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ORIGINAL ARTICLE

Transgenic α-1-antitrypsin secreted into the bloodstream from salivary glands is biologically active

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OBJECTIVES: Salivary glands are potentially a valuable target for gene therapeutics. Herein, we examined the expression and biochemical activity of human alpha-1-antitrypsin (hA1AT) produced in rodent submandibular glands after gene transfer.

METHODS: A serotype 5 adenoviral vector (Ad.hA1AT) was constructed and first characterized by dose response and time course studies using SMIE cells *in vitro*. hA1AT expression was analysed by ELISA and the biologic activity determined by the inhibition of human neutrophil elastase (hNE) and formation of hA1AT-hNE complexes. Ad.hA1AT was administered to submandibular glands of rats and mice. The levels and activity of hA1AT were analysed in saliva, serum and gland extracts. Treatment with endoglycosidase H and Peptide *N*-Glycosidase F was used to assess *N*-linked glycosylation.

RESULTS: Transgenic hA1AT, expressed in submandibular glands following Ad.hA1AT administration, was secreted into the bloodstream, *N*-glycosylated and biochemically active.

CONCLUSION: After *in vivo* gene transfer, rodent salivary glands can produce a non-hormonal, transgenic, secretory glycoprotein exhibiting complex and conformation-dependent biologic activity.

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Keywords: human α-1-antitrypsin; bioactivity; gene therapy; secretion; salivary glands

Introduction

Alpha1-antitrypsin (A1AT), the archetypal member of the SERPIN family of serine protease inhibitors (Morgan and Kalsheker, 1997), is a 52 kDa glycoprotein secreted by the liver, which inhibits a wide variety of proteases. In particular, it protects tissue from proteases produced by inflammatory cells, like neutrophil elastase (NE) in the lungs (Balfour-Lynn, 1999; Kohnlein and Welte, 2008), by forming a tight and stable complex with a 1 : 1 stoichiometry (Lomas and Carrell, 1993; Egelund *et al*, 1998; Sun and Yang, 2004; Kohnlein and Welte, 2008). Severe A1AT deficiency is inherited as an autosomal co-dominant disorder characterized by reduced serum levels of A1AT (Fregonese and Stolk, 2008). Low levels of A1AT in the lung interstitium render individuals susceptible to proteolytic damage from resident NE and at high risk for developing emphysema (Kohnlein and Welte, 2008). In addition, A1AT levels can affect cancer progression and the course of bacterial infections (Balfour-Lynn, 1999; Sun and Yang, 2004). Human (h) A1AT is normally glycoslylated at three asparagine residues (46, 83, 247; Mills *et al*, 2003), and these carbohydrate moieties provide both conformational and kinetic stability (Kwon and Yu, 1997). Indeed, point mutations in SERPIN family members, the best described of which occurs in hA1AT, lead to conformational pathologies, so-called serpinopathies (Gooptu and Lomas, 2009).

We have proposed the use of salivary glands as target sites for gene therapeutics (Baum *et al*, 1999, 2004; Perez *et al*, 2010), i.e., to function as an endogenous bioreactor supplying transgenic secretory proteins useful in treating upper gastrointestinal tract and systemic disorders. To date, we have shown that salivary glands of animal models can produce many such therapeutic secretory proteins, including several hormones, cytokines and growth factors (He *et al*, 1998a; Kok *et al*, 2003; Voutetakis *et al*, 2004, 2010; Lodde *et al*, 2006; Cotrim *et al*, 2007; Adriaansen *et al*, 2008; Sugito *et al*, 2009; Zheng *et al*, 2009). Mammalian salivary gland cells are organs easily accessible by intraoral duct cannulation, not critical-for-life, and able to produce and secrete large amounts of protein into both saliva and the bloodstream (Baum *et al*, 1999). Previously, we

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showed that immunoreactive hA1AT is secreted into the bloodstream from rat parotid and submandibular glands after gene transfer (Kagami *et al*, 1996). Because hA1AT's mechanism of action is much different from that of any previously expressed transgenic secretory protein produced in salivary glands, herein, we specifically have examined the biochemical activity of hA1AT expressed and secreted after transduction of mouse and rat submandibular glands by a serotype 5 adenoviral (Ad5) vector. We show that the transgenic hA1AT produced in salivary glands was secreted into the bloodstream, *N*-glycosylated and biologically active in both species.

Materials and methods

Animals

BALB/c male mice ($n = 6$ per treatment group) and Wistar male rats ($n = 4$–5 per treatment group) were obtained from Harlan–Sprague–Dawley (Walkersville, MD, USA) at 6 weeks of age. They were acclimated for 1 week before the start of experiments, and water and food were provided *ad libitum*. Animal experiments were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee and the National Institutes of Health Biosafety Committee.

Recombinant adenovirus construction

A first-generation, replication-deficient Ad5 vector containing the hA1AT cDNA was constructed. A hA1AT carrier plasmid (GeneCopoeia, Germantown, MD, USA) was digested using *Xmn*I and *Xho*I restriction enzymes. The resulting hA1AT cDNA (1257 bp) obtained was cloned into the Ad5 expression shuttle plasmid pACCMV-pLpA, containing both the CMV promoter/enhancer and simian virus 40 polyadenylation signal. The resulting plasmid, pACCMV-hA1AT, was co-transfected with pJM17 into C7 cells (a gift from Dr. J. Chamberlain; Amalfitano and Chamberlain, 1997), using a calcium phosphate precipitation protocol (Becker *et al*, 1994) to yield Ad.hA1AT. The generated Ad.hA1AT was amplified in 293 cells and purified as previously reported (Zheng *et al*, 2000; Baum *et al*, 2002; Sugito *et al*, 2009). Vector titers and the concentrations used herein were determined by real-time quantitative PCR (QPCR) using CMV promoter-specific primers as previously reported (Adriaansen *et al*, 2008).

In vitro *cell transductions*

In vitro studies were done using the rat submandibular cell line SMIE (He *et al*, 1998b). Cells were seeded at 80% confluence in 6-well plates in Dulbecco's modified Eagle's medium/10% fetal calf serum (Biosource, Camarillo, CA, USA) and later transduced in serum-free medium with Ad.hA1AT at a multiplicity of infection (MOI) ranging from 10 to 500. Medium was harvested 16 and 24 h later and stored at −80°C until assayed. All experiments were performed in duplicate.

Detection of hA1AT/human neutrophil elastase (hNE) complexes

As one of the approaches to determine the biologic function of the transgenic hA1AT produced *in vitro*, we analysed the formation of hA1AT/hNE complexes. We were unable, however, to use this assay with serum samples following vector delivery to submandibular glands *in vivo*, because of serum protein interference. Purified hA1AT (Sigma, St. Louis, MO, USA) and conditioned media samples were mixed with purified hNE (Calbiochem, Darmstadt, Germany) and incubated at 37°C for 15 min. The complex, intact and/or cleaved hA1AT, was detected by Western blot using a rabbit anti-hA1AT antibody (1 : 800; DakoCytomation, Glostrup, Denmark). Briefly, samples were mixed with loading buffer without β-mercaptoethanol and loaded on a 4–15% gradient Tris–HCl precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and electrophoresed at 100 V for 1 h. The proteins were then transferred onto nitrocellulose membranes, blocked in 4% non-fat milk and incubated with rabbit anti-hA1AT for 1 h at room temperature. Membranes were placed in a 1 : 5000 dilution of anti-rabbit–IgG horseradish peroxidase conjugated antibody (Amersham Life Science Inc., Arlington Heights, IL, USA) for 1 h. Finally, membranes were incubated for 1 min in chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to BioMax MR film (Kodak/Carestream Health, Rochester, NY, USA).

Anti-elastase activity assay

Enzymatic activity of hNE was followed by monitoring the cleavage of colorimetric elastase substrate I (Calbiochem) at 410 nm for 15 min. Fifty microliters of (2 μg ml^{-1}) hNE was mixed with 100 μl of sample (either conditioned media 1 : 10 or rodent serum 1 : 2000), diluted in reaction buffer (0.1 M Hepes, 0.5 M NaCl, 10% dimethyl sulfoxide, pH 7.5), and the reaction initiated by adding 50 μl substrate (2 mM), also diluted in reaction buffer. The initial activity was determined by calculating the slope of the product generated *vs* time. The inhibition of hNE was calculated relative to the control activity obtained in a blank sample.

Vector delivery into submandibular glands

The vector delivery into submandibular glands of rats or mice was performed essentially as previously described (Baum *et al*, 2002; Sugito *et al*, 2009) with minor modifications. Animals were anesthetized with a mixture of 60 mg ml^{-1} ketamine (Phoenix Scientific, St. Joseph, MO, USA) and 8 mg ml^{-1} xylazine (Phoenix Scientific) given intramuscularly (i.m.; 1 μl g^{-1} body weight). Cannulation of submandibular gland ducts was done with modified polyethylene tubing (Intramedic PE-10; BD Diagnostic Systems, Sparks, MD, USA), as described earlier (Baum *et al*, 2002). Atropine (i.m., 0.5 mg kg^{-1} body weight; Sigma) was administered to decrease salivary flow prior to vector delivery. After 10 min, 10^8, 10^9 or 2×10^9 vector genomes (vg)/gland (suspended in saline; 200 μl for rats and 50 μl for mice)

were administered by retrograde submandibular duct delivery. After 48 h, the animals were re-anesthetized and given a subcutaneous injection of 0.5 μg g^{-1} pilocarpine (PLP; Sigma) to induce saliva secretion. Whole saliva and blood were collected as previously described (Kagami *et al*, 1996) and, following separation of serum, samples were stored at −80°C until all assays were performed. Animals were euthanized in a CO_2 chamber, and death was ensured by bilateral thoracotomy. Submandibular glands were removed, cleaned, and each gland was cut longitudinally in two pieces. One part was snap frozen and the second was fixed in paraformaldehyde 4%.

Determination of hA1AT levels

Levels of hA1AT were measured using an ELISA, following the manufacturer's instructions (ALPCO Diagnostic, Salem, NH, USA). Before samples were measured, a spiking assay was done to establish the optimal dilutions of saliva, serum and extracted glandular protein samples. The total amount of hA1AT secreted was calculated using an average rat serum volume of 4 ml, an average mouse serum volume of 2 ml, and the exact volume of saliva collected after stimulation of secretion with PLP (Kagami *et al*, 1996).

Immunostaining

For immunohistochemistry, submandibular glands were removed, fixed in paraformaldehyde 4% diluted in phosphate-buffered saline (PBS; Electron Microscopy Sciences, Hatfield, PA, USA) and embedded in paraffin. Sections of 5 μm were de-waxed and rehydrated in a gradient of ethanol and washed in PBS. Antigen retrieval was performed by enzymatic digestion with 20 μg ml^{-1} Proteinase K in TE buffer pH 8.0 for 5 min at 37°C. Endogenous peroxidase quenching was performed using 3% v/v hydrogen peroxide in 100% methanol for 10 min at room temperature. Sections were then blocked with 10% goat serum in PBS for 30 min, incubated with 16 μg ml^{-1} rabbit antibody against hA1AT (DakoCytomation) in 10% bovine serum albumin in PBS for 1 h at room temperature and washed with PBS. Bound antibodies were visualized using Histostain-SP Kits LAB-SA detection System (Zymed Laboratories, San Francisco, CA, USA) followed by counterstaining with hematoxylin.

Endoglycosidase H (Endo H) and Peptide : N-Glycosidase F (PNGase F) digestion

Serum samples were stored at −80°C until glycosidase digestions were performed. For five transduced rats (two administered 1×10^9vg/gland; three administered 2×10^9vg/gland), albumin-free serum samples were obtained using the Promax Albumin Removal kit (Polyscience, Inc., Warrington, PA, USA), according to the manufacturer's instructions, with the following minimal modifications. The incubation time of serum with the magnetic particles was increased to 15 min and the elution volume was decreased to 20 μl per each 10 μl of serum treated. After elution from magnetic particles the protein samples were immediately digested with glycosidase as follows. One microliter of 10 × Glycoprotein Denaturing Buffer (0.5% SDS, 40 mM dithiothreitol; New England Biolabs, Beverly, MA, USA) was added to 9 μl of albumin-free serum protein. The mixture was incubated at 100°C for 10 min. Thereafter, for Endo H digestions, 2 μl of G5 10× reaction buffer (500 mM sodium citrate, pH 5.5), 6 μl of water and 2 μl Endo H (New England Biolabs) were added, and the reaction mix incubated at 37°C for either 4 or 12 h (no differences were seen in the extent of Endo H digestion achieved). For the PNGase F digestions, 2 μl of 10× G7 Reaction Buffer, 2 μl 10% NP40, 2.5 μl PNGase F and 3.5 μl of water were added and incubated at 37°C for 12 h. In parallel, a mock-digested sample without either glycosidase enzyme was performed as a control. Laemmli denaturating buffer (10 μl) was added to stop each reaction, and samples were heated at 95°C for 10 min. Samples were electrophoresed on 4–12% NuPAGE Bis-Tris denaturing gels (Invitrogen, Carlsbad, CA, USA), transferred to nitrocellulose membranes, incubated for 30 min at room temperature in blocking solution (5% w/v non-fat powdered milk in 1× Tris-buffered saline/0.1% Tween 20), and then incubated with primary antibodies (goat anti-hA1AT, 1:300 dilution; Novus Biologicals, Littleton, CO, USA) for 12 h at 4°C. Donkey anti-goat IgG Cy 3 (Amersham, Buckinghamshire, UK), at a 1 : 2500 dilution in 1× Tris-buffered saline, 0.1% Tween 20, was used as a secondary antibody. Images were obtained using the Typhoon 9410 (Amersham) and densitometric analyses used Scion Image for Windows (Scion Corp., Frederick, MD, USA). Quantified results are expressed as the percentage of intensity in the glycosidase generated immunopositive band relative to that seen in the control untreated sample from each animal.

Statistical analyses

Statistical analyses were done using Prisma 5 for windows version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data were tested using the Kruskal–Wallis test, followed by a Mann–Whitney *U* rank-sum test. A probability level of $P < 0.05$ was considered statistically significant. Data are expressed as mean values ± s.e.m.,

Results and discussion

In vitro *production of hA1AT*

After transduction of SMIE cells with Ad.hA1AT, high levels of hA1AT were found in the culture media and these levels were dose and time dependent (Figure 1a,b). The biochemical activity of hA1AT was determined by both the inhibition of hNE in a direct enzymatic assay, and by formation of a hA1AT-hNE complex detected by Western blot. In the dose-response experiments, an optimal response was detected at a MOI of 50 (approximately 1000 ng ml^{-1} hA1AT). At this dose the inhibition of hNE activity in the media was approximately 70% compared to that with media from the non-transduced cells. When a higher MOI was used (250 or 500), the amount of hA1AT produced decreased, which

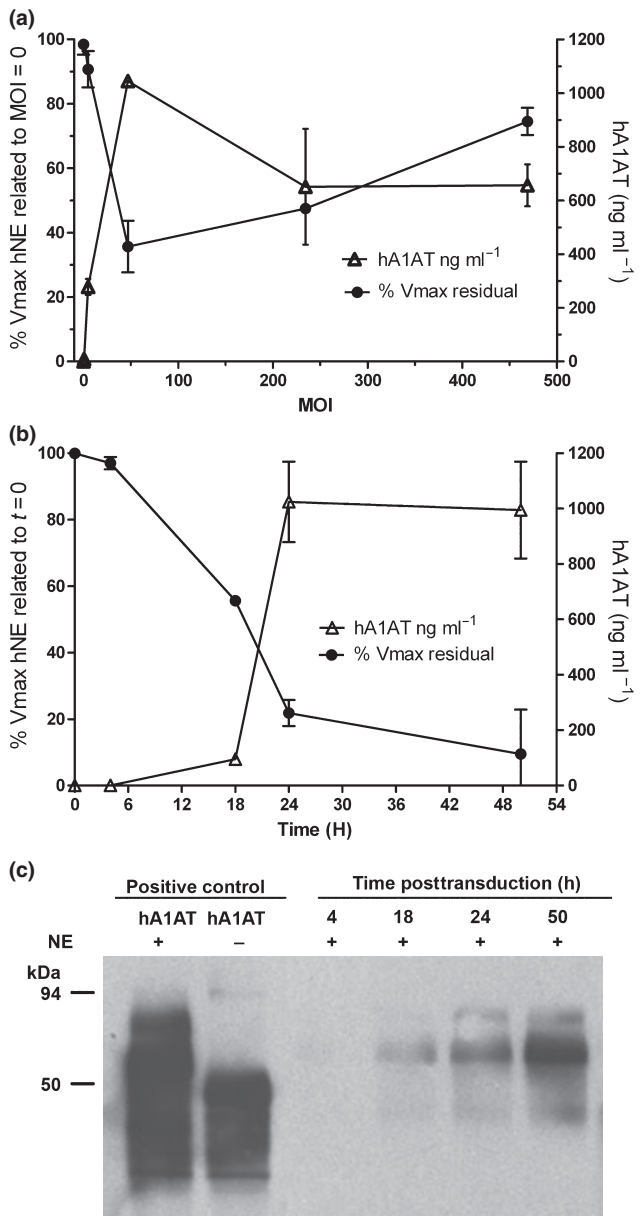

Figure 1 Secretion of human alpha 1 anti-trypsin (hA1AT) after transduction of SMIE cells *in vitro* with Ad.hA1AT. (**a**) hA1AT concentration and anti-elastase activity in culture media after transduction of SMIE cells with different vector doses. SMIE cells were incubated with the indicated multiplicity of infection (MOI) of Ad.hA1AT. After 48 h, medium was harvested and assayed for activity: anti-human neutrophil elastase (hNE) activity (circles) and hA1AT concentration by ELISA (triangles). (**b**) hA1AT concentration and anti-elastase activity in culture media at different times after transduction of SMIE cells with 50 vg of Ad.hA1AT/cell. The media was harvested at the indicated times and assayed for anti-elastase activity (circles) and hA1AT concentration by ELISA (triangles). The data shown in a and b are representative of two experiments performed in duplicate and displayed as mean ± SEM. (**c**) Detection of hA1AT/human neutrophil elastase (NE) complexes with culture media of SMIE cells, as a source of hA1AT, at different times after transduction. In the positive control, commercially available hA1AT was employed

was related to a decrease of the cell number attributable to Ad5 vector toxicity (data not shown). The time-course experiment was performed using a MOI of 50. The highest levels of hA1AT and the maximum inhibitory activity in the culture media (80% inhibition related to non-transduced cells) were reached at approximately 24 h post transduction. The formation of the hA1AT-hNE complex was detected, using samples of conditioned media as a source of hA1AT, as early as 18 h post transduction (Figure 1c). These initial experiments showed that the Ad.hA1AT vector directs the formation of immunologically recognized and biochemically functional hA1AT *in vitro*.

hA1AT production after administration of Ad.hA1AT to mouse and rat salivary glands in vivo

After demonstrating that Ad.hA1AT mediated the production of functional hA1AT *in vitro*, we next administered the vector (10^8 or 10^9 vg) to the submandibular glands of mice. In addition, mice treated with saline were used as controls (Figure 2a). After 24 h, no hA1AT was detected using an ELISA assay in both serum and saliva (data not shown). At 48 h post vector delivery, no hA1AT was detected in saliva at either dose. However, the levels of hA1AT found in serum were considerably elevated from all animals in the highest dose group (25.7 ± 4.68 ng ml^{-1}; mean ± s.e.m.; Figure 2a). Previously, we have shown that the secretion pattern of transgenic human parathyroid hormone and erythropoietin can be different in salivary glands of mice and rats (Adriaansen *et al*, 2008; Voutetakis *et al*, 2008). To determine if the secretion pattern of hA1AT from murine submandibular glands was different from that seen with rat glands, we delivered three doses (10^8, 10^9 or 2×10^9 vg/gland) of vector to rat submandibular glands. As shown in Figure 2b and Table 1, most of the total hA1AT secreted was found in serum. However, a low (< 10% serum values), but significant, increase of hA1AT also was seen in saliva with the PLP stimulation. We also analysed the levels of hA1AT in rat glandular protein extracts. When 10^9 vg were delivered, the total amount of hA1AT in gland extracts was 673 ng, and when 2×10^9 vg were used the total amount of hA1AT was 894 ng (Figure 2b). Interestingly, most of the transgenic hA1AT protein produced was not secreted (Table 1). This finding suggests that the targeted cells are inefficient in secreting hA1AT at these relatively high expression levels (Table 1), an observation that requires more detailed analysis in future to understand this fully.

Endo H and PNGase F digestion assay

In the secretory pathway, acquisition of endoglycosidase H (Endo H) resistance occurs shortly after arrival of *N*-linked glycoproteins in the Golgi complex (Rothman and Fine, 1980). Endo H cleaves the chitobiose core of only high mannose oligosaccharides (Maley *et al*, 1989). Conversely, PNGase F cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins (Maley *et al*, 1989). Using these characteristic enzymes, we examined the glycosylation of the hA1AT secreted from salivary glands to serum. As shown in Figure 3, and consistent with previous

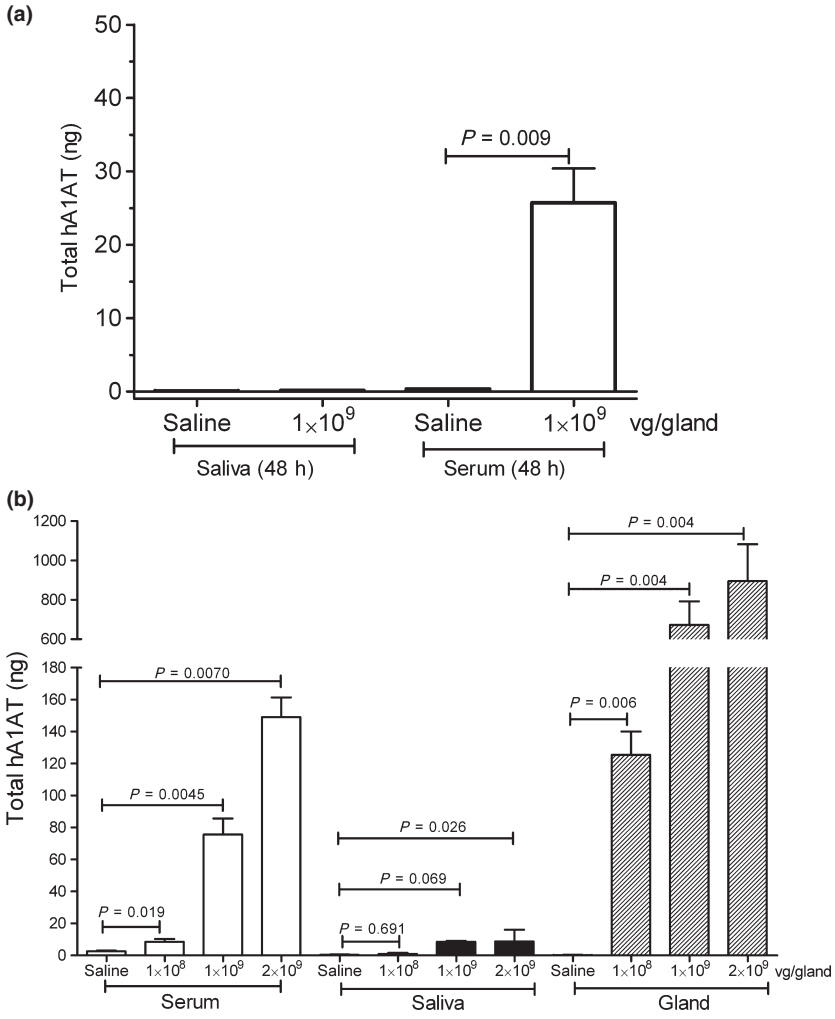

Figure 2 Distribution and biochemical activity of hA1AT in mice and rats following administration of Ad.hA1AT to submandibular glands by retrograde ductal instillation. (**a**) hA1AT in mouse serum and saliva 48 h after an infusion of 10^9 vg of Ad.hA1AT/gland (6 animals/group; analyses were made in triplicate; mean ± s.e.m.). (**b**) Distribution of hA1AT in rats following administration of Ad.hA1AT to submandibular glands. hA1AT was measured in rat serum, saliva and total protein extracts from submandibular glands 48 h after vector infusion. Data shown are the mean ± SEM of triplicate assays from four animals per group and representative of two independent experiments

Table 1 Distribution of hA1AT expressed after transduction of rat submandibular glands

Vector genomes/ gland	*% secretion from total ± s.e.m.*		*% non-secreted*	*Total Expressed* ng ± s.e.m.*
	Serum	*Saliva*		
1×10^8	16.5 ± 7.3	1.0 ± 0.5	82.5 ± 7.8	130.1 ± 36.1
1×10^9	10.7 ± 3.6	3.8 ± 1.6	85.5 ± 4.9	667.7 ± 76.6
2×10^9	13.7 ± 2.9	1.0 ± 0.9	85.3 ± 3.4	1101 ± 182

*The human alpha 1 anti-trypsin (hA1AT) concentration in serum and saliva was multiplied times an average volume of serum in rats (4 ml) and the specific volume of saliva collected, respectively. The amount in glands per μg protein was multiplied by the total amount of protein in glands.

reports (Sidhar *et al*, 1995), most (95%) of the hA1AT in serum was de-glycosylated by PNGase F (Figure 3), producing a digested band of ≈45 kDa. However, the hA1AT found in serum was totally resistant to treatment with Endo H. Note that the transgenic hA1AT in rat serum samples showed a small difference in size from that of purified, native hA1AT (approximately 54 kDa in the transgenic compared to approximately 56 kDa of pure protein; Figure 3a). However, the transgenic and native hA1ATs presented an almost identical reactivity pattern with the glycosidases used.

Biochemical activity of secreted, transgenic hA1AT

The biologic function of transgenic hA1AT produced *in vivo* was evaluated by measuring the inhibition of hNE catalytic activity by rodent serum samples. As noted earlier, because of serum protein interference, we could not use serum samples to assess the formation of stable hA1AT-hNE complexes. The hNE inhibitory activity of serum samples from vector-treated rats was significantly higher (approximately 75%) than that of serum from control rats at 48 h post transduction (approximately 35% *vs* approximately 20% inhibition of hNE; Figure 4). Given the amount of hNE used in these assays, and the concentration of hA1AT in rat serum (60 ng ml^{-1}), the level of inhibitory activity observed is comparable to what was expected. Generally, similar results were found with mouse serum samples (data not shown). Thus, the transgenic hA1AT secreted into the bloodstream from salivary glands of both species was biochemically active

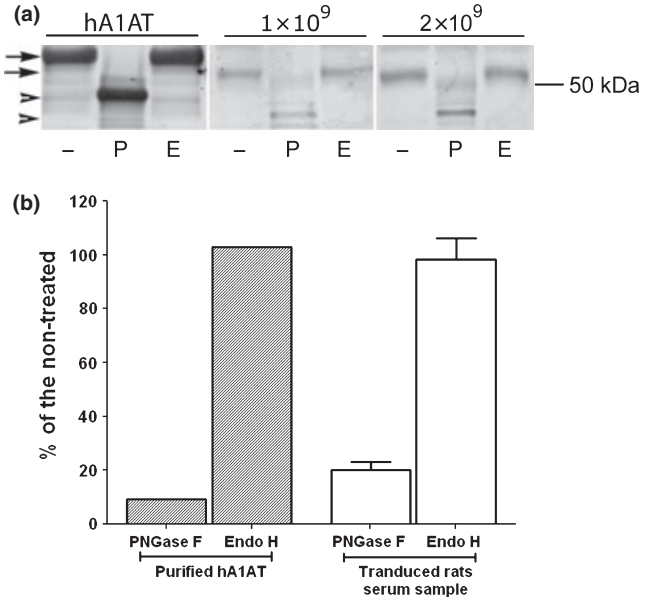

Figure 3 Endo H and PNGase F sensitivity of secreted transgenic hA1AT in serum. Albumin-free rat serum proteins were obtained from serum samples 48 h after vector delivery (10^9 and 2×10^9 vg/gland). Purified hA1AT and serum samples were denatured and incubated for 12 h with Endo H, PNGase F or mock incubated as described in Materials and methods. (**a**) Western blot showing representative results from five analysed (two rats administered 10^9 vg/gland and three rats administered 2×10^9 vg/gland). PNGase treatment generated an immunoreactive form of hA1AT with lower molecular weight than the non-treated (-) samples (arrows *vs* arrowheads), Endo H treatment does not have any effect on the migration position of secreted hA1AT. (**b**) Results of densitometric analyses of all samples treated. The percentage of the control level of hA1AT that is present in PNGase F- and Endo H-treated samples is shown. Results represent the mean ± s.e.m. obtained from all five animals analysed

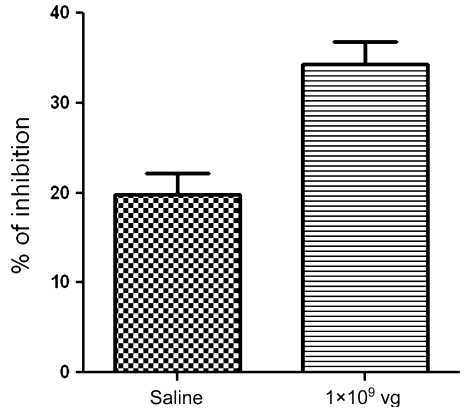

Figure 4 Biochemical activity of transgenic hA1AT in rat serum. The ability to inhibit human neutrophil elastase activity of transgenic hA1AT produced in and secreted from rat submandibular glands was assayed by measuring the anti-human neutrophil elastase activity found in rat serum 48 h after vector infusion with 10^9 vg/gland. Data shown are the mean ± s.e.m. of triplicate assays from five animals/group

Cellular localization of hA1AT in transduced salivary glands by immunohistochemistry

Two days after vector infusion, salivary glands from mice (data not shown) and rats were excised, fixed in paraformaldehyde, embedded in paraffin and labeled with an antibody directed against hA1AT. Even after stimulation with PLP, hA1AT was visible in all treated glands, clustered in groups of acinar cells and primarily in the cytoplasm of these cells, in a punctate pattern without any defined polarity (Figure 5). Sections from animals treated with saline or labeled with the irrelevant IgG control did not exhibit any immunostaining (data not shown).

In the present study, we have examined the biochemical activity of hA1AT expressed and secreted after transduction of mouse and rat submandibular glands with the Ad.hA1AT vector. As shown herein, the transgenic hA1AT secreted into the bloodstream of both species was biochemically active, able to inhibit the catalytic activity of hNE. While we have reported that salivary glands can produce many biologically active therapeutic human secretory proteins, all have been either hormones, growth factors or cytokines, including growth hormone, interleukin-10, erythropoietin (hEpo), vasoactive intestinal peptide, vascular endothelial growth factor, basic fibroblast growth factor, parathyroid hormone (hPTH), keratinocyte growth factor, TWEAK, and glucagon-like peptide 1 (He *et al*, 1998a; Kok *et al*, 2003; Voutetakis *et al*, 2004, 2010; Lodde *et al*, 2006; Cotrim *et al*, 2007; Adriaansen *et al*, 2008; Sugito *et al*, 2009; Zheng *et al*, 2009). All of these transgenic proteins function by binding to and activating a cell surface receptor, which then results in an amplified intracellular signaling cascade to elicit the appropriate biologic response, e.g., an increased hematocrit with transgenic hEpo or elevated serum calcium levels with hPTH (Voutetakis *et al*, 2004; Adriaansen *et al*, 2008).

The findings shown in this report represent the first time that a non-hormonal, transgenic secretory protein, exogenously produced after gene transfer to salivary glands, was proven to be biochemically active. Furthermore, that the transgenic hA1AT was biochemically functional is particularly significant because of its implications about the structure and processing of the secreted transgenic protein. Both the *N*-glycosylation of specific asparagine residues and the resultant stabilized protein conformation are essential to hA1AT's mechanism of anti-protease action (Lomas and Carrell, 1993; Kwon and Yu, 1997). Consequently, each molecule of hA1AT is able to bind directly to one molecule of hNE with a 1 : 1 stoichiometry, an interaction that in effect leads to the functional suicide of both molecules and involves no signal amplification (Egelund *et al*, 1998; Sun and Yang, 2004). We also show that the secreted hA1AT is PNGase F- and not Endo H-sensitive suggesting that this product is an end-point in the glycosylation pathway. The size difference in the secreted transgenic hA1AT from the native protein may in part be attributable to differences in the oligosaccharides added by salivary gland cells. However, the fact that biochemically active transgenic hA1AT is found in rat and mouse sera after Ad.hA1AT transduction strongly argues that this transgenic protein, which is known to be highly sensitive to mis-folding as a result of point mutations (Gooptu and Lomas, 2009), is correctly processed and folded prior to its secretion from the transduced salivary glands. Although the actual

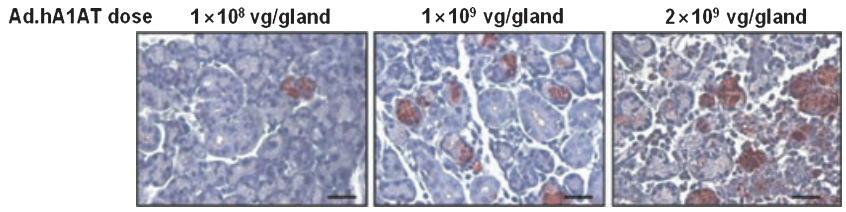

Figure 5 Detection and localization of hA1AT in transduced rat salivary glands. Submandibular glands were removed, fixed in paraformaldehyde and embedded in paraffin. Staining of tissue sections from transduced rat glands revealed a clustered accumulation of hA1AT inside acinar cells, with an apparent vesicular organization in the absence of any specific polar localization. Similar results were found in mouse glands (not shown). Scale bar corresponds to 20 μm

levels of hA1AT secreted into the bloodstream after rodent salivary gland transduction are too low to be clinically useful for an A1AT-deficiency (serum levels would need to be approximately 10^4-fold greater), the fact that salivary glands can produce and secrete a complex, conformation-dependent, functional protein with significant and important post translational modifications would appear to expand the possibilities for utilizing salivary gland gene transfer in novel applications of gene therapeutics.

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