Journal of Neurochemistry, 2005, **93**, 53–62 doi:10.1111/j.1471-4159.2004.02995.x

A chemically modified preparation of α2-macroglobulin binds β-amyloid peptide with increased affinity and inhibits Aβ cytotoxicity

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Abstract

Macromolecules that bind β-amyloid peptide (Aβ) and neutralize Aβ cytotoxicity offer a promising new approach for treating Alzheimer's disease. When the plasma protein, α2-macroglobulin (α2M), is treated with methylamine (α2M-MA), it undergoes conformational change and acquires Aβ-binding activity. In this study, we demonstrate that a chemically stabilized preparation of human α2M conformational intermediates (α2M-*cis*-Pt/MA) binds Aβ with greatly increased affinity, compared with α2M-MA. α2M-*cis*-Pt/MA was generated by reacting α2M with the protein cross-linking reagent, *cis*-Pt, followed by methylamine. Increased Aβ-binding to α2M-*cis*-Pt/MA was demonstrated by co-migration of radio-iodinated proteins in non-denaturing PAGE, chemical cross-linking, and co-immunoprecipitation. The apparent K_D for Aβ-binding to α2M-*cis*-Pt/MA was decreased 10-fold, compared with α2M-MA, to 29 nM. Native α2M demonstrated negligible Aβ-binding, as anticipated. α2M-*cis*-Pt/MA markedly counteracted Aβ-induced C6 cell apoptosis. Essentially complete inhibition of apoptosis was observed even when the Aβ was present at fourfold molar excess to α2M-*cis*-Pt/MA. Under equivalent conditions, α2M-MA inhibited apoptosis by 25 ± 6%. When Aβ and α2M-*cis*-Pt/MA were added to human plasma *in vitro*, significant binding was detected. No binding was observed when an equivalent concentration of native α2M or α2M-MA was added to plasma. We propose that α2M-*cis*-Pt/MA is a novel alternative to Aβ-specific antibodies, for studying the efficacy of Aβ-binding agents *in vitro* and *in vivo*.

Keywords: Alzheimer's disease, β-amyloid peptide, low-density lipoprotein receptor-related protein, α2-macroglobulin, transforming growth factor-β, tumor necrosis factor-α.

J. Neurochem. (2005) **93,** 53–62.

Specific mutations in amyloid precursor protein or in the presenilin genes, PS1 and PS2, promote production of β-amyloid peptide (Aβ; Citron *et al*. 1992, 1997; Johnston *et al*. 1994; Borchelt *et al*. 1996; Sudoh *et al*. 1998) and confer high risk for early onset Alzheimer's disease (AD; Tanzi *et al*. 1996). In sporadic late-onset AD, accumulation of Aβ may be related to failure of mechanisms that maintain Aβ homeostasis in the brain. Increased Aβ production, insufficient metabolism, and altered transport of Aβ across the blood–brain barrier may result in Aβ accumulation and contribute to the pathogenesis of the disease (Selkoe 1994; Sisodia and Price 1995).

In mouse models of AD, immunization with Aβ, to generate Aβ-specific antibodies, reduces plaque burden (Janus *et al*. 2000), decreases other AD-like pathologies (Schenk *et al*. 1999) and reverses cognitive impairment and memory deficits (Janus *et al*. 2000; Dodart *et al*. 2002). Macromolecules with Aβ-binding activity that are administered into the blood, including Aβ-specific antibodies and gelsolin, have been reported to function as 'Aβ sinks'. These agents shift the balance in Aβ transport away from the CNS and decrease the amount of Aβ deposited in plaques in the

Received August 22, 2004; revised manuscript received November 14, 2004; accepted November 16, 2004.

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Abbreviations used: Aβ, β-amyloid peptide; AD, Alzheimer's disease; BS^3, bis(sulfosuccinimidyl) suberate; BSA, bovine serum albumin; *cis*-Pt, *cis*-dichlorodiammineplatinum-II; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; LRP-1, low-density lipoprotein receptor-related protein-1; α2M, α2-macroglobulin; MA, methylamine; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

brain (Bard *et al*. 2000; DeMattos *et al*. 2001, 2002; Matsuoka *et al*. 2003).

α2-Macroglobulin (α2M) is a 718-kDa glycoprotein that functions as an extracellular protease inhibitor and as a growth factor carrier (LaMarre *et al*. 1991). The function of α2M, as a growth factor carrier, is regulated by conformational change, which may be induced by reaction with proteases or methylamine (α2M-MA). α2M that has undergone conformational change also binds Aβ and thereby inhibits Aβ fibril formation (Du *et al*. 1997; Hughes *et al*. 1998), neutralizes the cytotoxic activity of Aβ *in vitro* (Du *et al*. 1998; Van Uden *et al*. 2000; Fabrizi *et al*. 2001), and clears Aβ from extracellular spaces (Narita *et al*. 1997; Qiu *et al*. 1999; Kang *et al*. 2000). Clearance of Aβ depends on the function of the endocytic α2M receptor, low-density lipoprotein receptor-related protein-1 (LRP-1), which mediates cellular uptake and catabolism of activated α2M, with associated proteins, including growth factors and Aβ (LaMarre *et al*. 1991; Narita *et al*. 1997).

When α2M is treated with the bifunctional protein-cross-linking reagent, *cis*-dicholorodiammineplatinum-II (*cis*-Pt), and then with methylamine, in sequence, a preparation of stable conformational intermediates (α2M-*cis*-Pt/MA) is generated (Gonias and Pizzo 1981). In electron microscopy studies, α2M-*cis*-Pt/MA demonstrates structural heterogeneity; however, characteristics of both native α2M and α2M that has undergone complete conformational change are observed (Gonias and Figler 1989; Marshall *et al*. 1992). Most importantly, α2M-*cis*-Pt/MA binds specific cytokines/growth factors, including transforming growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α), with increased affinity, suggesting that the binding site(s) for these proteins are not optimally exposed or oriented in native α2M or in α2M that has undergone complete conformational change (Webb and Gonias 1997, 1998). The binding sites for TGF-β1 and Aβ have been identified in the primary structure of human α2M (Hughes *et al*. 1998; Webb *et al*. 1998; Mettenburg *et al*. 2002). These sites are entirely distinct; Aβ does not bind to the TGF-β1-binding site and TGF-β1 does not bind to the Aβ-binding site (Mettenburg *et al*. 2002).

The goal of the present study was to characterize the interaction of Aβ with α2M-*cis*-Pt/MA. We report that α2M-*cis*-Pt/MA demonstrates greatly increased Aβ-binding activity, compared with any other previously characterized α2M derivative. The K_D for Aβ-binding to α2M-*cis*-Pt/MA is 29 nM, which is decreased by a factor of 10 compared with α2M-MA. As a result of this increase in binding affinity, α2M-*cis*-Pt/MA demonstrates significantly enhanced capacity to neutralize the cytotoxic activity of Aβ *in vitro*. α2M-*cis*-Pt/MA can be generated in large quantities and retains the ability to bind to LRP-1 (Gonias and Pizzo 1983). We propose that α2M-*cis*-Pt/MA is a promising reagent for further studies on Aβ neutralization *in vivo*.

Methods

Proteins and reagents

Native α2M was purified from human plasma by the method of Imber and Pizzo (1981). α2M-MA was prepared by reacting α2M with 200 mM methylamine–HCl in 50 mM Tris–HCl, pH 8.2 for 12 h at 22°C. Excess methylamine was removed by dialysis against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). To generate α2M-*cis*-Pt/MA, native α2M was reacted with 1.6 mM *cis*-Pt for 6 h at 37°C and then with methylamine, as previously described (Gonias and Figler 1989). Under these conditions, platinum incorporation into α2M is approximately 4 mol/mol subunit (Gonias and Figler 1989).

$A\beta_{(1-40)}$ was purchased from Bachem (Torrance, CA, USA), dissolved in hexafluoroisopropanol, aliquoted, and dried for storage at − 20°C. The most recently studied $A\beta_{(1-40)}$ lot number was T-20824. Hexafluoroisopropanol abolishes aggregates so that homogeneous solutions of soluble monomeric Aβ are generated when the dried preparation is re-dissolved (Walsh *et al*. 1997; Dahlgren *et al*. 2002). $A\beta_{(40-1)}$ was also purchased from Bachem (lot number 0552342). For experiments, Aβ was re-dissolved in dimethyl sulphoxide (DMSO) and then diluted extensively in 50 mM Tris–HCl, pH 7.4 or in Dulbecco's modified Eagle medium (DMEM). $A\beta_{1-40}$ was radio-iodinated to a specific activity of 5–20 μCi/μg, in 50 mM Tris–HCl, pH 7.4, using Iodobeads (Pierce, Rockford IL, USA). Free $Na^{125}I$ was removed by chromatography on PD10 columns (Amersham Biosciences, Piscataway, NJ, USA). TGF-β1 was purchased from R & D Systems (Minneapolis, MN, USA) and radio-iodinated using chloramine-T, as previously described (Webb *et al*. 1998). ^{125}I-TNF-α and bis(sulfosuccinimidyl) suberate (BS^3) were purchased from Pierce. Cell culture media and heat-inactivated fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA).

Non-denaturing PAGE analysis of Aβ-binding to α2M

The interaction of α2M with Aβ is primarily non-covalent (Mettenburg *et al*. 2002). Therefore, non-denaturing polyacrylamide gel electrophoresis (PAGE) was used to compare the binding of Aβ to different forms of α2M. Non-denaturing PAGE preserves non-covalent interactions, although during electrophoresis, reversible complexes may partially dissociate (Gonias *et al*. 1994). ^{125}I-Aβ (1 nM) was incubated with different α2M derivatives (0.1 μM) in 50 mM Tris–HCl, pH 7.4, 0.1 mg/mL bovine serum albumin (BSA) for up to 4 h at 22°C. NaCl was omitted from the buffer to inhibit formation of insoluble fibrils/unordered aggregates (Dahlgren *et al*. 2002). Samples were subjected to non-denaturing PAGE, using the method described by Van Leuven *et al*. (1981a). The time of electrophoresis was limited to 90 min to minimize dissociation of complexes. α2M-associated radioactivity was determined by PhosphorImager analysis, using ImageQuant software. In the absence of α2M, ^{125}I-Aβ did not migrate near the α2M bands. The equivalent protocol was executed to assess the binding of ^{125}I-TNF-α to different forms of α2M.

Analysis of ^{125}I-Aβ-binding to α2M by co-immunoprecipitation

Various concentrations of native α2M, α2M-MA or α2M-*cis*-Pt/MA were coupled to rabbit anti-human α2M polyclonal antibody (Dako, Carpenteria, CA, USA) immobilized on Protein-A Sepharose beads (Amersham). The coupling time was 1 h at 22°C. The beads were

washed and then incubated with ^{125}I-Aβ (10 nM), alone or in the presence of a 50- to 200-fold molar excess of cold Aβ, for 90 min at 22°C in 50 mM Tris–HCl pH 7.4, 0.1 mg/mL BSA. After washing the beads three times again, the amount of ^{125}I-Aβ associated with the beads was determined by measuring radioactivity in a 1470 Wizard Gamma Counter (Perkin Elmer, Foster City, CA, USA).

Immunoblot analysis of Aβ-binding to α2M

To assess Aβ-binding to α2M without radio-iodination, we developed an immunoblotting method. Different forms of α2M were incubated with Aβ. The samples were then divided in two. One aliquot was denatured in sodium dodecyl sulfate (SDS), without further modification, so that only covalent interactions would be preserved. The other aliquot was treated with the homobifunctional cross-linker, BS^3 (5 mM) for 1 min. Pulse-exposure to BS^3 covalently stabilizes a fraction of the non-covalent Aβ-α2M complex. Because BS^3 is reacted with the protein mixture under pseudo first-order conditions, the fraction of covalently stabilized complex is constant and independent of the total amount of non-covalent complex (Crookston *et al*. 1994). Cross-linking was terminated by acidification with HCl and immediate addition of buffered SDS (1.0% v/v).

BS^3-cross-linked and vehicle (water)-treated samples were subjected to 5% SDS–PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% milk in PBS containing 0.1% Tween-20 and then probed with Aβ-specific monoclonal antibody 6E10 (Signet Laboratories, Dedham, MA, USA), diluted 1 : 5000 in blocking solution, for 12 h at 4°C. The membranes were washed extensively and then incubated with horseradish peroxidase-conjugated anti-mouse secondary IgG (1 : 5000, Amersham-Pharmacia, Piscataway, NJ, USA). Membranes were analyzed by enhanced chemiluminescence and densitometry. Some membranes were also probed for α2M using rabbit polyclonal α2M-specific IgG (Dako).

Binding affinity determinations by competition assay

^{125}I-Aβ (1.0 nM) and ^{125}I-TGF-β1 (1.0 nM) were incubated with combinations of α2M-*cis*-Pt/MA (50 nM) and α2M-MA (0.05–0.5 μM) for 4 h in 50 mM Tris–HCl, pH 7.4, 0.1 mg/mL BSA. Samples were pulse-exposed to BS^3 for 1 min and then subjected to SDS–PAGE under non-reducing conditions. α2M-MA migrated as a disulfide-linked dimer (360-kDa), as determined by Coomassie-staining, as anticipated (LaMarre *et al*. 1991). α2M-*cis*-Pt/MA migrated as tetramers (720 kDa) due to the intersubunit covalent cross-links. ^{125}I-Aβ and ^{125}I-TGF-β1, which were associated with the two forms of α2M, migrated in the same bands. Radioligand association with α2M-MA and α2M-*cis*-Pt/MA was determined, for each coincubation, by PhosphorImager analysis. A small correction was applied in some experiments, to account for low levels of radioligand that migrated with α2M-MA in the tetramer band. Results were plotted according to the following equation in the form $y = mx$, to estimate the K_D for the binding of the radioligand (L*) to α2M-*cis*-Pt/MA using Graphpad Prism 3.0:

$$[L^*\text{-}\alpha2M\text{-}cis\text{-}Pt/MA]/[L^*\text{-}\alpha2M\text{-}MA] = (1/K_D1) \times K_D2[\alpha2M\text{-}cis\text{-}Pt/MA_f]/[\alpha2M\text{-}MA_f]$$

[L*-α2M-*cis*-Pt/MA] is the amount of radioligand detected in complex with α2M-*cis*-Pt/MA and [L*-α2M-MA] is the amount of radioligand detected, in the same incubation, in association with α2M-MA. [α2M-MA$_f$] and [α2M-*cis*-Pt/MA$_f$] are the concentrations of free α2M-MA and α2M-*cis*-Pt/MA, respectively. K_D1 is the equilibrium dissociation constant for the binding of radioligand to α2M-*cis*-Pt/MA (the value to be determined). K_D2 is the dissociation constant for the binding of radioligand to α2M-MA. For TGF-β1, K_D2 is 80 nM (Crookston *et al*. 1994). For Aβ, K_D2 is 290 nM (Mettenburg *et al*. 2002). This treatment assumes that the BS^3-cross-linking efficiency is equivalent for α2M-MA and α2M-*cis*-Pt/MA (Crookston *et al*. 1994).

Effects of α2M on the cytotoxic activity of Aβ

To study the cytotoxic activity of Aβ and the effects of different forms of α2M on this cytotoxicity, we used the Cell Death Detection ELISA Plus kit (Roche, Indianapolis, IN, USA), which measures intracytoplasmic histone-associated DNA fragments. Aβ$_{(1-40)}$ and Aβ$_{(40-1)}$ were dissolved in DMSO and then diluted extensively into DMEM with 10% FBS, with or without α2M-MA or α2M-*cis*-Pt/MA, as indicated. The final Aβ concentration was 4 μM. In control incubations, vehicle (DMSO) was added instead of Aβ. In order to generate cytotoxic Aβ assemblies, solutions were pre-incubated for 24 h at 37°C, in a 5% CO_2 atmosphere. C6 rat glioma cells were purchased from the ATCC, plated at a density of 10 000 cells/well in 96-well plates, and cultured for 24 h. The cells were then treated with Aβ- or vehicle-containing solutions for 36 h. The medium was gently aspirated and the cells were processed for the ELISA assay, as described by the manufacturer. Reported results are in the units, Absorbance at 405 nm (mean ± SEM, $n = 4$).

Binding of Aβ to α2M in human plasma

^{125}I-Aβ (1 nM) was incubated with 20% human plasma (v/v), diluted into 50 mM Tris–HCl, pH 7.4 and supplemented with native α2M, α2M-MA, or α2M-*cis*-Pt/MA (0.1 μM), for 4 h at 22°C. The estimated concentration of native α2M in 20% plasma is 0.6 μM (LaMarre *et al*. 1991). Samples were subjected to non-denaturing PAGE for 90 min. The gels were dried and subjected to PhosphorImager analysis using Quantity One software (Bio-Rad, Hercules, CA, USA).

Results

α2M-*cis*-Pt/MA demonstrates increased ^{125}I-Aβ-binding activity

α2M that reacts with proteases or with methylamine undergoes conformational change and, as a result, demonstrates increased mobility by non-denaturing PAGE (Barrett *et al*. 1979; Van Leuven *et al*. 1981a). The Coomassie-stained gel in Fig. 1 shows the increase in mobility of α2M-MA, compared with native α2M. Treatment of α2M with *cis*-Pt, prior to methylamine, almost entirely blocked the shift in electrophoretic mobility. This effect has been previously attributed to the ability of *cis*-Pt to prevent α2M from

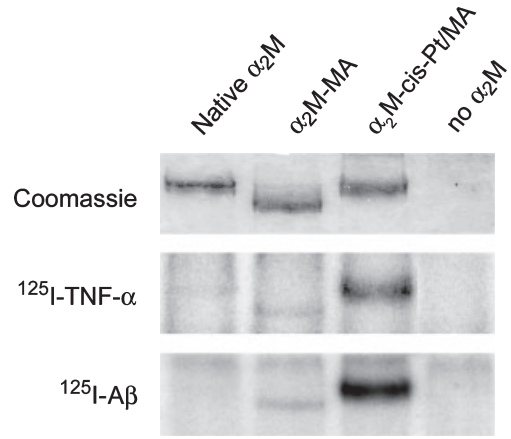

Fig. 1 Non-denaturing PAGE analysis of ligand-binding to α2M-*cis*-Pt/MA. Native α2M, α2M-MA, and α2M-*cis*-Pt/MA (0.1 μM) were incubated with radio-iodinated TNF-α (1.0 nM) or Aβ (1.0 nM) for 4 h. The reaction mixtures were then subjected to non-denaturing PAGE. The Coomassie-stained gel in the top panel compares the mobilities of the various α2M species. ^{125}I-TNF-α binding was determined by PhosphorImager analysis and is shown in the middle panel. ^{125}I-Aβ-binding was also determined by PhosphorImager analysis and is shown in the bottom panel.

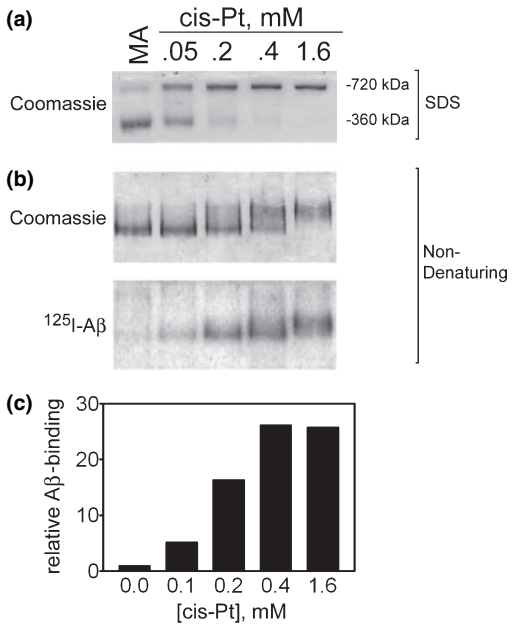

Fig. 2 The effects of *cis*-Pt concentration on α2M conformational change and Aβ-binding. Increasing concentrations of *cis*-Pt (0.05–1.6 mM) were incubated with α2M for 6 h prior to reaction with methylamine. (a) These samples and α2M-MA (MA) were subjected to SDS–PAGE under non-reducing conditions (the concentration of *cis*-Pt is shown). α2M was detected by Coomassie blue-staining. (b) The same preparations of α2M (0.1 μM) were incubated with ^{125}I-Aβ (1.0 nM) for 4 h. To detect binding, we performed non-denaturing PAGE. α2M was demonstrated by Coomassie blue-staining (upper panel) and bound ^{125}I-Aβ was detected by PhosphorImager analysis (lower panel). (c) The results shown in panel B were subjected to ImageQuant analysis. Binding is expressed relative to that observed with α2M-MA.

undergoing complete conformational change (Gonias and Pizzo 1981; Gonias and Figler 1989).

We incubated ^{125}I-TNF-α with equivalent concentrations of native α2M, α2M-MA or α2M-*cis*-Pt/MA and assessed binding based on co-migration of radioactivity with the α2M bands. α2M-*cis*-Pt/MA bound greatly increased amounts of ^{125}I-TNF-α, compared with the two other forms of α2M, confirming our previous observation (Webb and Gonias 1998).

Next, we utilized the same technique to compare the binding of ^{125}I-Aβ to native α2M, α2M-MA, and α2M-*cis*-Pt/MA. Aβ bound to α2M-MA but not to native α2M, confirming our previous results (Mettenburg *et al*. 2002). ^{125}I-Aβ also bound to α2M-*cis*-Pt/MA and the level of binding was substantially increased, compared with either previously characterized form of α2M. In order to adequately demonstrate the increase in ^{125}I-Aβ-binding to α2M-*cis*-Pt/MA, a short PhosphorImager exposure time was necessary, explaining why binding of ^{125}I-Aβ to α2M-MA appears low in Fig. 1, compared with our previously published figures (Mettenburg *et al*. 2002).

The binding site for Aβ has been localized to a single sequence in the α2M subunit (Hughes *et al*. 1998; Mettenburg *et al*. 2002); however, the binding site for TNF-α has not been identified. We examined ^{125}I-TNF-α-binding to α2M-*cis*-Pt/MA in the presence of increasing concentrations of Aβ (0.5–2.0 μM). No competition was observed (results not shown), suggesting that TNF-α does not bind directly to the Aβ-binding site in α2M.

In our standard preparation of α2M-*cis*-Pt/MA, platinum incorporation into α2M is limited (about 4 mol Pt/mol α2M subunit); however, the extent of platinum incorporation is related to the degree of inhibition of α2M conformational change (Gonias and Figler 1989). We varied the *cis*-Pt concentration, prior to reaction with methylamine, and assessed Aβ-binding. By SDS–PAGE, *cis*-Pt-treated α2M migrated as intact tetramers instead of disulfide-linked dimers, reflecting the presence of intersubunit cross-links (Fig. 2a). Conversion of dimers into tetramers was *cis*-Pt concentration-dependent. The ability of *cis*-Pt to inhibit the α2M slow-to-fast electrophoretic mobility transition, by non-denaturing PAGE, was also *cis*-Pt-concentration-dependent (Fig. 2b), as anticipated.

^{125}I-Aβ-binding to α2M-*cis*-Pt/MA was increased, compared with α2M-MA, irrespective of the *cis*-Pt concentration (Figs 2b and c). With lower *cis*-Pt concentrations, substantial amounts of ^{125}I-Aβ co-migrated with α2M that was apparently fast-form, suggesting that marked restriction of α2M conformational change is not essential for increased Aβ-

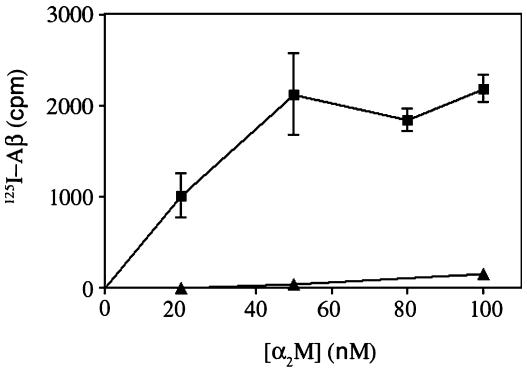

Fig. 3 Aβ-binding to α2M-MA and α2M-*cis*-Pt/MA as determined by co-immunoprecipitation. Protein A-Sepharose was loaded with α2M-specific antibody and then with the indicated concentrations of α2M-MA (?) or α2M-*cis*-Pt/MA (¦). ^{125}I-Aβ (10 nM) was incubated with the beads for 90 min. The beads were then washed three times. Binding was determined by the radioactivity that remained associated with the beads. ^{125}I-Aβ-binding to Protein A-Sepharose, which was loaded with α2M-specific antibody but not with α2M-MA or α2M-*cis*-Pt/MA (0 nM α2M), was subtracted in each experiment. This background was 400 ± 70 cpm.

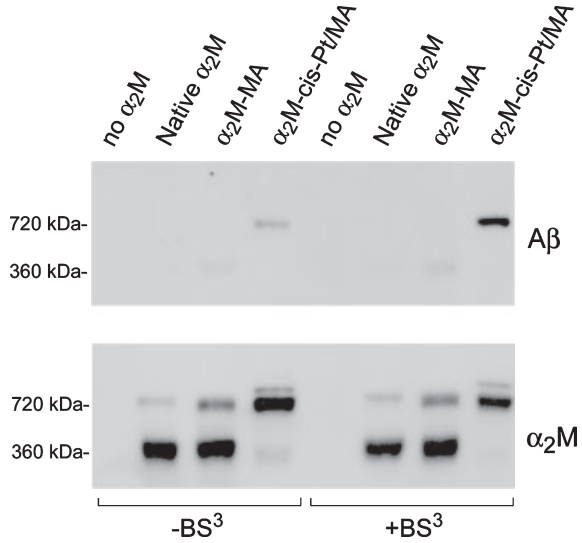

Fig. 4 Immunoblot analysis of Aβ-binding to α2M. Non-radiolabeled Aβ (50 nM) was incubated with native α2M, α2M-MA, or α2M-*cis*-Pt/MA (0.5 μM) for 4 h. BS^3 or vehicle (water) was added for 1.0 min. Cross-linking reactions were terminated by addition of HCl, followed by buffered SDS. The samples were subjected to SDS–PAGE under non-reducing conditions and immunoblot analysis with Aβ-specific antibody (upper panel). The same membrane was stripped and probed with α2M-specific antibody (lower panel).

binding. With higher *cis*-Pt concentrations, Aβ-binding to α2M-*cis*-Pt/MA was increased by over 20-fold, compared with α2M-MA, and the majority of the Aβ associated with slower-migrating forms of α2M. Relative changes in Aβ-binding detected by non-denaturing PAGE are only semi-quantitative because these complexes may partially dissociate during electrophoresis (Gonias *et al*. 1994).

As a second method to confirm that ^{125}I-Aβ-binding to α2M-*cis*-Pt/MA is increased, we loaded Protein A-Sepharose beads with α2M-specific antibody and then with increasing amounts of α2M-MA or α2M-*cis*-Pt/MA. ^{125}I-Aβ (10 nM) was incubated with the beads for 90 min. Binding was determined by co-precipitation of radioactivity. Greatly increased amounts of ^{125}I-Aβ precipitated with immobilized α2M-*cis*-Pt/MA, compared with α2M-MA (Fig. 3). The plateau in the graph depicting Aβ-binding to α2M-*cis*-Pt/MA may represent saturation of the beads with α2M-*cis*-Pt/MA.

We previously demonstrated that native α2M and recombinant fragments of α2M, which lack the Aβ-binding sequence, fail to bind ^{125}I-Aβ directly or compete with α2M-MA for Aβ-binding (Mettenburg *et al*. 2002). By contrast, α2M fragments that contain the Aβ-binding site fully compete with α2M-MA for ^{125}I-Aβ-binding. These prior studies indicate specificity in the Aβ–α2M–MA interaction. In the present study, we utilized the co-immunoprecipitation method to test for competition between ^{125}I-Aβ and unlabeled Aβ in binding to α2M-*cis*-Pt/MA. In four separate experiments with 10 nM ^{125}I-Aβ and 0.5–2.0 μM unlabeled Aβ, 45–55% competition was observed. The reason why greater competition was not observed is not completely understood; however, we hypothesize that high concentrations of Aβ may allow assembly of Aβ fibrils in association with the immobilized α2M-*cis*-Pt/MA. To rule out the possibility that Aβ radio-iodination affected our results, alternative methods for detecting Aβ-binding to α2M were developed and applied.

Immunoblot analysis of Aβ-binding to α2M-*cis*-Pt/MA

To study Aβ-binding to α2M without radio-iodinating Aβ, we modified the method of Crookston *et al*. (1994), in which pulse-exposure to BS^3 (5 mM for 1 min) is used to covalently stabilize non-covalent protein complexes before subjecting samples to SDS–PAGE. Because the cross-linking reaction is conducted under pseudo-first order conditions and only a small fraction of the non-covalent complex is stabilized, the amount of cross-linked complex is proportional to the total amount of non-covalent complex available. Figure 4 shows an immunoblotting experiment comparing the binding of Aβ (50 nM) to native α2M, α2M-MA and α2M-*cis*-Pt/MA. When the samples were treated with vehicle (water), instead of BS^3, prior to SDS–PAGE, trace levels of SDS–stable Aβ-α2M-MA complex were detected. The amount of SDS–stable Aβ-α2M-*cis*-Pt/MA was slightly greater. No complex was detected with native α2M.

Pulse exposure to BS^3 slightly increased the amount of Aβ co-migrating with α2M-MA, as would be anticipated if BS^3 covalently stabilized some non-covalent complex. The amount of Aβ detected in association with α2M-*cis*-Pt/MA

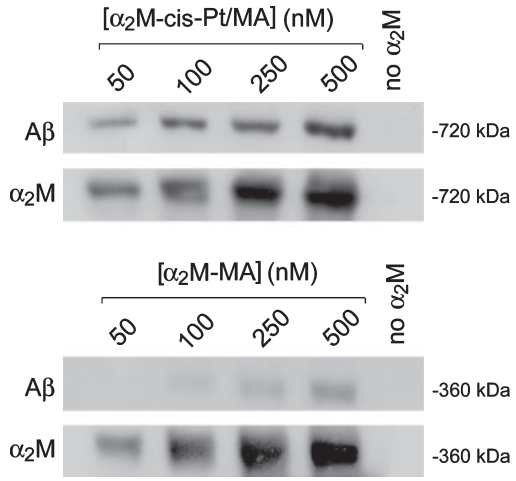

Fig. 5 Immunoblot analysis of Aβ-binding to increasing concentrations of α2M-MA and α2M-*cis*-Pt/MA. Non-radiolabeled Aβ (50 nM) was incubated with α2M-MA or *cis*-Pt/MA-treated α2M at the indicated concentrations. Each sample was treated with BS^3 for 1 min and then subjected to immunoblot analysis with Aβ-specific antibody. The membranes were then stripped and probed with α2M-specific antibody.

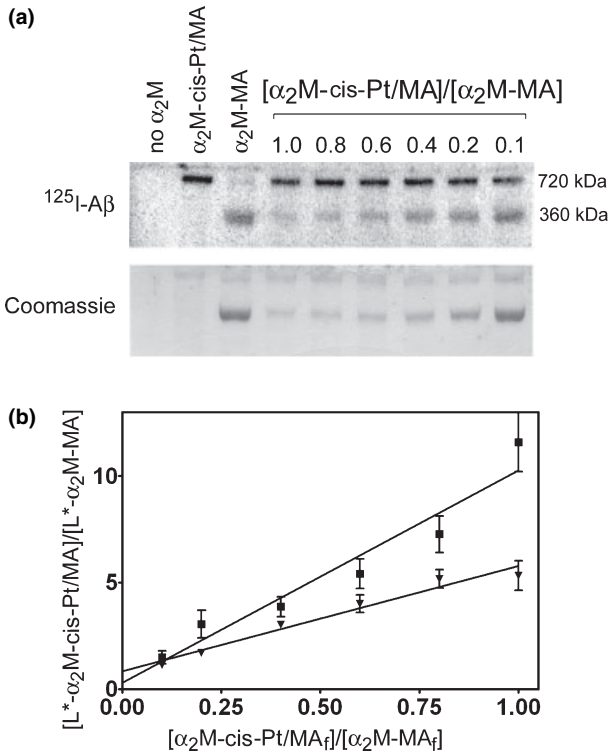

Fig. 6 Determination of the K_D for the interaction of Aβ and TGF-β1 with α2M-*cis*-Pt/MA by competition-binding assay. (a) ^{125}I-Aβ (1 nM) was incubated with mixtures of α2M-MA (50–500 nM) and α2M-*cis*-Pt/MA (50 nM) for 4 h [α2M-*cis*-Pt/MA]/[α2M-MA] is the ratio of α2M-*cis*-Pt/MA to α2M-MA in the original incubation mixture. Control lanes include ^{125}I-Aβ alone (no α2M) and ^{125}I-Aβ incubated with either 50 nM α2M-*cis*-Pt/MA or 500 nM α2M-MA. Samples were treated with BS^3 and subjected to SDS–PAGE. Aβ was detected by PhosphorImager analysis. α2M was detected by Coomassie blue-staining. (b) The distribution of Aβ between α2M-MA and α2M-*cis*-Pt/MA was determined and plotted according to the equation in the Methods. The results of four separate experiments were averaged to generate the graph (■). Equivalent experiments were performed in which ^{125}I-TGF-β1 (0.1 nM) was incubated with mixtures of α2M-MA (50–500 nM) and α2M-*cis*-Pt/MA (50 nM). The data from four experiments were analyzed to generate the graph that is shown (▼).

was greatly increased. Thus, relative Aβ-binding decreased in the following sequence: α2M-*cis*-Pt/MA > α2M-MA > native α2M, irrespective of whether binding was assessed by immunoblot analysis or by Aβ radio-iodination. In experiments that are not shown, we extended the BS^3 cross-linking time and detected increased Aβ-binding to α2M-MA and α2M-*cis*-Pt-MA, without a change in the relative Aβ-binding activity of the two α2M derivatives. We typically avoid prolonged BS^3 reaction times so that the cross-linker does not significantly shift equilibrium in favor of complex formation.

Figure 5 shows the results of experiments in which Aβ was incubated with increasing concentrations of α2M-MA or α2M-*cis*-Pt/MA. Binding was analyzed by BS^3-cross-linking and immunoblot analysis. Aβ-binding increased as a function of the α2M concentration, with both α2M species, but was substantially higher with α2M-*cis*-Pt/MA. To more directly compare the Aβ-binding activity of α2M-MA and α2M-*cis*-Pt/MA, we incubated ^{125}I-Aβ with both forms of α2M together (Fig. 6a). When the concentration of α2M-MA exceeded that of α2M-*cis*-Pt/MA by a factor of 10, ^{125}I-Aβ distributed nearly evenly between the two species. With lower concentrations of α2M-MA, ^{125}I-Aβ distributed mainly with α2M-*cis*-Pt/MA.

The results of four separate co-incubation experiments, like that shown in Fig. 6(a), were plotted according to the equation presented in the Methods (Fig. 6b). From these graphs, we derived an apparent K_D for the binding of ^{125}I-Aβ to α2M-*cis*-Pt/MA of 29 ± 9 nM. This value is decreased 10-fold, compared with the K_D for Aβ-binding to α2M-MA (290 nM). Because α2M-*cis*-Pt/MA is probably somewhat heterogeneous in nature, the K_D represents an average over the various species present. To confirm the efficacy of our coincubation method for determining apparent K_D values, equivalent experiments were performed with ^{125}I-TGF-β1 and α2M-*cis*-Pt/MA. The apparent K_D was 16 ± 7 nM, which is decreased fivefold compared with the K_D for TGF-β1-binding to α2M-MA and in excellent agreement with the K_D (14 nM) for TGF-β1-binding to α2M-*cis*-Pt/MA determined previously, by a different method (Webb *et al*. 1997).

Effects of α2M on Aβ-induced apoptosis in rat C6 glioma cells

Because α2M-*cis*-Pt/MA bound Aβ with increased affinity, we hypothesized that this form of α2M may show increased

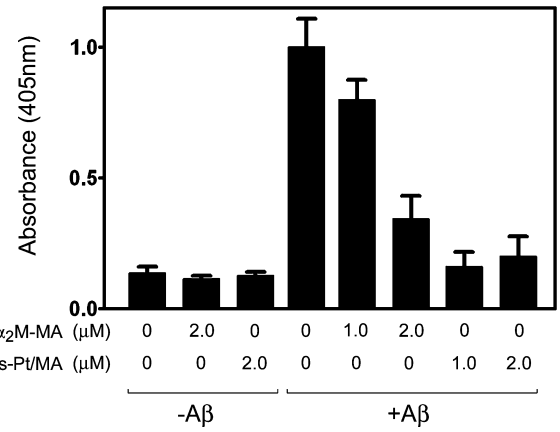

Fig. 7 Effects of α2M-*cis*-Pt/MA and α2M-MA on Aβ-induced C6 cell apoptosis. C6 glioma cells were plated at a density of 10 000 cells/well in 96-well plates. Aβ (4.0 μM) or vehicle-containing solutions, with or without α2M-MA or α2M-*cis*-Pt/MA, at the indicated concentrations, were added to the cultures for 36 h. Cell extracts were analyzed for intracytoplasmic mono- and oligonucleosomes by ELISA. Results are expressed as the absorbance at 405 nm (mean ± SEM, $n = 6$).

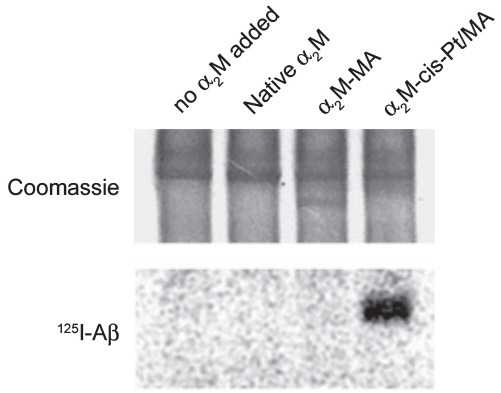

Fig. 8 Binding of Aβ to α2M in human plasma. ^{125}I-Aβ was incubated with 20% plasma, without additional supplementation (no α2M added), with 0.1 μM native α2M, 0.1 μM α2M-MA, or 0.1 μM α2M-*cis*-Pt/MA. Binding was detected by non-denaturing PAGE and PhosphorImager analysis (shown in the bottom panel). The Coomassie-stained gel shows α2M, the predominant fraction of which was derived from the plasma, and possibly other proteins that co-migrate with α2M in this electrophoresis system. The distinct band in the lane labeled 'α2M-MA' results from α2M-MA that was added to the plasma.

efficacy in neutralizing the cytotoxic activity of Aβ. To test this hypothesis, we studied the effects of Aβ on C6 cell apoptosis. The cytotoxic activity of Aβ against this cell line has been previously demonstrated (Van Uden *et al*. 2000).

C6 cells that were exposed to 4 μM Aβ for 36 h demonstrated substantially increased apoptosis, as determined by measuring intracytoplasmic mono- and oligonucleosomes by ELISA (Fig. 7). α2M-MA partially antagonized this activity. At 1 μM, α2M-MA inhibited apoptosis by 25 ± 7% ($n = 6$). At 2 μM, α2M-MA inhibited apoptosis by 73 ± 9%. In the same assay, α2M-*cis*-Pt/MA was substantially more effective, consistent with its ability to bind Aβ with increased affinity. α2M-*cis*-Pt/MA, at 1 μM, inhibited apoptosis, essentially to the level observed in the absence of Aβ (98 ± 6%). In control experiments, α2M-MA and α2M-*cis*-Pt/MA did not cause apoptosis independently of Aβ. In further control experiments, $Aβ_{(40-1)}$ also failed to induce apoptosis, as anticipated (results not shown).

α2M-*cis*-Pt/MA binds Aβ in a plasma milieu

We compared the ability of purified native α2M, α2M-MA, and α2M-cisPt/MA (0.1 μM) to bind ^{125}I-Aβ (1 nM) when added to human plasma (20%) *in vitro*. We estimate that the amount of native α2M, which was already present in the plasma, exceeded that which was added as a supplement by about 6-fold. Binding was detected by radioactivity co-migrating with the α2M bands by non-denaturing PAGE. As shown in Fig. 8, the sensitivity of the non-denaturing PAGE assay was insufficient to detect ^{125}I-Aβ-binding to native α2M that is endogenous in plasma, native α2M that was added as a supplement, and α2M-MA. By contrast, substantial binding was observed with α2M-*cis*-Pt/MA. Thus, α2M-*cis*-Pt/MA is unique amongst the forms of α2M studied here, in its capacity to effectively sequester Aβ in the presence of a full complement of plasma proteins.

Discussion

There are substantial data to support the hypothesis that naturally occurring α2M and its receptor, LRP-1, regulate Aβ homeostasis in the CNS. Both proteins have been linked to AD by genetic studies (Kang *et al*. 1997; Blacker *et al*. 1998; Lambert *et al*. 1998; Liao *et al*. 1998). Furthermore, α2M and LRP-1 may function as a system to shuttle Aβ from the extracellular spaces into cells for catabolism (Narita *et al*. 1997; Qiu *et al*. 1999; Kang *et al*. 2000). Because of this process, reduced LRP-1 expression has been associated with increased amyloid deposition (Kang *et al*. 2000). α2M and endothelial cell LRP-1 transport Aβ across the blood–brain barrier, away from the CNS and into the plasma (Shibata *et al*. 2000), which may substantially influence Aβ deposition in the brain (Ghersi-Egea *et al*. 1996). Aβ transport also occurs across the blood–brain barrier in the reverse direction, probably under the control of RAGE receptors (Deane *et al*. 2003). Endothelial cell LRP-1 may act independently of α2M in transporting Aβ across the blood–brain barrier and into the blood (Deane *et al*. 2004).

To the extent that transport of Aβ across the blood–brain barrier is reversible and controls Aβ deposition in the brain, agents that bind Aβ in the plasma may shift the balance in favor of Aβ removal from the CNS. Proof of principle was demonstrated using an Aβ-specific monoclonal antibody (DeMattos *et al*. 2001, 2002). When administered directly

into the blood of transgenic mice, which express human amyloid precursor protein V717F, the antibody increased plasma levels of Aβ by 1000-fold and decreased the Aβ burden in the brain. The studies presented here demonstrate that α2M-cis-Pt/MA has multiple properties that may be advantageous for Aβ-binding in the plasma. First, α2M-cis-Pt/MA binds Aβ with substantially increased affinity, compared with any other previously characterized α2M derivative. When added to human plasma *in vitro*, α2M-cis-Pt/MA demonstrates a unique capacity to sequester Aβ. Finally, α2M-cis-Pt/MA demonstrates substantially improved activity in counteracting the cytotoxic activity of Aβ, compared with α2M-MA.

α2M-cis-Pt/MA may be prepared in large amounts and is not independently toxic, because platinum(II) does not dissociate from α2M at an appreciable rate (Webb and Gonias 1998). When α2M-cis-Pt/MA is administered into the intraperitoneal space, in mice, the preparation transfers into the plasma, remains structurally intact, and retains total function as a growth factor-binding protein. Furthermore, α2M-cis-Pt/MA is cleared from the blood by LRP-1, albeit at a decreased rate compared with fully transformed species, such as α2M-MA (Gonias and Pizzo 1983). As a result, α2M-cis-Pt/MA survives for a period of time in the plasma but is ultimately catabolized by LRP-1 in the liver, apparently with associated proteins (LaMarre *et al*. 1991). The ultimate degradation of Aβ-α2M-cis-Pt/MA by a receptor that naturally functions in Aβ homeostasis may represent an advantage for α2M-cis-Pt/MA as an Aβ-neutralizing agent.

The structure of α2M includes a number of well-defined functional regions. An area termed the 'bait region' is located near the center of the 180-kDa subunit. The bait region is composed of a series of peptide bonds that may be cleaved by attacking proteases (Sottrup-Jensen *et al*. 1981). This process initiates α2M conformational change and protease trapping. Just C-terminal to the bait region is the sequence that mediates binding of TGF-β (Webb and Gonias 1998). A thiol ester bond is formed by the side-chains of Cys-949 and Gln-952 and functions in covalent binding of trapped proteases (Van Leuven *et al*. 1981b). The Aβ-binding site is located between aa 1314 and 1365 (Mettenburg *et al*. 2002). Finally, the recognition sequence for LRP-1 is centered in an alpha helix and includes residues 1370–1377 (Jenner *et al*. 1998).

Overall, intact α2M that has undergone conformational change due to reaction with proteases or methylamine adopts the shape of a hollow cylinder (Feldman *et al*. 1985). Although the partial conformational change that occurs in α2M-*cis*-Pt/MA has been extensively studied (Gonias and Pizzo 1981; Gonias and Figler 1989; Marshall *et al*. 1992), we do not fully understand why this α2M derivative demonstrates dramatically increased Aβ-binding activity. Even when α2M is pre-treated with the highest concentration of *cis*-Pt (1.6 mM), the amount of platinum(II) incorporation into α2M is limited (Gonias and Figler 1989). Furthermore, we observe significantly increased Aβ-binding to α2M that is treated with only 0.05 mM *cis*-Pt. Both reactive sites in *cis*-Pt (the Cl^- leaving groups) tend to react internally with nucleophilic amino acids in α2M (Gonias and Pizzo 1981). The resulting bonds are SDS-stable whereas the interaction of Aβ with α2M-*cis*-Pt/MA is primarily non-covalent. Thus, it is highly unlikely that Aβ interacts directly with the platinum(II) that incorporates into α2M-*cis*-Pt/MA. Instead, we view the advantage of α2M-*cis*-Pt/MA in Aβ-binding as resulting from optimal exposure of the Aβ recognition sequence in conformational intermediates. If this hypothesis is correct, then we conclude that the Aβ-binding site is entirely cryptic in native α2M, partially exposed in fully transformed α2M, and more optimally exposed in the intermediate conformations assumed by α2M-*cis*-Pt/MA.

We have analyzed the Aβ-α2M-*cis*–Pt/MA interaction because of the potential to learn more regarding how Aβ-binding macromolecules affect Aβ activity and because of the possible therapeutic implications of this work. It is also possible that α2M-*cis*-Pt/MA may model certain α2M structural changes that occur *in vivo*. α2M conformational intermediates have been identified in the plasma (Nelles *et al*. 1980). Furthermore, when α2M reacts with certain proteases, such as thrombin, the α2M adopts intermediate structures that resemble α2M-*cis*-Pt/MA by electron microscopy (Marshall *et al*. 1992). Whether α2M structures that bind Aβ with high-affinity, like α2M-*cis*-Pt/MA, are generated naturally *in vivo* is a topic for future investigation.

Acknowledgement

This work was supported in part by a grant from the National Institutes of Health, CA-53462.

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