Differences in the binding of transforming growth factor β1 to the acute-phase reactant and constitutively synthesized α-macroglobulins of rat

Donna J. WEBB,* Kendall P. CROOKSTON,* Nancy L. FIGLER,† Jonathan LAMARRE‡ and Steven L. GONIAS*†§

Departments of *Biochemistry and †Pathology, University of Virginia Health Sciences Center, Charlottesville, VA 22908, U.S.A. and ‡Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada N1G2W1

Human α_2-macroglobulin (α_2M) is a proteinase inhibitor and carrier of certain growth factors, including transforming growth factor β1 (TGF-β1). The constitutively synthesized homologue of human α_2M in the adult rat is α_1M. Rat α_2M is an acute-phase reactant, expressed at high levels in experimental trauma, pregnancy and in certain pathological conditions. The physiological role of rat α_2M is not known. In this investigation, we demonstrated that rat α_1M and rat α_2M bind TGF-β1. The equilibrium dissociation constants (K_D) for the binding of TGF-β1 to the native forms of α_1M and α_2M were 257 and 109 nM respectively. α_1M underwent conformational change when it reacted with methylamine. The resulting product bound TGF-β1 with higher affinity (32 nM). Methylamine-treated rat α_2M did not undergo conformational change and did not bind TGF-β1 with increased affinity. Previous studies suggest that the native conformation may be the principal form responsible for the cytokine-carrier activity of α_2M in plasma and serum-supplemented cell culture medium. To confirm that native rat α_2M is a more efficient TGF-β1 carrier than native α_1M, fetal bovine heart endothelial cell (FBHE) proliferation assays were performed. TGF-β1 (5 pM) inhibited FBHE proliferation, and native α_2M (0.3 μM) counteracted this activity whereas α_1M (0.3 μM) had almost no effect. Rat α_2M underwent conformational change when it reacted with plasmin incorporating 1.1 mol of plasmin/mol. α_2M–plasmin bound TGF-β1; the K_D (61 nM) was lower ($P < 0.01$) than that determined for the native α_2M–TGF-β1 interaction. These studies demonstrate that both rat α-macroglobulins are carriers of TGF-β1. The native form of rat α_2M probably has a predominant role, compared with native α_1M, as a TGF-β1 carrier in the plasma during the acute-phase response.

INTRODUCTION

The α-macroglobulin (αM) family of thiol-ester-bond-containing proteinase inhibitors includes at least two or three separate members in most mammalian species [1]. Human α_2M and many other αMs are tetramers, composed of four identical 180 kDa subunits that form up to two distinct proteinase-binding sites [2,3]. The 'bait region', near the centre of each αM subunit, includes a series of peptide bonds that may be cleaved by diverse proteinases [4,5]. Bait-region cleavage initiates a major conformational change in the αM that irreversibly 'traps' the proteinase in a non-dissociable complex [4,6]. αM conformational change also exposes a receptor-recognition site at the C-terminus of each subunit [7–9]. An α_2M receptor has been purified from liver, placenta and brain [10–12]. This receptor is identical with low-density-lipoprotein-receptor-related protein (LRP) [10,13].

αM thiol ester bonds may be directly cleaved by small primary amines such as methylamine [14–16]. Aminolysis of the thiol esters in human α_2M causes a conformational change equivalent to that induced by proteinases, as determined by a variety of methods including non-denaturing PAGE [2,17–20]. Methylamine-modified human α_2M (α_2M-MA) and α_2M–proteinase complexes demonstrate increased electrophoretic mobility relative to the native form and are therefore referred to as α_2M fast forms.

The reason why certain species express more than one αM is an unresolved problem, related to the more general question of αM function. The constitutively synthesized homologue of human α_2M in the rat is α_1M (molecular mass ~ 746 kDa). Concentrations of human α_2M and rat α_1M in the plasma range from 2 to 5 μM [21]. Rat α_2M (molecular mass ~ 716 kDa) is an acute-phase reactant, present at very low concentration in adult plasma, except in experimental trauma, pregnancy and certain pathological conditions [21–23]. In the acute-phase response, the plasma concentration of rat α_2M may increase to 6–12 μM, reflecting the presence of a type-II cytokine-responsive element in the rat α_2M promoter [23,24].

Differences in the structure/function of rat α_2M, relative to rat α_1M and human α_2M, have been demonstrated only in experiments with methylamine. Even though rat α_2M incorporates methylamine, it does not undergo conformational change, as assessed by non-denaturing PAGE [25,26]. Rat α_2M-MA is not recognized by LRP and thus not cleared from the circulation [26]. Furthermore, rat α_2M-MA retains proteinase-binding activity, unlike human α_2M-MA [26]. Although these results are intriguing, their significance is unclear since evidence for aminolysis of α_2M occurring *in vivo* has not been obtained.

Recent studies suggest that human α_2M may function in the immune response and in cellular growth regulation [27,28]. Human α_2M binds a number of growth factors and cytokines, including transforming growth factor β1 (TGF-β1) and TGF-β2 [29–31]. In human plasma, nearly all of the TGF-β1 is associated with α_2M [30,31]. The binding affinity of human α_2M for most cytokines, including TGF-β1, depends on the α_2M conformation; interactions with transformed α_2M species such as α_2M-MA are favoured [29]. Nevertheless, native α_2M probably functions as the primary carrier of many cytokines in the plasma because of its high concentration relative to that of transformed species [28–34]. Cytokines that bind to conformationally transformed human α_2M are cleared quickly from the circulation via LRP [32,33], whereas cytokines that bind to native α_2M are stabilized in the vascular compartment [28,33,34].

Abbreviations used: αM, α-macroglobulin; TGF-β1, transforming growth factor β1; LRP, low-density-lipoprotein-receptor-related protein; PNPGB, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; BS^3, *bis*(sulphosuccinimidyl)suberate; PEG, poly(ethylene glycol); TBS, Tris-buffered saline; IL-6, interleukin 6; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FBHE, fetal bovine heart endothelial.

§ To whom correspondence should be addressed.

The recently described biological activities of human α_2M [27,28] provide the opportunity to probe for novel differences in the function of the various αMs expressed by a single species. Identifying differences in the function of acute-phase and constitutively synthesized αMs may provide insight into the biological role of these proteins in general. In the present investigation, we performed new studies to compare the conformational states of rat α_1M and α_2M. Binding of TGF-β1 was then studied. Although both rat αMs bound TGF-β1, the apparent equilibrium dissociation constant (K_D) for the interaction of TGF-β1 with native α_1M was approx. 2.5 times higher than the corresponding K_D for TGF-β1 and native α_2M. In endothelial-cell-growth-inhibition assays, rat α_2M counteracted the activity of TGF-β1 to a greater extent than α_1M, as predicted by the equilibrium binding studies. The TGF-β1-binding affinity of rat α_2M was further increased when the α_2M was complexed with plasmin. These studies suggest an important role for α_2M as a carrier and regulator of cytokines in the plasma during the acute-phase response in rats.

MATERIALS AND METHODS

Reagents

Methylamine HCl, chloramine-T, 1,4-dithiothreitol, *p*-nitrophenyl *p'*-guanidinobenzoate (PNPGB), diethylaminoethyl-Cibacron Blue 3GA, PMSF, TosLysCH$_2$Cl, trypsin and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). *Bis*(sulphosuccinimidyl)suberate (BS^3) was purchased from Pierce (Rockford, IL, U.S.A.), Na^{125}I from Amersham (Arlington Heights, IL, U.S.A.), electrophoresis reagents from Bio-Rad (Hercules, CA, U.S.A.) and Sepharose CL4B from Pharmacia LKB (Piscataway, NJ, U.S.A.). ACA 22 chromatography resin was from IBF Biotechnics (Savage, MD, U.S.A.) and Carbowax poly(ethylene glycol) (PEG) 3350 was from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Low-molecular-mass urokinase plasminogen activator was purchased from Calbiochem, La Jolla, CA, U.S.A , D-Valyl-L-leucyl-L-lysine *p*-nitroanilide hydrochloride (S-2251) was from Kabi Vitrum (Stockholm, Sweden).

Trypsin was active-site-titrated with PNPGB by the method of Chase and Shaw [35]. Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN, U.S.A.) and radioiodinated as previously described [36]. Specific radioactivities of ^{125}I-TGF-β1 ranged from 100 to 200 μCi/μg. [Glu^1]Plasminogen was purified from human plasma by lysine–Sepharose chromatography [37]. Plasminogen was radioiodinated using Iodobeads as recommended by the manufacturer (Pierce). The specific radioactivity was 1.5 μCi/μg.

Preparation of rat α_1M immunoaffinity chromatography column

α_1M-specific polyclonal antibody was prepared in a New Zealand White rabbit following standard protocols [38]. The immunoglobulin fraction was purified from rabbit serum by chromatography on diethylaminoethyl-Cibacron Blue 3GA equilibrated in 20 mM Tris/HCl/28 mM NaCl, pH 8.0. Rabbit immunoglobulin does not bind to the matrix under these conditions. The immunoglobulin fraction was covalently linked to CNBr-activated Sepharose CL4B (5 mg of protein was bound per ml of Sepharose).

αM purification

Rat α_1M and α_2M were purified from male Sprague–Dawley or Wistar rats (350–450 g) using the procedure of Lonberg-Holm et al. [22] with some modifications. Rat α_1M was purified from normal rat plasma. Blood was collected from the aorta of anaesthetized rats into a cocktail of proteinase inhibitors (2 μM aprotinin, 5 μM soyabean trypsin inhibitor, 20 μM leupeptin, 0.6 mM benzamidine, 1 mM PMSF, 10 mM EDTA). The plasma was then fractionated by precipitation with 10% (w/v) PEG. After centrifugation, the supernatant was dialysed against 50 mM Tris/HCl/150 mM NaCl, pH 7.8 (TBS) and loaded on to a zinc–Sepharose CL4B column prepared by the method of Porath et al. [39]. α_1M was eluted in 50 mM sodium phosphate/150 mM NaCl, pH 6.0. Fractions containing α_1M were concentrated and further purified by gel-filtration chromatography on Ultrogel ACA 22.

Rat α_2M was purified from acute-phase plasma obtained 48 h after subcutaneous injection of croton oil (0.5 ml/100 g) in anaesthetized animals [40]. Blood was collected as described for α_1M. Plasma was fractionated with 6% (w/v) PEG; the supernatant was further fractionated with 14% (w/v) PEG. The precipitate from the second fractionation was resuspended in TBS and subjected to zinc-chelate and gel-filtration chromatography as described for α_1M. Any residual α_1M that contaminated the α_2M preparations was removed by negative affinity chromatography using the polyclonal α_1M-specific antibody–Sepharose column. Final preparations of rat α_1M and α_2M were homogeneous as determined by SDS/PAGE and Coomassie Blue staining. α_1M and α_2M concentrations were determined by absorbance at 280 nm using absorbance coefficients ($A^{1\%}_{1\,cm}$) of 9.8 and 9.2 respectively [21].

Rat α_1M-MA and α_2M-MA were prepared by dialysing the native proteins against 200 mM methylamine in 50 mM Tris/HCl, pH 8.2, for 6 h at 22 °C followed by extensive dialysis against PBS at 4 °C. α_1M, α_1M-MA, α_2M and α_2M-MA (0.6 μM) were each allowed to react with a 2.5-fold molar excess of trypsin for 10 min at 37 °C. The trypsin was then inactivated with TosLysCH$_2$Cl (0.6 mM).

Electron microscopy

α_2M, α_2M–trypsin, α_2M-MA, α_2M-MA–trypsin, α_1M and α_1M-MA were studied by transmission electron microscopy. Thin carbon films were floated for 60 s on 400 μl aliquots of each preparation at 10–30 μg/ml. The films were transferred into buffered 2% glutaraldehyde for 10 min and then washed with deionized water. Negative staining was performed with 2.0% uranyl formate for 60 s, and the films were air-dried on 300-mesh nickel grids. Electron microscopy was performed at 80 kV using a Zeiss 902 electron microscope. Images of elastically scattered electrons were photographed at a direct magnification of 46000 using a micro-dose focusing technique and Kodak SO-163 film.

Analysis of ^{125}I-TGF-β1 binding to α_1M and α_2M by non-denaturing PAGE

Native α_1M, α_1M-MA, α_1M–trypsin complex, trypsin-treated α_1M-MA, native α_2M, α_2M-MA, α_2M–trypsin complex and trypsin-treated α_2M-MA (each of 0.6 μM) were incubated with ^{125}I-TGF-β1 (0.3 nM) in PBS containing 15 μM BSA for 1 h at 37 °C. Samples were subjected to non-denaturing PAGE on a 5% slab for 3 h at 150 V using the buffer system described by Van Leuven et al. [20]. ^{125}I-TGF-β1 binding to α_1M and α_2M was assessed by co-migration of the growth factor with αM, as determined by autoradiography [38,33].

Non-denaturing PAGE provides qualitative evidence for ^{125}I-TGF-β1 binding to αMs; however, the amount of binding observed does not correlate with the affinity of the interaction

since αM–cytokine complexes dissociate during electrophoresis and the extent of dissociation is variable for different forms of αM [28].

Determination of apparent K_D values

The BS³ cross-linking method was used to determine K_D values for the binding of TGF-β1 to rat α_1M and α_2M [29,41,42]. Binding of a cytokine to αM is described by the following two-step mechanism:

$$A + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AC \xrightarrow{k_{+2}} AC^* \quad (1)$$

Free cytokine (C) associates reversibly with αM (A) to form a non-covalent αM–cytokine complex (AC). AC is converted into covalently stabilized complex (AC*) by thiol–disulphide exchange; however, with human α_2M, this reaction (k_{+2}) is slow compared with k_{-1} [29]. Therefore a rapid equilibrium assumption may be applied and the K_D for reversible binding of ^{125}I-cytokine to αM may be expressed according to the following simple relationship:

$$\frac{C}{AC} = K_D \frac{1}{A} \quad (2)$$

Unless otherwise indicated, various concentrations of αM (10 nM–5 μM) were incubated with ^{125}I-TGF-β1 (0.25–1.0 nM) in PBS with 150 μM BSA at 37 °C for 1 h. BS³ (in water) was then added to a final concentration of 5 mM. Water alone was added to identical control incubations. Each tube was incubated for exactly 60 s at 22 °C. The cross-linking reaction was terminated by acidification. Samples were denatured in 2.0% SDS for 30 min at 37 °C. Each sample was brought to a final concentration of 100 mM Tris/HCl/10% glycerol and subjected to SDS/PAGE. Dried gels were exposed to X-ray film and the amount of ^{125}I-TGF-β1 in each band was determined by slicing the gel into sections and measuring the radioactivity in each section using a γ-counter.

In the absence of BS³, ^{125}I-TGF-β1 that was covalently bound to α_1M or α_2M (AC*) co-migrated with the high-molecular-mass αM bands. When BS³ was added, additional ^{125}I-cytokine–αM complex was detected. The additional complex represented non-covalent αM–cytokine complex (AC) that was cross-linked by BS³ (AC_e). Since BS³ was added at a high concentration, AC cross-linking by BS³ occurred under pseudo-first-order conditions and the fraction of AC cross-linked (z) was constant for a given set of cross-linking conditions and independent of the concentration of αM–cytokine complex. Therefore $AC_e = zAC$.

AC_e was determined by counting gel slices and corrected for the presence of AC*, as determined from the control incubations without BS³. Experimentally detected free cytokine (C_e) included free cytokine and cytokine that was bound to αM but not cross-linked by the BS³.

$$C_e = C + (1-z)AC \quad (3)$$

By substituting expressions for C_e and AC_e into eqn. (2), a linear relationship in the form $y = mx + b$ is derived, as previously described [29]:

$$\frac{C_e}{AC_e} = \left(\frac{K_D}{z}\right)\left(\frac{1}{A}\right) + \left(\frac{1}{z} - 1\right) \quad (4)$$

The results of each experiment were plotted according to eqn. (4) and analysed by linear regression. The y intercept was used to calculate the BS³ cross-linking efficiency (z); this, together with the slope, was used to calculate the apparent K_D. Each constant reflects the mean ± S.E.M. of four to six separate experiments. In using the BS³ cross-linking method, one assumes that each αM expresses a single cytokine-binding site. A second assumption is that all αM molecules in a given preparation bind cytokine with equal affinity. We have previously shown that the BS³ cross-linking reagent does not cause a substantial shift in the equilibria for the binding of TGF-β1 to human α_2M [29].

Inhibition of endothelial cell proliferation

Fetal bovine heart endothelial (FBHE) cells were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 ng/ml acidic fibroblast growth factor and 80 ng/ml basic fibroblast growth factor. Cells were harvested at subconfluence with 0.5% trypsin/0.5 mM EDTA and passaged. FBHE proliferation assays were performed in dilute (0.2%) FBS as previously described [42,43]. Briefly, cells were plated in 24-well cell culture plates (2×10^4 cells per well) and incubated in DMEM supplemented with 10% FBS for 15 h. After the cultures had been washed, fresh DMEM supplemented with 0.2% FBS and TGF-β1 (5 pM) was added to each well. Some cultures were simultaneously treated with native rat α_1M or native rat α_2M (0.3 μM). After 30 h, [^{3}H]thymidine was added for an additional 18 h. Cells were harvested and [^{3}H]thymidine incorporation was assessed as previously described [42].

TGF-β1 binding to rat α_2M–plasmin complex

Plasminogen (9.8 μM) was activated with low-molecular-mass urokinase plasminogen activator (100 nM) for 20 min at 37 °C. The extent of activation was determined by the rate of hydrolysis of S-2251, using the following kinetic parameters: $k_{cat.}$, 11.7 s^{-1}; K_m, 0.18 mM [44]. Rat α_2M–plasmin complex was formed by incubating α_2M (0.35 μM) with plasmin (0.9 μM) for 30 min at 37 °C. The plasmin was then inactivated with PNPGB (0.1 mM). The stoicheiometry of plasmin binding to rat α_2M was determined by performing the identical incubation with ^{125}I-plasmin. The products of the reaction of α_2M with ^{125}I-plasmin were subjected to non-denaturing PAGE, SDS/PAGE and autoradiography. Dried gels were sliced into 3 mm slices and each section was counted in a γ-counter. ^{125}I-Plasmin binding was determined from the radioactivity associated with the α_2M bands in non-denaturing PAGE (total binding) and SDS/PAGE (covalent binding), after correction for recovery, as previously described [45].

TGF-β1 binding to rat α_2M–plasmin was assessed by incubating different concentrations of rat α_2M–plasmin (6–270 nM) with ^{125}I-TGF-β1 (0.8 nM) for 1 h at 37 °C. The BS³-cross-linking method was then used as described above.

RESULTS

Non-denaturing PAGE analysis of αM conformation

Rat α_1M and rat α_2M were allowed to react with methylamine or trypsin and subjected to non-denaturing PAGE (Figure 1). The native αMs migrated in single bands without evidence of contamination by conformational variants (which migrate more rapidly). α_1M-MA and α_1M–trypsin complex demonstrated increased mobility compared with native α_1M. The slight difference in the mobilities of α_1M-MA and α_1M–trypsin complex was eliminated when α_1M-MA was allowed to react with trypsin for 1 h.

Rat α_2M did not undergo conformational change when it reacted with methylamine, as determined by non-denaturing

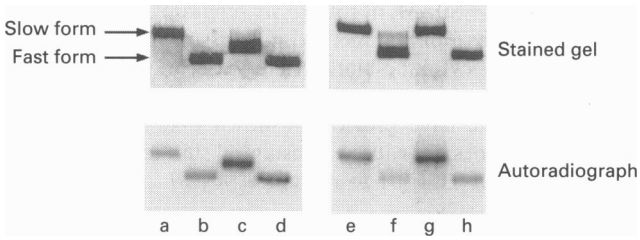

Figure 1 Binding of ^{125}I-TGF-β1 to rat αMs

^{125}I-TGF-β1 (0.3 nM) was incubated with rat αMs for 1 h at 37 °C. Samples were then subjected to non-denaturing PAGE and autoradiography. The Coomassie-stained gel and autoradiograph are shown. The αM derivatives are α_1M (lane a), α_1M–trypsin (lane b), α_1M-MA (lane c), α_1M-MA–trypsin (lane d), α_2M (lane e), α_2M–trypsin (lane f), α_2M-MA (lane g) and α_2M-MA–trypsin (lane h). The mobility of native αM is designated slow form and the mobility of trypsin-modified αM is designated fast form.

PAGE, even though methylamine is incorporated into the rat α_2M thiol esters [26]. When rat α_2M-MA reacted with trypsin, a complete shift in electrophoretic mobility was observed, as previously reported [26]. Rat α_2M–trypsin complex and rat α_2M-MA–trypsin complex demonstrated equivalent mobility.

Non-denaturing PAGE analysis of TGF-β1 binding to the rat αMs

^{125}I-TGF-β1 bound to native α_1M and α_2M, co-migrating with both αMs in the non-denaturing polyacrylamide gel (Figure 1). The autoradiography bands completely aligned with the Coomassie-stained bands. An increase in TGF-β1 binding was observed when α_1M was modified by methylamine. This result may reflect: (i) higher affinity binding of ^{125}I-TGF-β1 to α_1M-MA than to native α_1M; (ii) a lower rate of ^{125}I-TGF-β1–α_1M-MA dissociation during electrophoresis; or (iii) increased covalent stabilization of TGF-β1–α_1M-MA complex (AC* formation).

Rat α_1M–trypsin and α_1M-MA–trypsin complex bound slightly higher levels of ^{125}I-TGF-β1 than did native α_1M. These results are difficult to interpret as we have previously demonstrated that TGF-β1 binding to human α_2M–trypsin complex is affected by the stoicheiometry of trypsin associated with α_2M [46]. For the experiments shown in Figure 1, the stoicheiometries of the αM–trypsin complexes were not determined.

The three modified forms of rat α_2M bound TGF-β1. The extent of TGF-β1 binding to rat α_2M-MA was slightly increased despite the lack of conformational change in α_2M. TGF-β1 binding to the trypsin-treated α_2M variants was slightly decreased. In control experiments, we demonstrated that free ^{125}I-TGF-β1 does not migrate near the areas of the gel occupied by any of the αM variants (results not shown).

Electron-microscopy studies of α_1M and α_2M

αMs from many mammalian species present similar images on electron microscopy [47]. Native αMs are typically heterogeneous in appearance. The diverse assortment of native αM images results largely from variability in orientation of the adsorbed molecules on the carbon film [48]. Flexibility in the native αM structure may also contribute to the observed imaging heterogeneity. By contrast, conformationally transformed αMs yield a relatively consistent and reproducible image resembling the letter H or the Russian letter Ж [47].

The TGF-β1-binding studies shown in Figure 1 suggested that native rat α_2M may differ from rat α_2M-MA. Therefore electron microscopy studies were performed to compare the structures of these two species. Figure 2(a) shows the heterogeneous appearance of native rat α_2M, as expected. Reaction with trypsin converted most of the rat α_2M into easily recognized H structures (Figure 2b). By contrast, almost no H structures were detected in the rat α_2M-MA preparation. By visual inspection, the assortment of images present in the rat α_2M-MA electron micrographs was indistinguishable from that present in the native α_2M electron micrographs. Reaction of rat α_2M-MA with trypsin converted the entire population into H images. These studies demonstrate that non-denaturing PAGE provides an accurate assessment of rat α_2M conformation.

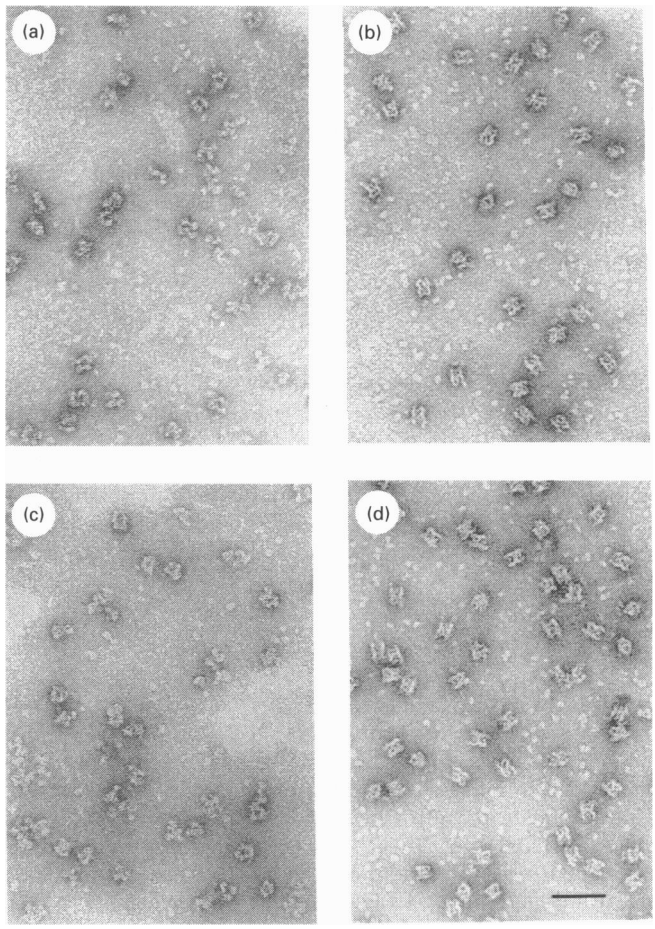

Figure 2 Electron micrographs of rat α_2M derivatives

Negatively stained images are shown of native rat α_2M (**a**), α_2M–trypsin (**b**), α_2M-MA (**c**) and α_2M-MA–trypsin (**d**). The bar represents 45 nM.

In separate studies, we examined rat α_1M-MA and α_1M–trypsin complex by electron microscopy. Although the mobilities of these preparations in the non-denaturing PAGE system were slightly different, visual inspection of the electron micrographs did not provide evidence for a correlating difference in structure (results not shown). The two preparations were both composed almost entirely of H-like images.

Binding of TGF-β1 to rat α_1M and α_2M under apparent equilibrium conditions

We previously demonstrated that the interaction of TGF-β1 with human α_2M to form non-covalent complex reaches apparent equilibrium within 15 min at 37 °C [29]. In this study, we

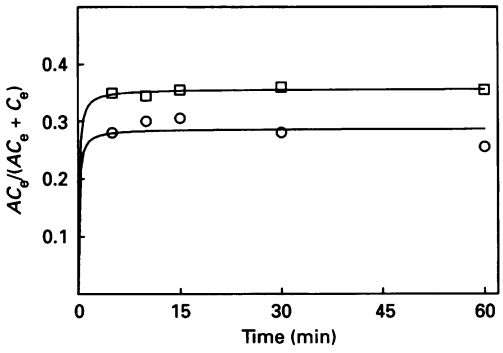

Figure 3 ^{125}I-TGF-β1 binding to rat α_2M as a function of time

^{125}I-TGF-β1 (0.3 nM) was incubated with 0.7 μM native rat α_2M (○) or rat α_2M-MA (□) at 37 °C for the indicated times. Binding was determined by the BS^3-stabilization method ($n = 2$). The amount of TGF-β1–α_2M complex cross-linked by BS^3 (AC_e) is expressed as a fraction of the total TGF-β1 minus the amount of covalent complex ($AC_e + C_e$).

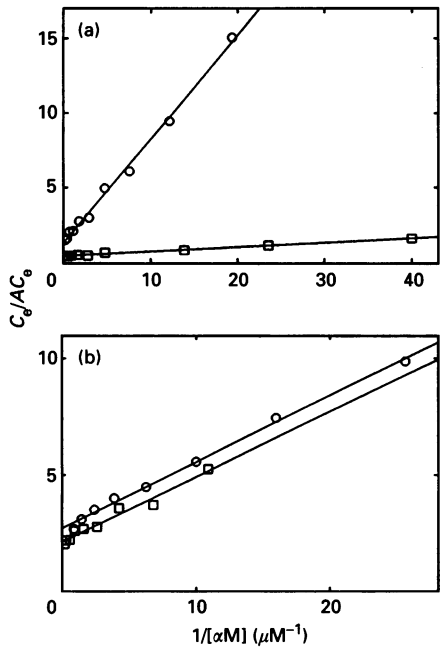

Figure 4 Determination of K_D values for ^{125}I-TGF-β1 binding to rat α_1M and α_2M

The results of representative experiments showing binding of ^{125}I-TGF-β1 to rat α_1M (**a**) and α_2M (**b**) are presented. Binding results for native αM (○) and αM-MA (□) were plotted according to eqn. (4). K_D values from four to six separate experiments for each αM were averaged to obtain the results presented in Table 1.

incubated ^{125}I-TGF-β1 with native rat α_2M or α_2M-MA for various periods of time. Binding was determined by the BS^3-cross-linking method. Within 10 min, the level of AC_e reached an apparent maximum with both forms of rat α_2M (Figure 3). Comparable results were obtained with rat α_1M (results not shown).

^{125}I-TGF-β1 was incubated with different concentrations of rat α_1M, α_1M-MA, α_2M, or α_2M-MA at 37 °C. After cross-linking with BS^3, AC_e, C_e and AC^* were determined by SDS/PAGE. C_e/AC_e was plotted against $1/A$, according to eqn. (4) (Figure 4) and K_D values were determined (summarized in Table 1). The K_D for TGF-β1 binding to native α_1M (257 nM) was 8-fold higher than the K_D for TGF-β1 binding to α_1M-MA. In this respect, rat α_1M was similar to human α_2M, since human α_2M-MA binds TGF-β1 with higher affinity than native human α_2M [29]. The native form of rat α_2M bound TGF-β1 with higher affinity than rat α_1M; however, rat α_2M showed no further increase in TGF-β1-binding affinity after reaction with methylamine. These results support the hypothesis that conformational change is necessary for the increased growth-factor-binding activity of methylamine-modified αMs. Furthermore, these results suggest that TGF-β1 may bind preferentially to native rat α_2M when equal concentrations of native α_2M and native α_1M are present.

Covalent binding of TGF-β1 to rat α_1M-MA and α_2M-MA was slightly increased compared with that observed when the native forms of these αMs were studied (Table 1). This was an expected result, since covalent binding results from thiol–disulphide exchange which occurs principally with the free Cys residues generated by thiol ester aminolysis [27–29]. Increased covalent stabilization may explain the apparent increase in ^{125}I-TGF-β1 binding to rat α_2M-MA (compared with native α_2M) observed by non-denaturing PAGE.

Table 1 Equilibrium dissociation constants, BS^3-cross-linking efficiencies (z) and covalent binding of ^{125}I-TGF-β1 to rat αMs

Results were determined using the BS^3-stabilization method. Means ± S.E.M. are shown for K_D and z. $AC^*/(AC^* + AC)$ represents the fraction of complex that was covalently stabilized without BS^3 and is expressed as the complete range of values for ten different α_2M concentrations in n separate experiments ($10n$ samples).

αM	K_D (nM)	z	$AC^*/(AC^* + AC)$	n
α_1M	257 ± 26	0.44 ± 0.02	0.03–0.08	5
α_1M-MA	32 ± 4	0.61 ± 0.05	0.07–0.30	6
α_2M	109 ± 11	0.29 ± 0.02	0.00–0.03	5
α_2M-MA	94 ± 16	0.37 ± 0.02	0.01–0.13	4

Table 2 Effects of native rat α_1M and native rat α_2M on the activity of TGF-β1 in an endothelial cell proliferation assay

FBHE cells were incubated with TGF-β1 (5 pM), native rat α_1M (300 nM), native rat α_2M (300 nM) or TGF-β1 + αM. After 30 h, 1 μCi/ml [^{3}H]thymidine was added and incubations were continued for an additional 18 h. [^{3}H]Thymidine incorporation was then determined. For each set of experimental parameters, incorporation is expressed as a percentage of that measured in the absence of TGF-β1 and αM.

Agents added	[^{3}H]Thymidine incorporation (%)
TGF-β1	45 ± 4
α_1M	102 ± 4
α_2M	97 ± 6
α_1M + TGF-β1	56 ± 4
α_2M + TGF-β1	90 ± 5

FBHE cell proliferation

To probe the hypothesis that native rat α_2M is the preferred carrier of TGF-β1 compared with native α_1M, we studied the neutralization of TGF-β1 by α_1M and α_2M in FBHE cell proliferation assays. TGF-β isoforms inhibit [^{3}H]thymidine incorporation in FBHE cells and we have previously shown that human α_2M counteracts the activity of TGF-β in this system [42]. Residual TGF-β activity in the presence of human α_2M is inversely correlated with the human α_2M concentration and the

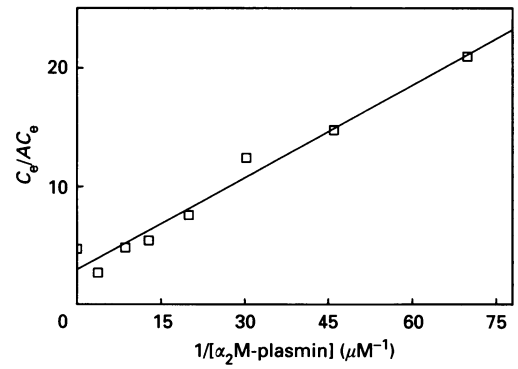

Figure 5 Determination of the K_D value for ^{125}I-TGF-β1 binding to rat α_2M–plasmin

The results of a representative experiment showing binding of ^{125}I-TGF-β1 to rat α_2M–plasmin are shown. Data were plotted according to eqn. (4). K_D values from four separate experiments were averaged to obtain the results presented in the text.

affinity of the TGF-β–human α_2M interaction [42,43]. In this study, FBHE cells were incubated with 5 pM TGF-β1, in the presence and absence of native rat α_1M or native rat α_2M (0.3 μM). The TGF-β1 concentration was near the reported IC_{50} for this assay [43] and, in the absence of αMs, [^{3}H]thymidine incorporation was decreased by 55% (Table 2). Native α_1M had only a slight effect on the activity of TGF-β1. By contrast, native α_2M almost completely neutralized the activity of TGF-β1. Neither αM significantly affected endothelial cell [^{3}H]thymidine incorporation in the absence of TGF-β1. The effects of native α_1M and α_2M on TGF-β1 activity in the FBHE proliferation assay are predicted by the K_D determinations and suggest an enhanced role for native rat α_2M, relative to native α_1M, as a growth-factor carrier and regulator in the acute-phase response.

TGF-β1 binding to rat α_2M–plasmin

Our studies demonstrated that rat α_2M-MA is not an appropriate model of the conformationally transformed α_2M structure. The results in Figure 1 suggested that rat α_2M–trypsin might bind less ^{125}I-TGF-β1 than native α_2M; however, we have previously shown that binding of cytokines to αM–trypsin complexes is highly dependent on whether 1 or 2 mol of trypsin are bound per mol of αM [33,46]. It is difficult to prepare a homogeneous preparation of α_2M–trypsin complex in binary stoicheiometry. Furthermore, α_2M–trypsin is not a physiologically significant α_2M–proteinase complex. For these reasons, we studied the binding of TGF-β1 to rat α_2M–plasmin complex. It is well documented that αMs bind plasmin primarily in 1:1 stoicheiometry [45,49], and our studies demonstrated that rat α_2M–plasmin complex formed with incorporation of 1.1 ± 0.1 mol of plasmin/mol of α_2M ($n = 3$). The majority of the plasmin (90%) was covalently associated with the rat α_2M, as determined by SDS/PAGE. Rat α_2M–plasmin complex demonstrated increased mobility on non-denaturing PAGE, confirming that the α_2M underwent conformational change (results not shown).

The apparent equilibrium analysis of ^{125}I-TGF-β1 binding to rat α_2M–plasmin is shown in Figure 5. The K_D was 61 ± 4 nM ($n = 4$). This value is significantly lower than that determined for the binding of ^{125}I-TGF-β1 to native α_2M ($P < 0.01$). The cross-linking efficiency (z) and range of covalent binding [$AC^*/(AC^* + AC)$] were 0.23 ± 0.02 and 0.01–0.02 respectively. These results indicate that conformational change in rat α_2M increases binding affinity for TGF-β1, consistent with the results obtained with other αMs.

DISCUSSION

In the normal adult rat, the plasma concentration of α_2M is approximately 15–150 nM [23]; however, during the acute-phase response, this may increase to 6–12 μM whereas the α_1M concentration remains constant. Interleukin 6 (IL-6) is the major inducer of rat hepatocyte α_2M synthesis *in vitro* [50] and *in vivo* [51]. Two copies of the type-II IL-6-responsive element have been identified in the promoter of the rat α_2M gene, accounting for the observed regulation of gene expression [52]. Other cytokines that induce expression of the rat α_2M gene include interleukin 11 and leukaemia inhibitory factor [23]. All of these cytokines may activate the same nuclear factor that binds to the type-II elements [23].

We hypothesized that inducible αMs provide enhanced cytokine/growth-factor carrier activity when the organism is challenged by disease, inflammation or foreign antigens. To explore this hypothesis, we chose to study binding of TGF-β1 to rat α_2M since extensive information is available on the TGF-β1–human α_2M interaction [28]. Numerous cytokines other than TGF-β1 also bind human α_2M, including platelet-derived growth factor, factor (BB) [29,33,53], interleukin 1β [54,55], IL-6 [56], tumour necrosis factor α [57] and neurotrophins [41]. Available data suggest that the α_2M-binding sites for many of these diverse cytokines may be equivalent or overlapping [28]. Although cytokines bind to human α_2M with widely variable affinity, eight of the nine cytokines studied by our laboratory to date, including TGF-β1, associate preferentially with the same human α_2M conformational variants and derivatives [28,29,41]. Therefore TGF-β1 was considered to be an appropriate model for initial studies on cytokine–αM interactions in rat.

The results of this investigation demonstrate that rat α_2M, in its native conformation, is preferred over native α_1M as a carrier of TGF-β1. The K_D for the TGF-β1–α_2M interaction was lower than that for the TGF-β1–α_1M interaction, and rat α_2M was a more efficient inhibitor of TGF-β1 bioactivity. Previous studies suggest that native αMs may be the most important cytokine carriers in the plasma [28,33,34]. Although conformationally transformed αMs bind most cytokines with higher affinity, these species are present in the blood at very low concentration because of rapid receptor-mediated plasma clearance ($t_{1/2}$ 3–5 min) [58]. ^{125}I-TGF-β1 and ^{125}I-platelet-derived growth factor (BB), when injected intravascularly in mice, associate largely with α_2M in the native conformation [28,32–34]. Furthermore, neutralization of TGF-β activity by serum in certain cell culture assays has been attributed to the native form of α_2M intrinsic to the serum [42,43]. Thus the ability of native rat α_2M to bind TGF-β1 with higher affinity than native rat α_1M may be important in determining TGF-β1 distribution in acute-phase plasma. A more complex pattern may arise if sufficient proteinases are generated to increase plasma levels of conformationally transformed αMs .

Given equal concentrations of α_1M and α_2M in the plasma during an acute-phase response, the K_D values predict that 70% of the TGF-β1 will associate with α_2M and 30% with α_1M at equilibrium. This prediction includes an assumption, shown to be valid for normal (non-acute-phase) plasma [33], that other TGF-β1-binding proteins do not contribute substantially to the plasma distribution of the growth factor. We speculate that the structural characteristics of native rat α_2M that increase binding affinity for TGF-β1 may also mediate increased binding of other cytokines such as TNF-α and IL-6.

At least two models have been proposed to explain why conformationally transformed αMs bind most cytokines with increased affinity [27,28]. First, conformational change may unmask or properly orient specific amino acids that contribute to the TGF-β1-binding site. Alternatively, thiol ester aminolysis or hydrolysis, which occurs during human α_2M conformational change, may provide critical thiol group(s) to participate in the binding site. Thiol-ester-derived cysteine residues are certainly involved in the slow covalent stabilization reaction (AC $\rightarrow$ AC*) since human α_2M has no other free cysteines. By contrast, we have shown that human α_2M-MA, when alkylated to block the thiol-ester-derived thiol groups, binds TGF-β1 and TGF-β2 with unchanged affinity [59], arguing against a role for the thiol groups in the non-covalent binding interaction. The studies presented here provide further evidence that release of thiol groups is not responsible for the increased TGF-β1-binding affinity of conformationally transformed αMs. Treatment of rat α_2M with methylamine disrupts the thiol esters revealing thiol groups [26], but in the absence of conformational change, does not increase the affinity for TGF-β1. By contrast, rat α_2M undergoes conformational change when it reacts with plasmin, and an increase in TGF-β1-binding affinity is observed.

Cytokines that associate with human α_2M-MA or α_2M–proteinase complexes may be rapidly taken up by cells that express LRP [32,60]. Therefore the fate of a cytokine that binds to α_2M is determined by the conformation of the carrier. Conformationally transformed αMs may be most important as cytokine carriers locally, when proteinases accumulate to high levels at sites of inflammation [27]. The rat αMs both bound TGF-β1 with higher affinity after conformational change. The K_D determined with α_1M-MA was actually lower than that determined with α_2M–plasmin; however, this finding should be interpreted with caution. Plasmin is a large proteinase that may partially interfere with the TGF-β1-binding site in α_2M in much the same way that a second molecule of trypsin in human α_2M–trypsin complex inhibits binding of TGF-β1 [46]. Detailed studies comparing the cytokine-binding affinities of methylamine- and proteinase-modified αMs are not available at this time. Nevertheless, it is reasonable to speculate that, although conformational change in αMs increases affinity for TGF-β1 and other cytokines, the extent of the increase may be somewhat influenced by the reagent (amine/proteinase) used to transform the αM.

In conclusion, this study has demonstrated that the two αMs from rat bind TGF-β1. For both, conformational change increases TGF-β1-binding affinity. The native form of rat α_2M binds TGF-β1 with higher affinity and is more efficient than native rat α_1M at neutralizing TGF-β1 in a bioassay. Thus rat α_2M may be an important cytokine carrier in the acute-phase response.

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