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Functional analysis of plasma α_2 -macroglobulin from Alzheimer's disease patients with the *A2M* intronic deletion

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Abstract

α_2 -Macroglobulin (α_2 M) is an abundant plasma/extracellular space protein implicated in clearance of amyloid β ($A\beta$), a key constituent of Alzheimer's disease (AD) plaques. α_2 M also regulates proteinase and growth factor activities. In recent years, there have been >30 genetic studies debating the controversial role of a five-base-pair intronic deletion in the *A2M* gene in late-onset AD. However, little is known about potential effects of the deletion upon α_2 M function. In this study, we examined the subunit and conformational structure of α_2 M in AD plasma samples, and its capacity to bind trypsin, transforming growth factor- β 1, and $A\beta$. Plasma from patients homozygous for the deletion (DD) showed normal α_2 M subunit size, conformation, and proteinase inhibitory activity. Interestingly, plasma α_2 M from two DD patients showed markedly increased TGF- β 1 binding. Moreover, methylamine-treated DD plasma samples showed modest, but significant, elevations in $A\beta$ binding to α_2 M* compared with samples from patients lacking the deletion. These observations suggest a possible functional basis by which the *A2M* deletion may influence multifactorial AD pathogenesis.
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Introduction

Mutations or polymorphisms in four genes (*AβPP*, *PS-1*, *PS-2*, and *APOE*) are well accepted risk factors for Alzheimer's disease (AD)¹ (Schellenberg et al., 2000; Rogava et al., 2001). However, these genes account for only a minor percentage of Alzheimer's disease cases, and other genes on

chromosome 12 have been implicated in AD (Pericak-Vance et al., 1997; Luedeking-Zimmer et al., 2002). In particular, polymorphisms in genes encoding α_2 -macroglobulin (α_2 M) (Blacker et al., 1998; Liao et al., 1998) and its receptor, the low-density lipoprotein receptor-related protein (LRP1) (Kang et al., 1997; Bullido et al., 2000), have been intensively studied as potential risk factors for late-onset Alzheimer's disease.

α_2 M is a large, homotetrameric protein secreted by hepatocytes, fibroblasts, neurons, and glia and is found at micromolar concentrations in extracellular spaces and in plasma (reviewed in Chu and Pizzo, 1994). When a proteinase cleaves a peptide sequence, termed the "bait-region" within the α_2 M solenoid structure, α_2 M's internal thioesters undergo hydrolysis, resulting in a major conformational change, entrapment of the proteinase, and exposure of cryptic LRP1-binding sites (Kolodziej et al., 2002). This more

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¹ Abbreviations used: AD, Alzheimer's disease; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; α_2 M, α_2 -macroglobulin; α_2 M*, the faster migrating conformation formed from proteinase or methylamine treatment; *A2M*, the α_2 M gene; $A\beta$, amyloid- β peptide_(1–40); CTL, nondemented control subjects; TGF- β 1, transforming growth factor- β 1; DD, homozygous for the pentanucleotide intronic deletion in the *A2M* gene that is associated with late-onset AD in some populations; II, intact at the DD locus.

compact conformation (α_2M^*), which migrates faster by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions, can also be formed by treatment of α_2M with methylamine. α_2M^* -bound proteinases remain enzymatically active, but are sterically shielded from both protein substrates and proteinase inhibitors that are too large to penetrate the internal cavities of α_2M^* .

In addition to its proteinase regulatory activity, α_2M has the ability to noncovalently or covalently bind growth factors (Chu et al., 1991; Chu and Pizzo, 1994; Crookston et al., 1994; Webb et al., 1996). One of the more extensively studied interactions involves regulation of transforming growth factor- β (TGF- β) isoforms. In general, binding of TGF- β to α_2M suppresses TGF- β activity (Danielpour and Sporn, 1990; LaMarre et al., 1990), although α_2M^* can synergize with TGF- β 1 in some systems (Stouffer et al., 1993; Webb et al., 1995). Alterations in TGF- β isoform distribution have been noted in regions of AD pathology (Flanders et al., 1995; Peress and Perillo, 1995), and there is evidence to suggest several mechanisms by which TGF- β influences processes central to AD pathogenesis (reviewed in Masliah et al., 2001).

Alzheimer's disease brain tissues typically contain numerous ubiquitin-labeled aggregates of τ and A β plaques admixed with neuritic processes (Chu et al., 2000; Mirra and Hyman, 2002). α_2M , LRP1, and several other LRP1 ligands have all been immunolocalized to A β plaques (Van Gool et al., 1993; Rebeck et al., 1995). α_2M may also target bound growth factors and other proteins for endolysosomal processing/degradation, after uptake by LRP1 (Chu and Pizzo, 1993, 1994). Indeed, α_2M binds A β , prevents A β fibril formation, and mediates its clearance by LRP1 (Narita et al., 1997; Du et al., 1998; Qiu et al., 1999; Shibata et al., 2000; Van Uden et al., 2000; Lauer et al., 2001; Mettenberg et al., 2002). Thus, there are numerous mechanisms by which alterations in α_2M function(s) may affect processes central to AD pathogenesis.

In 1998, an intronic deletion of five base pairs in the *A2M* gene near the 5' splice acceptor site of exon 18 was genetically linked to late-onset AD (Blacker et al., 1998). However, this association has been highly controversial. Subsequent studies supporting an association with AD have included Korean, Spanish, and Caribbean hispanic populations (Alvarez et al., 1999; Rudrasingham et al., 1999; Dodel et al., 2000; Kovacs, 2000; Romas et al., 2000; Verpillat et al., 2000; Jhoo et al., 2001). A large number of studies in other populations have failed to confirm an association (for example, Chen et al., 1999; Crawford et al., 1999; Hu et al., 1999; Korovaitseva et al., 1999; Shibata et al., 1999; Bullido et al., 2000; Koster et al., 2000), although some of these do show an excess of the homozygous deletion in AD patients (Singleton et al., 1999; Gibson et al., 2000; Wang et al., 2001). A recent study involving the intact allele in combination with a codon 1000 polymorphism was interpreted as supporting population-based susceptibilities (Zappia et al., 2002). These contradictory findings may

implicate an alternative gene in linkage disequilibrium with α_2M . Alternatively, the *A2M* deletion allele was found to modify the effects of interleukin-6 polymorphisms upon AD risk, indicating the importance of considering gene–gene interactions in multifactorial diseases such as AD (Bagli et al., 2000).

While there have been occasional studies indicating that the intronic deletion does not result in a major polypeptide truncation (Rogaeva et al., 1999; Gibson et al., 2000), little is known about potential effects of the deletion upon α_2M function. In this study, we examined the functional conformation of α_2M in plasma samples from patients homozygous for the intact (II) or the deletion (DD) allele and assessed the ability of α_2M in these plasma samples to bind trypsin, TGF- β 1, and A β .

Materials and methods

Plasma samples

Subjects were recruited from the University of Pittsburgh Alzheimer's Disease Research Center, between 1994 and 1998, following the consent procedures approved by the University of Pittsburgh Institutional Review Board. A set of 555 late-onset AD patients and 446 normal control (CTL) subjects were genotyped for the *A2M* deletion polymorphism as described previously (Wang et al., 2001). For this study, only patients who were homozygous for either the intact *A2M* gene (II) or the 5-bp intronic deletion (DD) were used. CTL samples were from healthy patients who had no evidence of dementia, cerebrovascular disease, or head injury. Plasma samples were stored at -80°C and aliquoted to avoid more than two freeze–thaw cycles. Total plasma protein concentration was determined using the Pierce Coomassie protein assay or by bicinchoninic acid protein assay.

All available DD plasma samples were analyzed along with a set of II plasma samples matched for patient age, diagnosis, and storage time. There were 14 AD cases, of which 57% were the DD genotype, and 10 CTL cases, of which 30% were DD. The II and DD plasma sets did not differ significantly in patient age (73.3 ± 2.3 years vs 73.8 ± 3.5 years, mean \pm SEM) or plasma storage time (51.0 ± 4.8 months vs 56.7 ± 5.4 months, mean \pm SEM). There was no correlation between plasma storage time and total protein concentration, proteinase inhibitory capacity, or α_2M band intensity on any of the gels/blots, and there were no significant differences in total plasma protein concentrations according to genotype, diagnosis, or plasma storage time. With regard to ApoE genotype, 33.3% of AD-II cases showed the 3,3 genotype and compared to 37.5% of the AD-DD cases, with the remainder having the 3,4 genotype except for one 4,4 sample. Eighty percent of the CTL cases were 3,3, with one 3,4 and one 4,4 sample.

Reagents

Fresh frozen plasma was obtained from the Central Blood Bank of Pittsburgh, Pittsburgh, PA, and α_2M was purified by zinc chelate chromatography as described previously (Imber and Pizzo, 1981; Chu and Pizzo, 1994). Thiol titrations were performed to monitor the amount of trypsin-releasable free thiol, which can be used to calculate the conformational composition of purified α_2M , as described previously (Chu and Pizzo, 1994). For growth factor and amyloid binding studies, purified α_2M or diluted plasma samples were sometimes dialyzed against 200 mM methylamine in 50 mM Tris buffer, pH 8.2, to induce conformational change to α_2M^* . Samples were dialyzed extensively against PBS to remove methylamine.

TGF- β_1 was purchased from R&D Systems (Minneapolis, MN) and radioiodinated by the method of Ruff and Rizzino (Ruff and Rizzino, 1986). Lyophilized ^{125}I -amyloid- $\beta_{(1-40)}$ was purchased from Bachem (Peninsula Laboratories, San Carlos, CA) and reconstituted in 1 ml of water, according to the manufacturer's recommendations. Aliquots were stored at -20°C and thawed only once prior to use.

PAGE and Western blotting

Plasma samples (2 μl of a 1:200 dilution) were analyzed by SDS-PAGE on 5–15% polyacrylamide gels under reducing or nonreducing conditions (Chu and Pizzo, 1993) and transferred to Immobilon-P membranes (Millipore). Membranes were blocked overnight in 5% (w/v) nonfat dry milk in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), and probed using a polyclonal α_2M -specific antibody (Carpenteria, CA) (1:18,000 in PBS-Tween; Dako), followed by anti-rabbit IgG-horseradish peroxidase (1:10,000 in PBS-Tween; Amersham Life Sci). Immunoblots were detected using ECL reagent (Amersham Pharmacia Biotech) and Kodak X-OMAT film.

Plasma samples (2 μl) were also analyzed on 4–15% polyacrylamide gels under nondenaturing conditions that optimize separation of α_2M conformation as previously described (Salvesen and Enghild, 1993). Gels were stained with Coomassie blue. Densitometry of Western blots and native gels was performed using Kodak Digital Science 1.0 (Kodak, Rochester, NY). Purified native and trypsin-treated α_2M and selected plasma samples were included in each gel as internal standards.

α_2M protease binding assay

The method of Ganrot, modified to a 96-well format, was used to determine the proteinase inhibitory capacity of α_2M in the plasma samples (Ganrot, 1966). This assay is based on the unique ability of α_2 -macroglobulins to bind and sterically shield proteinases from soybean trypsin inhibitor,

while still allowing access of the bound proteinases to small colorimetric substrates. It can be used in plasma and other mixed protein samples to distinguish α_2M from other trypsin inhibitors. Plasma samples were centrifuged at 1300 rpm in a microfuge for 20 min at 4°C , diluted 3/50 into 100 mM Tris-Cl, 20 mM CaCl_2 , pH 8.3, and added to 96-well plates such that 1.5 μl of plasma was added per well. A total of 1.5 μg of active trypsin (active-site titrated as described previously (Chase and Shaw, 1967)) was added to each well and allowed to incubate at 22°C for 5 min. Samples were then treated with 0.07 BAEE units of soybean trypsin inhibitor (SBTI, Sigma) for 5 min to quench the activity of unbound trypsin. α_2M -associated trypsin was detected with the chromogenic substrate, $N\alpha$ -benzoyl-DL-arginine *p*-nitroamide (2.4 nM), by following the absorbance kinetically at 405 nm in a plate reader (Molecular Devices). Simultaneous monitoring at 340 nm confirmed that there were no changes in turbidity to suggest clotting or precipitation during the assay. Samples were analyzed in triplicate. Standard curves were generated using purified α_2M , determined to be >95% in the native conformation by thiol titration on the day of the assay. Each sample was also analyzed without SBTI as a control. Substrate hydrolysis without SBTI was statistically greater than that observed when SBTI was added (paired *t* test, $P < 0.004$), indicating the presence of residual free trypsin after saturation of available plasma trypsin inhibitors.

Nondenaturing PAGE analysis of ligand-binding to α_2M

Equivalent amounts of native and methylamine-treated human plasma samples from AD patients and one CTL subject were diluted into PBS and incubated with 1 nM ^{125}I -A β or 0.1 nM ^{125}I -TGF- β_1 for 2 h at room temperature in siliconized tubes. The samples were separated by nondenaturing PAGE using the van Leuven gel system (Barrett et al., 1979), which preserves most noncovalent α_2M -growth factor interactions (Gonias et al., 1994). The gels were stained with Coomassie blue and dried. Radioligands bound to α_2M were detected using a PhosphorImager and analyzed with ImageQuant. Methylamine-activated, purified human α_2M was included in each assay as an internal control.

Statistics

Snedecor's *F* test, single-factor ANOVA, Student's two-tailed *t* test, and regression analysis were used to assess the effects of genotype and/or diagnosis on the data, using statistics packages in Microsoft Excel, SPSS 11.0, and GraphPad Prism. Equal volumes of plasma were analyzed in each experiment. The data were also normalized by total protein concentration for statistical analysis, yielding equivalent results. In addition, ligand binding results were

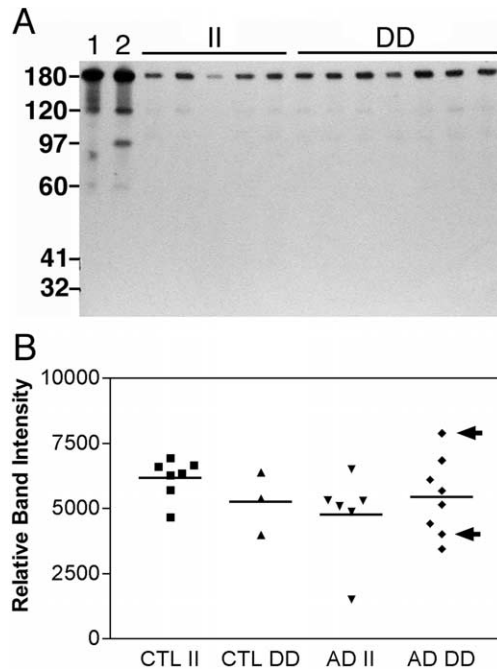


Fig. 1. SDS-PAGE and immunoblot analysis of α_2M in plasma samples. (A) Plasma samples analyzed under reducing conditions shows appropriate migration of the α_2M subunit at 180 kDa. Shown is a representative gel illustrating a subset of the 24 plasma samples. The genotypes of the plasma samples are indicated above the lanes. Lane 1 contains purified α_2M and lane 2 contains purified α_2M treated with a subsaturating amount of trypsin. Note that autolytic fragmentation results in minor bands of 120 and 60 kDa (lane 1). Truncation or cleavage at the bait region, which is located approximately at the center of each monomer, would result in migration at ~ 95 kDa (lane 2). (B) Densitometric analysis compiling data from all plasma samples analyzed via three independent gels, with each data point representing the averaged values of a single plasma sample. Band intensities from different blots were standardized using the densities of repeated standard samples. ANOVA revealed no significant differences between any of the groups, whether analyzed by plasma volume or normalized to plasma protein concentration. The two plasma samples showing altered TGF- β binding (see Fig. 3) are indicated by arrows.

standardized to account for α_2M content within each plasma sample, as determined by Western blot analysis.

Results

The major conformational and subunit structure of α_2M is preserved in DD plasma samples

α_2M is a homotetramer of approximately 720 kDa. When analyzed by SDS-PAGE under nonreducing conditions, α_2M from all of the samples showed the expected migration as a 360-kDa disulfide-linked dimer (not illustrated). Under reducing conditions, the intact 180-kDa α_2M subunit was observed (Fig. 1A). In agreement with previously published studies (Rogaeva et al., 1999), there was no shift in mobility of the α_2M subunit to suggest a major deletion or truncation

associated with the DD genotype. Moreover, there were no distinct proteolytic fragments identified, suggesting that α_2M^* generated by reaction with proteinases was cleared normally by LRP1. Densitometric analysis revealed no significant differences in α_2M band intensity by diagnosis and/or genotype (Fig. 1B). This was true whether analyzed by equivalent plasma volumes or when values were normalized to plasma protein concentrations.

The biological activities of α_2M are tightly regulated by its conformational state. Because minor alterations in the bait region sequence may result in conformational destabi-

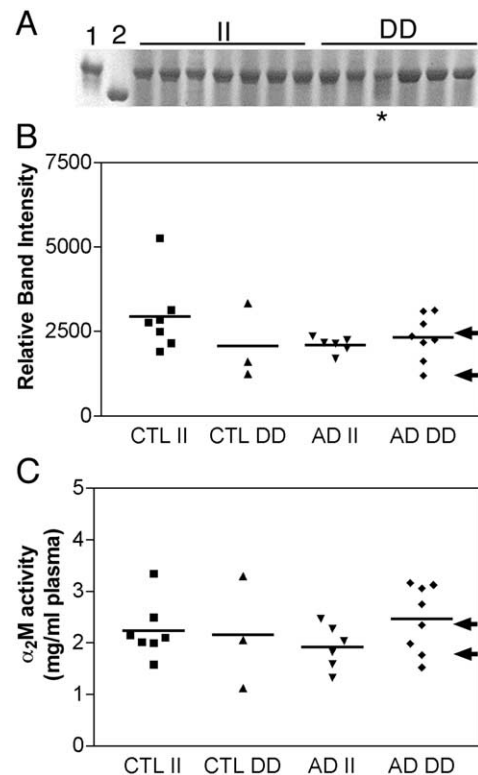


Fig. 2. Analysis of the functional α_2M conformation in plasma samples. (A) Coomassie-stained native polyacrylamide gel shows roughly equivalent amounts of native α_2M among samples. Shown is a representative gel illustrating a subset of the 24 plasma samples. The genotype is indicated above the lanes. Lane 1 contains purified α_2M and lane 2 contains purified α_2M treated with a saturating amount of trypsin. The asterisk indicates the only plasma sample (of 24) that showed a discernable faint α_2M^* band. (B) Densitometric analysis compiling data from three independent gels, with each data point representing the averaged values of a single plasma sample. Band intensities from different blots were standardized using the densities of repeated standard samples. ANOVA revealed no significant differences between any of the groups, whether analyzed by plasma volume or normalized to plasma protein concentration. The two plasma samples showing altered TGF- β binding (see Fig. 3) are indicated by arrows. (C) Proteinase inhibitory activity of plasma α_2M as assessed by capacity to entrap trypsin. Ganrot data were compiled from four independent experiments using the same batch of purified, titrated α_2M for the standard curves. ANOVA revealed no significant differences between the indicated groups, whether analyzed by plasma volume or normalized to plasma protein concentration. The two plasma samples showing altered TGF- β binding (see Fig. 3) are indicated by arrows.

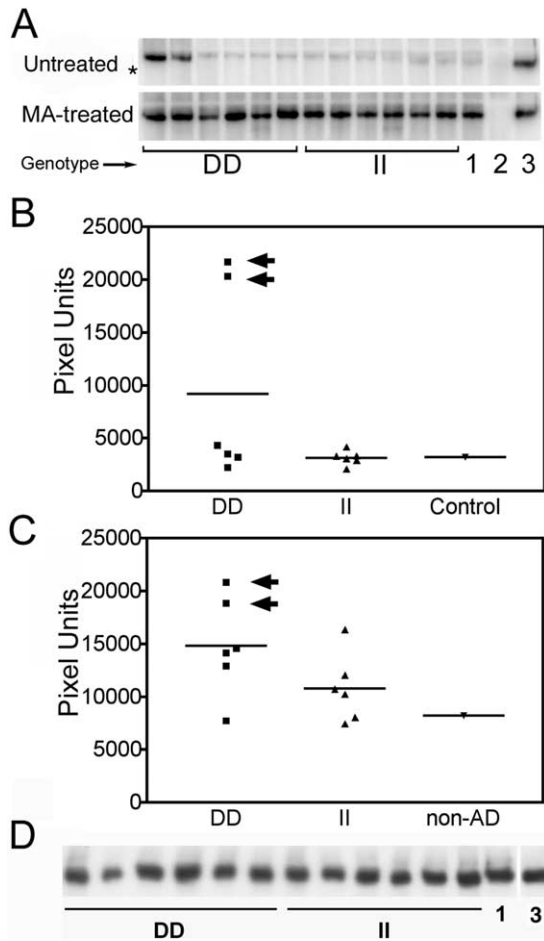


Fig. 3. Interaction of $^{125}\text{I-TGF-}\beta 1$ with plasma $\alpha_2\text{M}$ samples. (A) Representative autoradiograph of native and methylamine-treated plasma samples from AD patients incubated with 0.1 nM $^{125}\text{I-TGF-}\beta 1$ for 2 h and then subjected to nondenaturing PAGE. The genotypes are indicated beneath the lanes. The asterisk at the left indicates the position of migration of $\alpha_2\text{M}^*$. A non-AD, II control sample is included (lane 1). $^{125}\text{I-TGF-}\beta 1$ alone did not migrate near $\alpha_2\text{M}$ (lane 2). Methylamine-activated purified human $\alpha_2\text{M}^*$ ($\alpha_2\text{M}^*\text{-MA}$) is the positive control for migration of $\alpha_2\text{M}^*$ (lane 3). $^{125}\text{I-TGF-}\beta 1$ bound to all native samples. However, two of the DD-genotyped samples (arrows in B and C) bound significantly elevated levels compared to all other samples ($P = 0.01$). Methylamine treatment of the plasma samples to form $\alpha_2\text{M}^*$ resulted in increased $^{125}\text{I-TGF-}\beta 1$ in all the other samples, eliminating these differences ($P = 0.40$). B and C show the densitometric analysis of $^{125}\text{I-TGF-}\beta 1$ binding to $\alpha_2\text{M}$ and to $\alpha_2\text{M}^*$ in methylamine treated plasma, respectively. Each data point represents $^{125}\text{I-TGF-}\beta 1$ bound to $\alpha_2\text{M}$ in plasma from a single patient with the indicated genotype and is the average of data obtained from four independent gels ($r = 0.92\text{--}0.99$). Densitometric data are standardized by $\alpha_2\text{M}$ concentration and relative to the internal $\alpha_2\text{M}^*\text{-MA}$ control. (D) Western blot analysis of native $\alpha_2\text{M}$ samples as loading control for $^{125}\text{I-TGF-}\beta 1$ binding studies. See also Table 1 for densitometric analysis of band intensities and statistical analysis.

lization, we analyzed the plasma samples using nondenaturing PAGE, which resolves native $\alpha_2\text{M}$ from the faster migrating $\alpha_2\text{M}^*$. Fig. 2A is a representative Coomassie-stained, nondenaturing gel. Although occasional samples of both genotypes showed low levels of intermediate forms, only 1 of the 24 plasma samples showed a barely detectable

$\alpha_2\text{M}^*$ band (asterisk). Densitometry indicated no significant differences by diagnosis and/or genotype (Fig. 2B).

Capacity of $\alpha_2\text{M}$ to entrap trypsin is preserved in DD plasma samples

Given the location of the mutation between exons encoding the bait region for proteinase cleavage and entrapment, we determined the trypsin inhibitory capacity of the $\alpha_2\text{M}$ in the plasma samples. The Ganrot assay was used to quantitate levels of $\alpha_2\text{M}$ in its native, proteinase-inhibitory conformation, based upon the unique mechanism of proteinase inhibition by $\alpha_2\text{M}$. As shown in Fig. 2C, the total proteinase inhibitory capacity of $\alpha_2\text{M}$ did not differ significantly among plasma samples categorized by genotype and/or diagnosis. There was a significant correlation between the proteinase-binding activity of the $\alpha_2\text{M}$ in each sample and the band intensity of the native conformation of $\alpha_2\text{M}$ observed by Coomassie gel analysis ($r^2 = 0.66$, $P < 0.0005$). This indicates that the $\alpha_2\text{M}$ in the plasma samples not only migrated predominantly as native $\alpha_2\text{M}$, but also displayed normal trypsin inhibitory function.

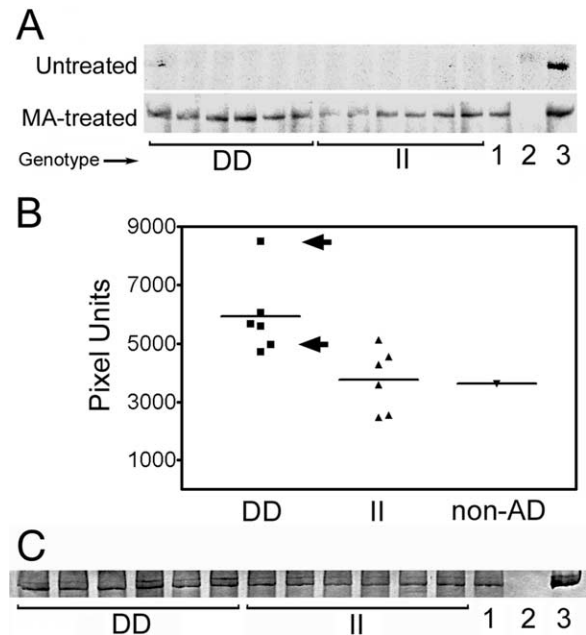


Fig. 4. Interaction of $^{125}\text{I-A}\beta$ with plasma $\alpha_2\text{M}$ samples. Radioligand binding experiments were performed by incubating native and methylamine-treated plasma samples with 1 nM $^{125}\text{I-A}\beta$ for 2 h. These samples were then subjected to nondenaturing PAGE and phosphorimager analysis. (A) Representative radiograph in which $^{125}\text{I-A}\beta$ binds only to the activated conformation of $\alpha_2\text{M}$. Lanes 1–3 are as described in Fig. 3, above. (B) In this system, there is a moderate, but significant increase in $^{125}\text{I-A}\beta$ binding to methylamine-activated $\alpha_2\text{M}$ derived from patients with the DD genotype compared to II samples ($P = 0.007$). Each data point represents the average of four experiments ($r = 0.84\text{--}0.92$). (C) Coomassie-stained gel of MA-treated $\alpha_2\text{M}$ samples as loading control for $^{125}\text{I-A}\beta$ binding studies. See also Table 1 for densitometric analysis of band intensities and statistical analysis.

Table 1
Properties of the two DD samples with increased TGF- β 1 binding to native plasma α_2 M

	DD No. 1	DD No. 2	Mean DD	Mean II
Protein assay for Ganrot (mg/ml)	107.9	98.3	116.7 \pm 19	99.7 \pm 24
α_2 M (mg/ml plasma)	2.4	1.8	2.4 \pm 0.75	2.1 \pm 0.51
α_2 M (μ g/mg prot)	18.3	16.4	21.1 \pm 8	22.1 \pm 7
Protein assay for gels (mg/ml)	119.7	100	113.9 \pm 13	106.6 \pm 19
Reduced band intensity	7897	4021	5396 \pm 1364	5524 \pm 1421
Reduced band/ μ g prot loaded	6599	4019	4813 \pm 1349	5310 \pm 1632
Native band intensity	2446	1242	2330 \pm 239	2644 \pm 261
Native band/ μ g prot loaded	270.4	167.1	335.4 \pm 125	399.7 \pm 107
Coomassie gel intensity for ligand blots	80.9	90.9	92.1 \pm 2.6	96.8 \pm 4.8
Western blot for ligand blots	4.6	2.8	4.4 \pm 0.4	4.4 \pm 0.2
TGF- β binding to native*	20324	21680	9204 \pm 3745	3147 \pm 236
TGF- β binding to MA-treated	12904	20831	14830 \pm 1888	10430 \pm 1167
A β -binding to native	ND	ND	ND	ND
A β -binding to MA-treated**	4975	8496	5921 \pm 551	3743 \pm 378

Note. Single-factor ANOVA followed by pairwise comparisons using Students' *t* test indicates * $P < 0.001$ for the first two DD samples of Fig. 3A compared to other DD samples and to the II samples, but no significant differences are observed between the DD group as a whole and the II group in terms of TGF- β 1 binding to native plasma samples; ** $p = 0.007$ for A β binding to MA-treated DD plasma vs MA-treated II plasma. Analysis by single-factor ANOVA indicates no other significant differences. ND, not detected

¹²⁵I-TGF- β 1 binding to native α_2 M is significantly increased in some AD-DD plasma samples

The TGF- β binding site is located just C-terminal to the bait region sequence of α_2 M (Gonias et al., 2000). Binding of radiolabeled TGF- β 1 to either native α_2 M or methylamine-treated α_2 M* was analyzed (Fig. 3A). Although TGF- β 1 characteristically binds with higher affinity to the faster migrating α_2 M* conformation (asterisk and lane 3), in untreated plasma samples, two of the six AD-DD samples showed increased ¹²⁵I-TGF- β 1 binding to slower migrating α_2 M forms (Fig. 3A and B). α_2 M in the other AD-DD plasma samples bound growth factor equivalently to the α_2 M in the II plasma samples. The increased binding of ¹²⁵I-TGF- β 1 to the two native DD samples cannot be explained by uneven loading of samples (Fig. 3D). Moreover, the same results were obtained from four independent ligand binding experiments ($r = 0.92$ – 0.99). As indicated by arrows in Figs. 1B, 2B, 2C, 3C, these two samples did not differ significantly from the II plasma samples in terms of any other measured parameters (Table 1), suggesting that the effect was probably not due to preexisting fast forms in these two native samples.

α_2 M* in methylamine-treated plasma samples demonstrated the characteristic increase in electrophoretic mobility. Under these conditions, the remaining samples of both genotypes showed the expected increase in ¹²⁵I-TGF- β 1 binding, and significant differences among the samples were no longer observed (Fig. 3C).

Increased binding of ¹²⁵I-A β to α_2 M is observed in methylamine-treated DD plasma samples*

¹²⁵I-A β binding to native and methylamine-treated plasma α_2 M was determined by nondenaturing PAGE anal-

ysis (Fig. 4A). ¹²⁵I-A β did not bind to native α_2 M in any of the untreated plasma samples (Fig. 4A, top). Binding to methylamine-treated purified human α_2 M* was observed, as anticipated (lane 3). After the plasma samples were treated with methylamine to convert the α_2 M to its α_2 M* conformation, all plasma samples bound ¹²⁵I-A β (Fig. 4A, bottom). The α_2 M* in DD-genotyped samples bound more ¹²⁵I-A β than the α_2 M* in II samples under these conditions (Fig. 4B). Although differences between DD and II binding of A β were statistically significant ($P < 0.05$ by Student's two-tailed *t* test), there are no significant differences between DD and II samples in terms of Western blot band intensities, Coomassie stain intensities, total protein concentration, SDS-gel band intensities, native gel band intensities, or Ganrot assay values, regardless of whether data were normalized by plasma volume or total protein concentrations (Table 1). As the A β binding site is located in the C-terminal portion of the α_2 M subunit (Mettenburg et al., 2002), we cannot exclude the possibility that A β binding may also be influenced by the codon 1000 polymorphism. However, there were no trends in ¹²⁵I-A β binding when II samples were analyzed by the codon 1000 polymorphism.

Discussion

α_2 M is an abundant, multifunctional binding protein that regulates proteolytic, inflammatory, and trophic processes. Proteinase- or methylamine-induced conformational change to α_2 M* results in altered growth-factor binding affinities (Gonias et al., 1994) and exposure of C-terminal residues that mediate interactions with A β (Mettenburg et al., 2002) and the α_2 M-receptor, LRP1 (Sottrup-Jensen et al., 1986). In this study, we found no significant effects of the intronic splice-acceptor site deletion upon overall tertiary and qua-

ternary assembly of the protein nor its ability to entrap trypsin. Although we did not directly examine conformational regulation of receptor binding sites, the fact that the large majority of $\alpha_2\text{M}$ in each plasma sample migrated with unchanged mobility when analyzed by native PAGE suggests that LRP1-mediated clearance of $\alpha_2\text{M}^*$ is also intact. This interpretation is supported by the absence of proteinase-cleaved subunits in Western blots. It should be noted that the major source of plasma $\alpha_2\text{M}$ is the liver, and the possibility of brain-specific splicing alterations was not addressed in this study. However, other groups have not observed evidence of exon skipping in mRNA isolated from liver or brain (Rogaeva et al., 1999; Gibson et al., 2000). We therefore conclude that effects of the deletion mutation on $\alpha_2\text{M}$ structure, if present, are subtle. However, AD-DD plasma samples did display alterations in TGF- β 1 and A β binding, suggesting possible mechanisms by which $\alpha_2\text{M}$ could influence multifactorial processes contributing to AD pathogenesis.

We found that two of the six DD-genotyped AD plasma samples available for radioligand binding experiments demonstrated markedly elevated binding of TGF- β to a slower migrating form of $\alpha_2\text{M}$. The observations cannot be explained by increased loading of $\alpha_2\text{M}$, as loading controls indicate a nonsignificant trend for less $\alpha_2\text{M}$ loaded in these two samples. The enhanced binding is also not likely to be due simply to storage-induced conversion to $\alpha_2\text{M}^*$ for the following reasons: there was no A β binding to these same plasma samples until after methylamine activation; these plasma samples possessed equivalent trypsin inhibitory capacity to other samples; and II plasma samples which contained intermediate forms did not show increased TGF- β 1 binding. We cannot, of course, exclude the possibility of some other factor such as oxidation or posttranslational modification being the factor involved, particularly given the limited sample size, but these two samples are no different from the II samples in other parameters examined. The difference in TGF- β 1 binding was largely dissipated by conformational activation of plasma to form $\alpha_2\text{M}^*$. Although the sample size is small, the potential biologic impact of enhanced TGF- β 1 binding to native or intermediate $\alpha_2\text{M}$ forms is likely to be significant due to receptor targeting considerations. While $\alpha_2\text{M}^*$ along with any bound proteins is rapidly cleared by LRP1, native or intermediate forms of $\alpha_2\text{M}$ would be expected to survive much longer and preferentially compete with other TGF- β -binding proteins, including the TGF- β s receptor signaling.

Although there was no evidence of major structural alterations in the DD samples relative to the II samples, small alterations in the sequence near the bait region domain and growth factor binding domain cannot be ruled out as a source of increased growth factor binding. Although DD- $\alpha_2\text{M}$ was fully functional as a trypsin inhibitor, saturating levels of trypsin were used to assess inhibitory capacity, and these conditions would not detect more subtle alterations in

affinity or rate. Moreover, trypsin can cleave the bait region at several sites (Sottrup-Jensen et al., 1981), so small changes in the bait region primary sequence might not affect its interaction with $\alpha_2\text{M}$. Hypothetically, sequence changes could result from increased use of nearby alternative splice acceptor sites that may be unmasked by the deletion, even if exon skipping does not occur (Berget, 1995). Differences in the efficiency of the splicing machinery and or other modulating effects of genetic background may regulate percentage use of the usual or alternative sites.

Even in the absence of potential primary sequence changes, increased TGF- β 1 binding may also result from posttranslational or oxidative modifications or to conformational alterations that indirectly benefit TGF- β 1 binding to $\alpha_2\text{M}$. Alterations in synthesis, half-life, or turnover may promote differences in posttranslational modifications. An interesting form of $\alpha_2\text{M}$ can be generated in vitro using chemical modifications (Webb and Gonias, 1997). This derivative is similar in conformation to the native protein, yet demonstrates higher binding affinities for both TGF- β 1 and TGF- β 2 compared with both native $\alpha_2\text{M}$ and $\alpha_2\text{M}^*$. Although the occurrence of similar intermediate forms is unconfirmed in vivo, minimally modified $\alpha_2\text{M}$ forms may have significantly altered growth-factor-binding activities.

In parallel experiments, the interaction of ^{125}I -A β with $\alpha_2\text{M}$ in plasma samples was investigated. There was no ^{125}I -A β binding to $\alpha_2\text{M}$ in any of the plasma samples that had not been treated with methylamine (Fig. 4A). These data suggest that possible $\alpha_2\text{M}$ conformational alterations associated with increased TGF- β 1 binding in native, untreated plasma do not extend to exposure of C-terminal sequences that mediate other important interactions. This observation also confirms that little disruption to the structure of $\alpha_2\text{M}$ in the plasma samples was incurred by storage at -80°C . However, upon activation with methylamine, $\alpha_2\text{M}^*$ in DD-genotyped plasma samples bound a significantly greater amount of ^{125}I -A β than $\alpha_2\text{M}^*$ from II samples (Fig. 4B), even when standardized to $\alpha_2\text{M}$ concentration in the individual plasma samples. These altered binding interactions involving A β and TGF- β 1 may have important consequences in modulating the pattern and severity of pathological changes associated with AD, particularly as TGF- β 1 overexpression appears to favor deposition of vascular rather than parenchymal amyloid (Wyss-Coray et al., 2001).

In summary, we found that plasma $\alpha_2\text{M}$ from AD patients homozygous for the DD allele showed enhanced interactions with TGF- β 1 and A β , despite showing no significant alterations in subunit structure, quaternary conformation, or trypsin inhibitory capacity. The biochemical basis for these differences is not understood and should be the focus of future investigation given its potential relevance to pathophysiologic mechanisms regulating deposition of vascular and parenchymal amyloid in AD patients.

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