The flow-through result was then purified with protein A-conjugated beads that had been equilibrated with phosphate-buffered saline (PBS) containing 0.5 mol/L NaCl. The filtered cell culture medium was passed through a column containing the protein A resin, and 1-mL fractions of purified protein were collected. Glycine (pH 2.7) was used as the elution buffer, and the fractions were neutralized immediately with 100 μ L of 1 mol/L Tris base.

Western Blotting

For the detection of recombinant AAT-Fc protein, aliquots of the purified protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels. The resolved proteins were transferred to a nitrocellulose membrane and Western blotting analysis was performed using rabbit anti-human AAT (YbdY, Seoul Korea) and horse radish peroxidase-conjugated goat anti-human IgG Fc antibodies (Sigma-Aldrich, St. Louis, MO, USA). The protein-antibody complexes were detected by using Supex (Neuronex, Seoul, Korea) and an LAS-4000 imaging device (Fujifilm, Japan).

Inhibitory Effect of AAT-Fc on the Enzymatic Activity of Elastase

Elastase (20 ng; Athens Research and Technology, Athens, GA, USA) and IL-32 γ (20 ng; YbdY) were incubated in 20 μ L PBS (pH 7.4) at room temperature for 10 min in the presence or absence of

AAT-Fc. After completion of the reaction, the samples were subjected to SDS-PAGE and Western blotting analysis. A human IL-32-specific polyclonal antibody (YbdY) was used for detecting cleaved IL-32 γ . A similar experiment was performed, and the reactions were used to assess the effect of AAT-Fc on the elastase-mediated cleavage of IL-32 γ by using an IL-32 γ -specific enzyme-linked immunosorbent assay (ELISA), instead of Western blotting analysis.

Cytokine Assays

To examine the effect of AAT-Fc on TNF α - or LPS-induced cytokine production, peripheral blood mononuclear cells (PBMCs) were preincubated with plasma-derived AAT or AAT-Fc for 1 h and then stimulated with fresh medium (0.2 mL) containing human TNF α (YbdY) or LPS (Sigma-Aldrich). Raw 264.7 cells were seeded in 96-well plates at a density of 1×10^5 cells per well 1 d before stimulation. The plated cells were preincubated for 1 h with plasma-derived AAT or AAT-Fc and then stimulated with LPS, human IL-32, human IL-33 or mouse IL-33 overnight. The cell culture supernatant of human PBMCs and mouse Raw 264.7 cells was harvested on the next day for the cytokine assay. Human IL-6 and mouse TNF α levels were measured in the supernatant by using sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For measuring tissue cytokine levels, liver and pancreas tissues of STZ-treated mice (see below) were homogenized in cell lysis buffer containing 1% Triton X-100, and the IL-6 levels were measured as described above.

STZ-Induced Diabetic Mice

Female Balb/c mice 6 wks of age from Orient Biology (Seoungnam Kyeonggi-do, South Korea) were housed under a 12-h light-dark cycle at 23°C and given restricted access to food and water. Animal experiments were approved by the Institutional Animal Care and Use Committee at Konkuk University. For induction of diabetes, the mice (n = 5 in each group)

were fasted for 3 h before intraperitoneal injection with a STZ solution (Sigma-Aldrich; 120 mg/kg body weight) in 0.05 mol/L sodium citrate buffer (pH 4.5); these solutions were prepared immediately before use. After injection, the mice were fasted for another 3 h and were then provided ad libitum access to a diet. To induce sufficient levels of hyperglycemia, the mice were treated with the same dose of STZ after 3 d. The mice were then tested daily for blood glucose levels after the initial injection. Blood glucose levels were determined by testing a drop of blood from an incision in the tip of the tail with a handheld blood glucose meter (Sollartek Products, Alameda, CA, USA). For nonfasting blood glucose readings, a blood glucose level >250 mg/dL was set as the lower limit, indicative of the presence of diabetes (%).

Statistical Analysis

The data were expressed as mean \pm standard error of the mean (SEM). The statistical significance of differences was analyzed using the unpaired two-tailed Student *t* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Expression of Recombinant Fc-Human AAT

Currently, several forms of AAT that have been purified from human plasma are available. However, we sought to produce a standardized recombinant form of AAT from CHO cells. The intact human *AAT* gene, encoding a protein of 418 amino acids, under the control of the chicken β -actin promoter, was transfected into CHO cells and stable transfectants were isolated. Although we were able to confirm the transfection of the human *AAT* gene by RT-PCR, compared with a mock transfection of CHO cells, recombinant AAT protein was not detectable in the cell culture supernatant (data not shown). We further examined the cell lysate of *AAT*-transfected CHO cells and found that most of the recombinant AAT protein remained inside the cells, despite

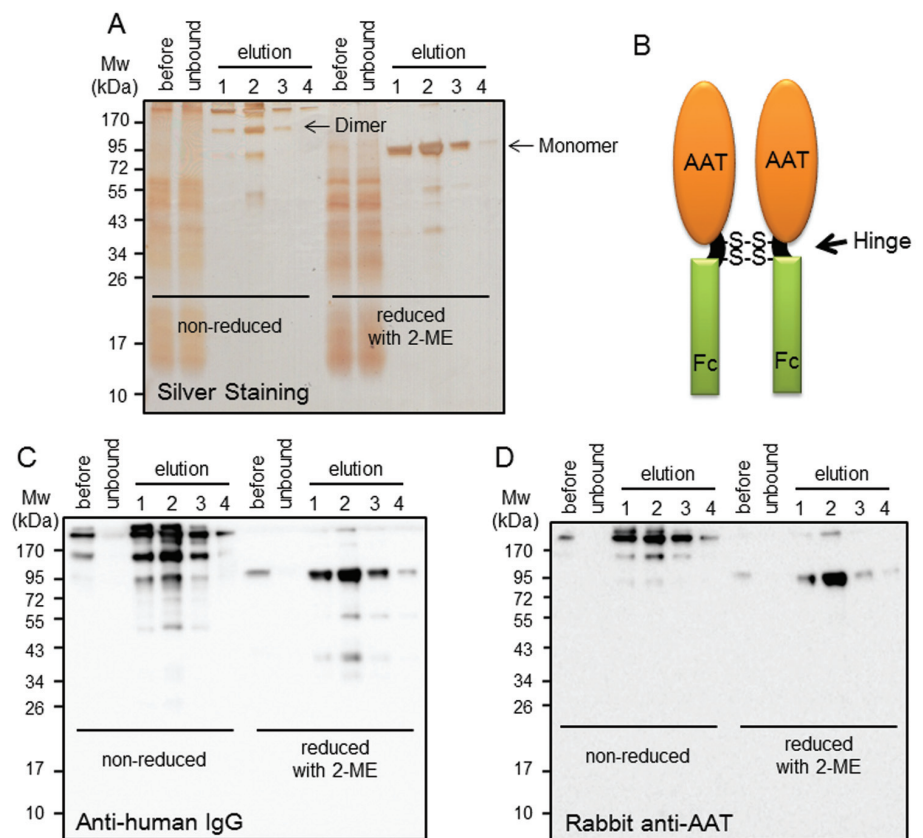


Figure 1. Purification of recombinant human AAT-Fc. AAT-Fc was purified by using protein A affinity chromatography. The eluted fractions were analyzed by silver staining and Western blotting under the reduced (with 2-mercaptoethanol (2-ME)) and nonreduced conditions. (A) The silver staining results indicated that the molecular size of the nonreduced AAT-Fc protein was approximately 160 kDa (dimer; major band), whereas that of the reduced AAT-Fc protein was 85 kDa (monomer). (B) Schematic representation of AAT-Fc in the dimeric form, connected by two disulfide bonds in the hinge region. Western blotting with anti-human IgG1 (C) and rabbit anti-human AAT antibodies (D) indicated the presence of AAT-Fc protein in the eluates. Representative data from one of five independent experiments are shown.

the presence of a typical hydrophobic signal peptide at the N-terminus of AAT.

To circumvent the retention of recombinant AAT protein in CHO cells, we fused the human *AAT* gene to a DNA fragment encoding the Fc domain of human IgG1 and transfected the Fc-fused human *AAT* gene into CHO cells for further analysis. Interestingly, a large amount of recombinant AAT-Fc was released into the cell culture supernatant, which was confirmed by silver staining and Western blotting analysis (Figure 1). We purified recombinant AAT-Fc by using protein A affinity chromatography, and the purified

AAT-Fc was analyzed by SDS-PAGE and silver staining analysis (Figure 1A). In addition, we performed Western blotting analysis of the purified AAT-Fc protein by using two different antibodies: anti-human IgG (Figure 1C) and anti-human AAT (Figure 1D). A schematic illustration of the recombinant AAT-Fc visualized by silver staining is depicted in Figure 1B. Under the nonreduced conditions, recombinant AAT-Fc appeared as several large bands above 170 kDa and as bands at approximately 160, 85 and 55 kDa; whereas under the reduced conditions, a major band was seen at 85 kDa. An anti-human

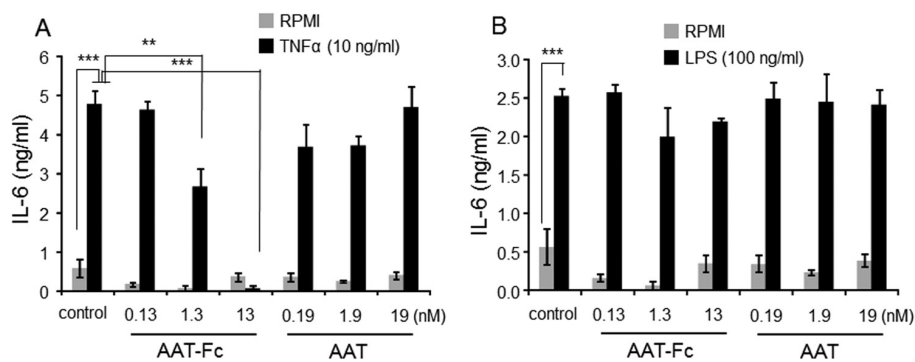


Figure 2. Dose-dependent suppression of TNF α -induced IL-6 production in human PBMCs. (A) AAT-Fc inhibited TNF α -induced IL-6 production in a dose-dependent manner, and AAT-Fc used at a concentration of 13 nmol/L abolished IL-6 production completely. However, the same concentration of plasma-derived AAT has no effect on IL-6 induction. (B) Unlike the effects of AAT on TNF α -induced IL-6 production, both AAT-Fc and AAT failed to suppress LPS-induced IL-6 production. The mean \pm SEM values of three independent experiments have been shown. ** $p < 0.01$; *** $p < 0.001$ ($n = 3$ per group).

IgG antibody (Figure 1C) recognized most of the bands that were detected on performing silver staining. We also used a rabbit anti-human AAT polyclonal antibody to confirm the presence and purity of the recombinant AAT-Fc protein, and the results were similar to those obtained with anti-IgG1; however, the rabbit anti-human AAT polyclonal antibody recognized only the 170-kDa band under the nonreduced conditions or the 85-kDa band under the reduced conditions (Figure 1D).

AAT-Fc Suppresses TNF α -Induced IL-6 Production in Human PBMCs

Besides the classic biological activity of AAT (that is, inhibition of serine proteinase enzyme activity), plasma-derived human AAT possesses antiinflammatory activity. Therefore, we examined whether AAT-Fc possesses a similar biological property. Human PBMCs were pretreated with AAT-Fc or plasma-derived AAT for 1 h and then stimulated with TNF α overnight. The next day, the cell culture medium was harvested and IL-6 concentrations were assessed by using ELISA. Stimulation with TNF α (10 ng/mL) increased IL-6 production significantly (Figure 2A). The induction of IL-6 was suppressed by pretreatment with AAT-Fc in a dose-dependent manner, and a high con-

centration of AAT-Fc (13 nmol/L) completely abolished IL-6 production. However, the same concentration of plasma-derived AAT failed to suppress IL-6 production (Figure 2A). We further investigated LPS-induced IL-6 production, and the results showed that both AAT-Fc and plasma-derived AAT failed to inhibit LPS-induced IL-6 production in human PBMCs (Figure 2B).

AAT-Fc Inhibits TNF α Production Induced by Various Cytokines

Further investigation of the antiinflammatory effect of AAT-Fc was performed with mouse Raw 264.7 cells stimulated with various cytokines, including LPS. AAT-Fc reduced human IL-32 γ -, human IL-33- and mouse IL-33-induced TNF α production in mouse Raw 264.7 cells, but the same concentration of plasma-derived AAT failed to suppress TNF α production (Figure 3). However, AAT-Fc failed to suppress LPS-induced TNF α production, consistent with the effect on IL-6 production in human PBMCs (Figure 2B).

Human AAT-Fc Decreased Blood Glucose Levels in the STZ-Induced Diabetic Mouse Model

Previous studies evaluated the antiinflammatory function of plasma-derived AAT and found that AAT prolonged islet

graft survival and decreased hyperglycemia in the STZ-induced diabetic mouse model. We evaluated the effects of AAT-Fc on hyperglycemia in STZ-induced diabetic mice. AAT-Fc (2 mg/kg body weight) was administered through intraperitoneal injection on d 0. A low dose of STZ was injected twice (on d 1 and 3) to induce diabetes, and blood glucose levels were monitored (Figure 4). At d 6 (3 d after the second injection), dramatic enhancement of the blood glucose level was observed (Figure 4). On d 13, AAT-Fc-injected mice had lower glucose levels than the nontreated control mice, but the difference was not statistically significant. On d 15 and 17, the blood glucose level of AAT-Fc-treated mice was significantly lower than that of nontreated control mice (Figure 4). To investigate the mechanism underlying AAT-Fc-mediated effects on inflammation in the mouse model of STZ-induced diabetes, we examined the levels of inflammatory cytokines in the pancreas and liver; the IL-6 level was found to be lower in AAT-Fc-treated mice than in control mice not treated with AAT-Fc (Figure 4B).

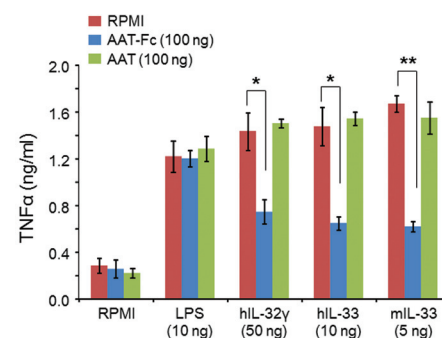


Figure 3. Cytokine-induced TNF α production in mouse Raw 264.7 cells is suppressed by AAT-Fc. Human IL-32 γ -, human IL-33- and mouse IL-33-induced TNF α production was suppressed by AAT-Fc, but AAT-Fc had no effect on LPS-induced mouse TNF α . The plasma-derived AAT had no effect on the suppression of TNF α . The mean \pm SEM values of two independent experiments have been shown. * $p < 0.05$; ** $p < 0.01$ ($n = 3$ per group).

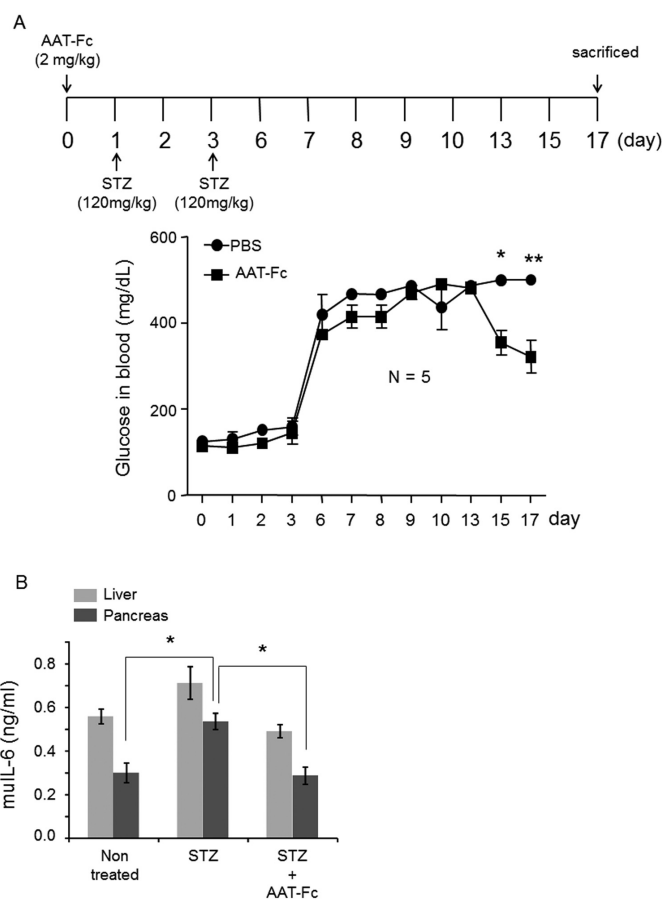


Figure 4. AAT-Fc suppresses hyperglycemia in the STZ-induced mouse model of diabetes. (A) Six-week-old BALB/c mice were administered AAT-Fc (2 mg/kg body weight), and then a low dose of STZ (120 mg/kg body weight) was injected twice, on d 1 and 3. The blood glucose level was monitored daily. After the second injection on d 3, the blood glucose level increased significantly. At d 15 and 17, mice that received AAT-Fc had decreased blood glucose levels. (B) At d 10 after the STZ injection, the mice were sacrificed, and the IL-6 level was measured in the liver and pancreas. The mean \pm SEM values have been shown ($n = 5$ per group). * $p < 0.05$; ** $p < 0.01$.

Biochemical Analysis of Elastase Activity in the Presence of AAT-Fc

AAT is a serine proteinase inhibitor that blocks the enzymatic activity of neutrophil elastase. We examined whether AAT-Fc inhibits this elastase. Plasma-derived AAT and AAT-Fc (two different concentrations) were preincubated with elastase for 20 min at room temperature and then added to tubes containing recombinant IL-32 γ , an elastase substrate. After 15 min of incubation at room temperature, the samples were subjected to Western blot analysis. Elastase fragmented the 40-kDa IL-32 γ ,

resulting in the formation of a cleaved IL-32 γ at 20 kDa (Figure 5); AAT treatment at a 1:5 molar ratio completely abolished the enzymatic activity of elastase (Figure 5), compared with that of elastase untreated with AAT (Figure 5). The same experiment performed with AAT-Fc at a higher molar ratio (1:25) failed to block the cleavage of IL-32 γ by elastase (Figure 5).

We further evaluated the effect of AAT on elastase activity by using an IL-32 γ -specific ELISA for measuring the stability of IL-32 γ . In this experiment, IL-32 γ (100 ng/mL) was mixed with only

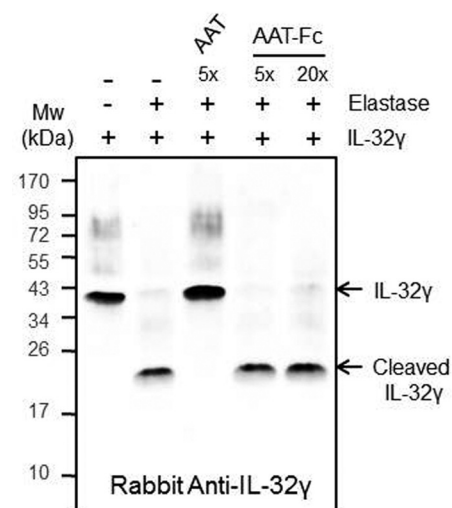


Figure 5. Western blot for examining the inhibitory effect of AAT-Fc against elastase. IL-32 γ (20 ng) was mixed with elastase (20 ng) in the presence or absence of plasma-derived AAT or AAT-Fc. The plasma-derived AAT blocked IL-32 γ cleavage in a 1:5 molar ratio, but AAT-Fc failed to inhibit IL-32 γ cleavage. The molar ratio of elastase and AAT in each reaction is indicated on the top. The elastase-cleaved IL-32 γ product appeared as a band of approximately 20 kDa. Representative data from one of four independent experiments are shown.

elastase or with elastase that had been preincubated with either plasma-derived AAT or AAT-Fc for 15 min at room temperature, followed by measurement of IL-32 γ concentrations by ELISA. Plasma-derived AAT protected IL-32 γ from elastase-mediated cleavage (black bar, Figure 6A), whereas only 15% of the IL-32 γ remained in the absence of AAT (open bar, Figure 6A). We examined the effect of AAT-Fc with the same assay and found that, unlike plasma-derived AAT, AAT-Fc failed to protect IL-32 γ from elastase-mediated cleavage (Figure 6B).

DISCUSSION

To our knowledge, this study is the first to report the production of much recombinant AAT-Fc and the characterization of its biological activity *in vitro* and *in vivo*. We designed a mammalian ex-

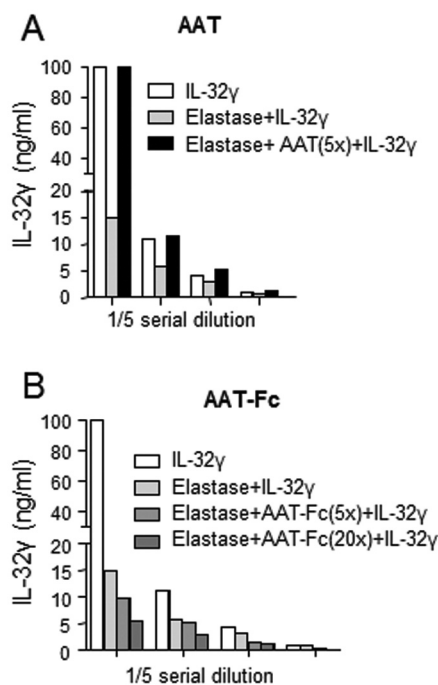


Figure 6. Quantitative analysis of the proteinase inhibitory activity of AAT on elastase, with IL-32 γ as the substrate. The IL-32 γ concentrations of reactions containing IL-32 γ (100 ng/mL) alone or reactions containing IL-32 γ with elastase in the presence or absence of plasma-derived AAT (A) or AAT-Fc (B) were measured using ELISA, as described in Materials and Methods. The molar ratio of elastase and plasma-derived AAT in the reaction is indicated. In the absence of plasma-derived AAT, only 15% of IL-32 γ remained at the end of the reaction, whereas plasma-derived AAT completely blocked IL-32 γ cleavage (A). However, AAT-Fc failed to block IL-32 γ cleavage by elastase (B). Representative data from one of five independent experiments are shown.

pression vector to express an Fc-fusion protein containing the constant domain of human IgG1 (CH2 and CH3), including two cysteine residues in the hinge region, fused to the C-terminus of human AAT to facilitate the dimerization of the AAT-Fc molecule (Figure 1). As shown by silver staining and Western blot analyses, the recombinant purified AAT-Fc protein appeared as a dimer under the nonreduced conditions, whereas it migrated as a monomer under the reduced conditions

(Figure 1, indicated by 2-mercaptoethanol [ME]).

Plasma-derived AAT augmentation therapy has been approved only for treatment of AAT deficiency in select adults with severe pulmonary emphysema. Recently, augmentation therapy has proven to be highly efficacious in small cohorts of patients with uncommon AAT deficiency-related diseases such as fibromyalgia, systemic vasculitis, relapsing panniculitis and bronchial asthma (25–27). In addition to these studies, a series of mouse model studies has described the efficacy of AAT under other conditions, including diabetes mellitus, organ transplant rejection and acute myocardial ischemia-reperfusion injury (1,3,4,6,28,29). These results have led to an expanding number of medical applications of AAT, such as allogeneic hematopoietic cell transplantation in patients with acute rejection (30,31).

Therefore, it would be useful and timely to implement new strategies for producing AAT. In this study, we produced recombinant AAT-Fc in CHO cells, tested the antiinflammatory effect of AAT-Fc on TNF α - or LPS-induced cytokine production in human PBMCs and compared the functions of plasma-derived AAT and our recombinant AAT-Fc. The recombinant AAT-Fc sufficiently suppressed TNF α -induced IL-6 production but failed to suppress LPS-induced IL-6. However, the same concentration of AAT failed to inhibit the activities of both TNF α and LPS; similar results were obtained in mouse Raw 264.7 cells. Previous studies have used AAT at concentrations that are at least several hundred times higher than that used in this experiment (4,20,21); this difference likely accounts for the lack of inhibition of TNF α and other inflammatory cytokines activities in our assays.

Recent studies on the antiinflammatory effects of AAT have suggested that these effects are independent of the inhibitory activity of serine proteinase (4,19,21) and that the ZZ allele mutation, which causes the formation of polymeric AAT (19), affects innate immunity, including inhibi-

tion of the T-cell immunity related to adaptive immunity (1,4). Nevertheless, growing evidence proposes that AAT possesses antiinflammatory functions that are independent of its inhibitory effects on serine proteinases. We also examined the effect of plasma-derived AAT and AAT-Fc on the enzymatic activity of elastase. Interestingly, plasma-derived AAT specifically blocked IL-32 γ cleavage by elastase, whereas AAT-Fc could not block it. We cannot exclude AAT-Fc-mediated inhibition of other serine proteinases, since we evaluated the enzymatic activity of elastase on a single substrate (IL-32 γ).

Numerous studies on plasma-derived AAT have shown that it protects insulin-producing β -cells in islets under different circumstances such as allogeneic islet transplantation (1,6,8,32,33) and treatment of NOD mice (2,5,7,28). Moreover, clinical studies have suggested that circulating AAT in plasma is associated with type 1 and type 2 diabetes (9,11–13,34–38). These studies led us to examine whether AAT-Fc exerts similar effects in the STZ-induced mouse model of diabetes. During earlier time points after induction of diabetes, there was no difference between nontreated control and AAT-Fc-treated mice. However, at 15 d after the first injection of STZ, the AAT-Fc-treated mice had significantly improved blood glucose levels compared with the mice treated with STZ alone; furthermore, these levels were sustained until d 17. These data suggest that AAT-Fc protects insulin-producing β -cells from STZ-induced diabetes. The mechanism underlying AAT-Fc-mediated antiinflammatory effects probably involves the suppression of IL-6 production in the pancreas, subsequently resulting in reduced hyperglycemia in the STZ-induced mouse model of diabetes.

CONCLUSION

To our knowledge, this study is the first to report a method for the production of much soluble recombinant AAT-Fc protein. The recombinant AAT-Fc protein was synthesized in CHO cells as a chimeric protein fused to the constant region of IgG1 and was purified by using

protein A affinity chromatography. The AAT-Fc antiinflammatory activity was evaluated by monitoring the suppression of IL-6 and TNF α by using *in vitro* assays. In addition, AAT-Fc protein was found to prevent hyperglycemia and inflammatory processes in the STZ-induced mouse model of diabetes. Further studies on AAT-Fc in various autoinflammatory disease models will help identify and develop other clinical applications of AAT-Fc, including the potential replacement of plasma-derived AAT in therapy.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Lewis EC, Shapiro L, Bowers OJ, Dinarello CA. (2005) Alpha1-antitrypsin monotherapy prolongs islet allograft survival in mice. *Proc. Natl. Acad. Sci. U. S. A.* 102:12153–8.
- Song S, et al. (2004) Recombinant adeno-associated virus-mediated alpha-1 antitrypsin gene therapy prevents type 1 diabetes in NOD mice. *Gene Ther.* 11:181–6.
- Lu Y, et al. (2006) Alpha1-antitrypsin gene therapy modulates cellular immunity and efficiently prevents type 1 diabetes in nonobese diabetic mice. *Hum. Gene Ther.* 17:625–34.
- Shahaf G, et al. (2011) Alpha-1-antitrypsin gene delivery reduces inflammation, increases T-regulatory cell population size and prevents islet allograft rejection. *Mol. Med.* 17:1000–11.
- Zhang B, et al. (2007) Alpha1-antitrypsin protects beta-cells from apoptosis. *Diabetes.* 56:1316–23.
- Lewis EC, et al. (2008) Alpha1-antitrypsin monotherapy induces immune tolerance during islet allograft transplantation in mice. *Proc. Natl. Acad. Sci. U. S. A.* 105:16236–41.
- Koulmanda M, et al. (2008) Curative and beta cell regenerative effects of alpha1-antitrypsin treatment in autoimmune diabetic NOD mice. *Proc. Natl. Acad. Sci. U. S. A.* 105:16242–7.
- Pileggi A, et al. (2008) Alpha-1 antitrypsin treatment of spontaneously diabetic nonobese diabetic mice receiving islet allografts. *Transplant. Proc.* 40:457–8.
- Lisowska-Myjak B, Pachecka J, Antoniewicz B, Krawczyk A, Jozwik A. (1995) Alpha-1-antitrypsin, albumin and whole protein in meconium and stools during the first days of life in the neonate [in Polish]. *Pediatr. Pol.* 70:819–26.
- Sharma K, et al. (2005) Two-dimensional fluorescence difference gel electrophoresis analysis of the urine proteome in human diabetic nephropathy. *Proteomics.* 5:2648–55.
- Lisowska-Myjak B, Pachecka J. (2003) Antigenic and functional levels of alpha-1-antitrypsin in serum during normal and diabetic pregnancy. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 106:31–5.
- Lisowska-Myjak B, Pachecka J, Kaczynska B, Miskurka G, Kadziela K. (2006) Serum protease inhibitor concentrations and total antitrypsin activity in diabetic and non-diabetic children during adolescence. *Acta Diabetol.* 43:88–92.
- Hashemi M, Naderi M, Rashidi H, Ghavami S. (2007) Impaired activity of serum alpha-1-antitrypsin in diabetes mellitus. *Diabetes Res. Clin. Pract.* 75:246–8.
- Yaghmaei M, et al. (2009) Serum trypsin inhibitory capacity in normal pregnancy and gestational diabetes mellitus. *Diabetes Res. Clin. Pract.* 84:201–4.
- Kueppers F, Briscoe WA, Bearn AG. (1964) Hereditary deficiency of serum alpha-L-antitrypsin. *Science.* 146:1678–9.
- Eriksson S. (1964) Pulmonary emphysema and alpha1-antitrypsin deficiency. *Acta. Med. Scand.* 175:197–205.
- Blanco I, Lara B, de Serres F. (2011) Efficacy of alpha1-antitrypsin augmentation therapy in conditions other than pulmonary emphysema. *Orphanet. J. Rare Dis.* 6:14.
- Cox DW, Woo SL, Mansfield T. (1985) DNA restriction fragments associated with alpha 1-antitrypsin indicate a single origin for deficiency allele PI Z. *Nature.* 316:79–81.
- Carroll TP, et al. (2010) Evidence for unfolded protein response activation in monocytes from individuals with alpha-1 antitrypsin deficiency. *J. Immunol.* 184:4538–46.
- Nita I, Hollander C, Westin U, Janciauskiene SM. (2005) Prolastin, a pharmaceutical preparation of purified human alpha1-antitrypsin, blocks endotoxin-mediated cytokine release. *Respir. Res.* 6:12.
- Al-Omari M, et al. (2011) Acute-phase protein alpha1-antitrypsin inhibits neutrophil calpain I and induces random migration. *Mol. Med.* 17:865–74.
- Subramaniyam D, et al. (2008) TNF-alpha-induced self expression in human lung endothelial cells is inhibited by native and oxidized alpha1-antitrypsin. *Int. J. Biochem. Cell Biol.* 40:258–71.
- Janciauskiene S, et al. (2008) Alpha1-antitrypsin inhibits the activity of the matrilysin catalytic domain in vitro. *Am. J. Respir. Cell Mol. Biol.* 39:631–7.
- Ryu CJ, Padlan EA, Jin BR, Yoo OJ, Hong HJ. (1996) A humanized antibody with specificity for hepatitis B surface antigen. *Hum. Antibodies Hybridomas.* 7:113–22.
- Eden E, et al. (1997) Atopy, asthma, and emphysema in patients with severe alpha-1-antitrypsin deficiency. *Am. J. Respir. Crit. Care Med.* 156:68–74.
- Griffith ME, Lovegrove JU, Gaskin G, Whitehouse DB, Pusey CD. (1996) C-antineutrophil cytoplasmic antibody positivity in vasculitis patients is associated with the Z allele of alpha-1-antitrypsin, and P-antineutrophil cytoplasmic antibody positivity with the S allele. *Nephrol. Dial. Transplant.* 11:438–43.
- King MA, et al. (1996) Alpha 1-antitrypsin deficiency: evaluation of bronchiectasis with CT. *Radiology.* 199:137–41.
- Ma H, et al. (2010) Intradermal alpha1-antitrypsin therapy avoids fatal anaphylaxis, prevents type 1 diabetes and reverses hyperglycaemia in the NOD mouse model of the disease. *Diabetologia.* 53:2198–204.
- Toldo S, et al. (2011) Alpha-1 antitrypsin inhibits caspase-1 and protects from acute myocardial ischemia-reperfusion injury. *J. Mol. Cell. Cardiol.* 51:244–51.
- Marcondes AM, et al. (2011) Inhibition of IL-32 activation by alpha-1 antitrypsin suppresses alloreactivity and increases survival in an allogeneic murine marrow transplantation model. *Blood.* 118:5031–9.
- Tawara I, et al. (2012) Alpha-1-antitrypsin monotherapy reduces graft-versus-host disease after experimental allogeneic bone marrow transplantation. *Proc. Natl. Acad. Sci. U. S. A.* 109:564–9.
- Kalis M, Kumar R, Janciauskiene S, Salehi A, Cilio CM. (2010) Alpha 1-antitrypsin enhances insulin secretion and prevents cytokine-mediated apoptosis in pancreatic beta-cells. *Islets.* 2:185–9.
- Kuttler B, et al. (2007) Ex vivo gene transfer of viral interleukin-10 to BB rat islets: no protection after transplantation to diabetic BB rats. *J. Cell Mol. Med.* 11:868–80.
- Heywood DM, Mansfield MW, Grant PJ. (1996) Levels of von Willebrand factor, insulin resistance syndrome, and a common vWF gene polymorphism in non-insulin-dependent (type 2) diabetes mellitus. *Diabet. Med.* 13:720–5.
- Laakso M, Malkki M, Kekalainen P, Kuusisto J, Deeb SS. (1995) Polymorphisms of the human hexokinase II gene: lack of association with NIDDM and insulin resistance. *Diabetologia.* 38:617–22.
- Lisowska-Myjak B, Sygitowicz G, Wolf B, Pachecka J. (2001) Serum alpha-1-antitrypsin concentration during normal and diabetic pregnancy. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 99:53–6.
- Sandstrom CS, et al. (2008) An association between type 2 diabetes and alpha-antitrypsin deficiency. *Diabet. Med.* 25:1370–3.
- Talmud PJ, et al. (2003) Progression of atherosclerosis is associated with variation in the alpha1-antitrypsin gene. *Arterioscler. Thromb. Vasc. Biol.* 23:644–9.