Binding of transforming growth factor- β 1 to methylamine-modified α_2 -macroglobulin and to binary and ternary α_2 -macroglobulin–proteinase complexes

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The binding of ^{125}I -labelled transforming growth factor- β 1 (TGF- β 1) to human α_2 -macroglobulin ($\alpha_2\text{M}$) was studied by native PAGE and autoradiography. TGF- β 1 bound preferentially to $\alpha_2\text{M}$ -methylamine and minimally, if at all, to native $\alpha_2\text{M}$. Preparations of $\alpha_2\text{M}$ -proteinase complex were generated by incubating a standard concentration of $\alpha_2\text{M}$ (0.4 μM) with different concentrations of trypsin, chymotrypsin or neutrophil elastase (0.04–2.0 μM). The ^{125}I -TGF- β 1-binding activity depended on the initial ratio of active proteinase to $\alpha_2\text{M}$, or r value, used to form the $\alpha_2\text{M}$ -proteinase complex. With all three proteinases, r values of 2 or greater yielded preparations with unchanged or decreased TGF- β 1-binding activity relative to native $\alpha_2\text{M}$. By contrast, r values near 1 yielded preparations with significantly increased TGF- β 1-binding activity. The results of [^3H]thymidine-incorporation studies performed in mouse keratinocytes were consistent with the ^{125}I -TGF- β -binding experiments. $\alpha_2\text{M}$ -trypsin and $\alpha_2\text{M}$ -chymotrypsin prepared at an r value of 1.0 counteracted the activity of TGF- β 1, whereas the equivalent complexes prepared at an r value of 3.0 had no effect. As determined by SDS/PAGE, ^{125}I -TGF- β 1 binding to $\alpha_2\text{M}$ -methylamine was at least 80 % non-covalent. Reaction of $\alpha_2\text{M}$ -methylamine with iodoacetamide or 5,5'-dithiobis-(2-nitrobenzoic acid) decreased the percentage of covalent binding but had no effect on total binding. Neuraminidase treatment had no effect on the binding of ^{125}I -TGF- β 1 to $\alpha_2\text{M}$ -methylamine. Cleavage of the 'bait regions' in $\alpha_2\text{M}$ -methylamine by prolonged treatment with trypsin also had no effect. These studies suggest that TGF- β 1 binding to $\alpha_2\text{M}$ is enhanced by conformational change in the proteinase inhibitor resulting from reaction with proteinase or amine. If both proteinase-binding sites in a single $\alpha_2\text{M}$ molecule are occupied, TGF- β 1-binding activity is decreased or perhaps eliminated.

INTRODUCTION

Human α_2 -macroglobulin ($\alpha_2\text{M}$) is a large plasma glycoprotein (approx. 718 kDa) that inhibits proteinases from all four major classes [1]. The native proteinase inhibitor consists of four identical subunits covalently linked into dimers by disulphide bonds and into intact tetramers by strong non-covalent interactions [2,3]. The sequence of $\alpha_2\text{M}$ includes a series of peptide bonds that are readily cleaved by proteinases of diverse specificity [1,4,5]. These peptide bonds, located near the centre of the $\alpha_2\text{M}$ subunit, form what is termed the 'bait region'. Peptide bond cleavage in the $\alpha_2\text{M}$ bait region causes exposure of β -glutamyl- γ -cysteinyl thioester bonds (one per subunit), which react rapidly with nucleophiles, including proteinase lysine ϵ -amino groups or water [5]. As the result of bait-region cleavage and thioester lysis, $\alpha_2\text{M}$ undergoes a major conformational change, trapping the proteinase in a non-dissociable complex [6–8]. Up to 2 mol of some proteinases (including trypsin, chymotrypsin and elastase) can bind per mol of $\alpha_2\text{M}$ [5,8–11], suggesting that presence of two distinct proteinase-binding sites (one per $\alpha_2\text{M}$ half-molecule).

Small primary amines such as methylamine react directly with $\alpha_2\text{M}$ thioester bonds, causing a conformational change that is analogous to the change caused by proteinase [7]. As the result of thioester cleavage, 4 mol of titratable thiol groups is exposed per mol of $\alpha_2\text{M}$ (one per subunit) [12]. $\alpha_2\text{M}$ conformational change may be detected by native PAGE; the mobility of unmodified $\alpha_2\text{M}$ (slow form) is less than that of $\alpha_2\text{M}$ -

methylamine or $\alpha_2\text{M}$ -proteinase complex (fast form) [9]. $\alpha_2\text{M}$ -methylamine and $\alpha_2\text{M}$ -proteinase complexes are recognized by specific receptors on macrophages, hepatocytes and fibroblasts [13]. These receptors mediate the rapid clearance of the altered protein from the circulation.

$\alpha_2\text{M}$ is a potent inhibitor of matrix metalloproteinases [14–16]; however, the physiological function of $\alpha_2\text{M}$ as a proteinase inhibitor remains vague. Recent investigations have demonstrated that $\alpha_2\text{M}$ binds a number of growth factors and cytokines, including nerve growth factor [17,18], platelet-derived growth factor [19,20], basic fibroblast growth factor [21], interleukin-1 β [22,23], interleukin-6 [24], transforming growth factor- β 1 (TGF- β 1) [25,26], transforming growth factor- β 2 [27,28], tumour necrosis factor- α [29] and insulin [30]. These studies suggest that $\alpha_2\text{M}$ plays an essential role in cellular growth regulation.

The binding of cytokines to $\alpha_2\text{M}$ has not been completely characterized; however, the nature of the interaction varies significantly with different cytokines. Interleukin-1 β binds exclusively to fast-form $\alpha_2\text{M}$ via a mechanism that primarily involves thiol/disulphide interchange [22,23]. Platelet-derived growth factor apparently binds to slow-form and fast-form $\alpha_2\text{M}$ [20]. Insulin binds covalently to $\alpha_2\text{M}$ only when present during $\alpha_2\text{M}$ conformational change [30]. In this reaction, the insulin behaves as a nucleophile attacking newly exposed thioester bonds and forming γ -glutamylamide linkages.

The interaction of $\alpha_2\text{M}$ with TGF- β 1 has been studied in a number of laboratories [25–28,31]. TGF- β 1 is a member of a

Abbreviations used: $\alpha_2\text{M}$, α_2 -macroglobulin; TGF- β 1, transforming growth factor- β 1; PNPGB, p -nitrophenyl p' -guanidinobenzoate hydrochloride; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); MeOSuc-, 3-carboxy-3-methoxypropionyl-; Suc-, 3-carboxypropionyl-; -NH-Np, p -nitroanilide.

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large family of multifunctional cytokines that express proliferative and anti-proliferative activity (for review see ref. [32]). The active form of TGF- β 1 is a 25 kDa disulphide-linked homodimer [33], which is secreted by cells as part of a large non-covalent latent complex [34]. One level of TGF- β 1 activity regulation probably involves activation of the latent form, a process that may be mediated by proteinases, endoglycosidases or changes in pH [33,35,36]. Once the active 25 kDa factor is released from the latency complex, other macromolecules such as α_2 M or beta-glycan [37] may bind TGF- β 1, altering availability of the cytokine to cellular receptors. α_2 M is the major TGF- β 1-binding protein in serum [25]. Receptor binding of 125 I-TGF- β 1 to A549 human carcinoma cells is inhibited by α_2 M [25,28]. Purified α_2 M also decreases the mitoinhibitory activity of TGF- β 1 towards hepatocytes in culture [27]. Finally, TGF- β 1- α_2 M-methylamine complexes are rapidly cleared from the circulation of mice via the α_2 M receptor, suggesting a possible role for the α_2 M receptor in the regulation of growth-factor activity *in vivo* [38].

A number of reports have suggested that TGF- β 1 binds preferentially to α_2 M fast form; however, this conclusion has been based on studies with α_2 M-methylamine [26,31,38]. LaMarre *et al.* [31] demonstrated that the TGF- β 1-binding activity of α_2 M-plasmin complex is increased compared with native α_2 M; however, the binding activity of α_2 M-trypsin complex was significantly decreased [26,31]. Therefore α_2 M fast form characteristics other than conformation must be important in determining TGF- β 1-binding activity.

In the present investigation we examined the characteristics of α_2 M that might affect TGF- β 1-binding activity. Our results demonstrate that the stoichiometry of the α_2 M-proteinase complex (mol of proteinase/mol of α_2 M) is important in determining TGF- β 1-binding activity. Binary (1:1) complexes of α_2 M with trypsin, chymotrypsin and human neutrophil elastase show enhanced binding of TGF- β 1 relative to native α_2 M. When the α_2 M is saturated with the same proteinases to form ternary (2:1) α_2 M-proteinase complexes, TGF- β 1 binding is unchanged or decreased, confirming earlier reports. Under most physiological conditions the concentration of α_2 M probably exceeds that of proteinases, and binary α_2 M-proteinase complexes are principally formed [8]. Therefore binary α_2 M-proteinase complexes may be highly significant as regulators of growth-factor activity *in vivo*.

MATERIALS AND METHODS

Proteins and reagents

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride (PNPGB), methylamine hydrochloride, iodoacetamide, bovine trypsin, α -chymotrypsin and BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). MeOSuc-Ala-Ala-Pro-Val-NH-Np (elastase substrate), MeOSuc-Ala-Ala-Pro-Val-CH₂Cl (elastase inhibitor), Suc-Ala-Ala-Pro-Phe-NH-Np (chymotrypsin substrate), chymostatin (chymotrypsin inhibitor) and neuraminidase were purchased from Boehringer Mannheim (Mannheim, Germany). Human sputum neutrophil elastase was obtained from Elastin Products Co. (Pacific, MO, U.S.A.).

Human α_2 M was purified by the method of Imber & Pizzo [39]. α_2 M-methylamine was prepared by incubating α_2 M with 200 mM-methylamine in 50 mM-Tris/HCl buffer, pH 8.2, for 6 h at 22 °C. The preparation was then extensively dialysed against phosphate-buffered saline in 20 mM-sodium phosphate (150 mM-NaCl buffer, pH 7.4). Free thiol groups in α_2 M-methylamine were modified by incubation with 2.0 mM-iodoacetamide or 0.5 mM-DTNB for 1 h at 22 °C. The final preparations were then dialysed exhaustively against phosphate-buffered saline.

Pig TGF- β 1 was purchased from R&D Systems (Minneapolis, MN, U.S.A.) and labelled with Na 125 I (Amersham, Arlington Heights, IL, U.S.A.) by the method of Ruff & Rizzino [40]. 125 I-TGF- β 1 was separated from free Na 125 I by chromatography on Sephadex G-25 equilibrated in 4 mM-HCl/100 mM-NaCl containing 1 mg of BSA/ml. The specific radioactivity ranged from 50 to 100 μ Ci/ μ g of protein. 125 I-TGF- β 1 prepared in this laboratory and 125 I-TGF- β 1 purchased from R&D Systems interact with α_2 M identically [38].

Protein concentrations

The concentration of α_2 M in purified preparations was determined from the absorbance at 280 nm by using an absorption coefficient (1.0%, 1 cm) of 8.93 [41]. Trypsin was active-site-titrated with PNPGB as previously described [42]. Concentrations of α -chymotrypsin were determined by measuring the hydrolysis of Suc-Ala-Ala-Pro-Phe-NH-Np in 0.1 M-Tris/HCl buffer, pH 8.2, containing 10 mM-CaCl₂ and using the constants $K_m = 0.043$ mM and $k_{cat} = 45$ s $^{-1}$ [43]. The concentration of active elastase was determined by measuring the rate of MeOSuc-Ala-Ala-Pro-Val-NH-Np hydrolysis in 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.01% Tween-20 and using the constants $K_m = 0.14$ mM and $K_{cat} = 17$ s $^{-1}$ [44].

DTNB titrations

The thiol group content of various α_2 M preparations was determined by titration with DTNB as described by Ellman [45]. The molar absorption coefficient for 3-carboxy-4-nitrophenolate ion at 412 nm is 13 600 M $^{-1}$ ·cm $^{-1}$.

Electrophoresis

Native PAGE was performed on 5% slabs with the buffers described by Van Leuven *et al.* [46]. In this system free 125 I-TGF- β 1 is not recovered in the gel. SDS/PAGE was performed on 5% gels with the Hepes/imidazole buffer system, pH 7.4, of McLellan [47]. Binding of 125 I-TGF- β 1 to α_2 M was detected by autoradiography, and quantified by slicing each lane into 3 mm slices. The radioactivity in the slices was determined in a γ -radiation counter.

Formation of α_2 M-proteinase-TGF- β 1 complexes

Native α_2 M (0.4 μ M) was treated with various concentrations of trypsin, chymotrypsin or elastase (0.04–2.0 μ M) in phosphate-buffered saline for 10 min at 22 °C. Proteinases that are bound to α_2 M retain activity towards small substrates [48]. Therefore low-molecular-mass proteinase inhibitors (as listed above) were added to each incubation mixture in order to inactivate residual free proteinase and proteinase bound to α_2 M. The final concentration of each low-molecular-mass inhibitor was 200 μ M. The incubation time was 10 min.

125 I-TGF- β 1 (0.7–2.0 nM) was incubated with the α_2 M-proteinase preparations, native α_2 M or α_2 M-methylamine for 1.5 h at 37 °C. Complex-formation was assessed by native PAGE as described above. In some experiments α_2 M-proteinase complexes were purified by gel-filtration chromatography on Superose-6 equilibrated in phosphate-buffered saline (0.4 ml/min). Binding of 125 I-TGF- β 1 by the purified α_2 M-proteinase complexes was unchanged. These results suggest that the uncomplexed proteinases and the low-molecular-mass proteinase inhibitors did not affect the binding of TGF- β 1 to α_2 M.

Evaluation of covalent binding of TGF- β 1 to α_2 M

125 I-TGF- β 1 (2.0 nM) was incubated with native α_2 M, α_2 M-methylamine, iodoacetamide-treated α_2 M-methylamine and DTNB-treated α_2 M-methylamine (0.7 μ M) for 1.5 h at 37 °C. 125 I-TGF- β 1- α_2 M complexes were then purified by chromato-

graphy on Superose-6. Recovery of α_2 M in chromatography fractions was monitored by the absorbance at 280 nm. Total binding of TGF- β 1 (covalent and non-covalent) was determined by measuring the radioactivity eluted in the high-molecular-mass fractions with α_2 M. As determined previously [38], more than 90 % of the 125 I-TGF- β 1 is recovered from Superose-6 when the cytokine is bound to α_2 M. Purified 125 I-TGF- β 1- α_2 M complexes were immediately subjected to SDS/PAGE in order to quantify covalent binding by autoradiography and gel slicing.

Neuraminidase treatment of α_2 M

Native α_2 M and α_2 M-methylamine (0.6 μ M) were incubated with 0.01 unit of neuraminidase in phosphate-buffered saline at 37 °C for 15 min. 125 I-TGF- β 1 (2.0 nM) was then added to each solution and incubation was continued for an additional 1.5 h. The effect of neuraminidase on α_2 M was examined by native PAGE after the initial 15 min incubation and after the complete 105 min incubation. Binding of 125 I-TGF- β 1 to the various forms of α_2 M was determined by autoradiography and gel slicing. The active form of TGF- β 1 is not a glycoprotein [32].

TGF- β 1 activity *in vitro*

BALB/MK mouse keratinocytes were kindly provided by Dr. Harold Moses (Vanderbilt University). These cells were plated in 24-well cell-culture plates at 40000 cells per well in Minimal Essential Medium (GIBCO) containing 0.05 mM-Ca²⁺, 8.0 % (v/v) dialysed fetal bovine serum and epidermal growth factor (4 ng/ml), as described by Perkett *et al.* [49]. After 24 h, the medium in each well was replaced with fresh medium containing TGF- β 1 (0.5 ng/ml) and native α_2 M, α_2 M-methylamine or α_2 M-proteinase complex (50 μ g/ml). The cells were incubated at 37 °C for an additional 22 h, and then [³H]thymidine (2.5 μ Ci) was added to each well for 2 h. The cells were washed with 10 % (w/v) trichloroacetic acid and solubilized with 0.2 M-NaOH. Radioactivity was determined in a scintillation counter.

TGF- β 1 inhibited [³H]thymidine incorporation by 60–80 %. The effect of α_2 M on the activity of TGF- β 1 was expressed as the percentage increase in [³H]thymidine incorporation relative to cells that were incubated with only TGF- β 1 (no α_2 M). Values represent the means $\pm$ S.E.M. ($n = 6$). Native α_2 M, α_2 M-methylamine and α_2 M-proteinase did not affect [³H]thymidine incorporation in the absence of TGF- β 1.

RESULTS

Binding of TGF- β 1 to α_2 M-trypsin

Previous studies demonstrated that the TGF- β 1-binding activity of α_2 M-trypsin is unchanged or decreased compared with native α_2 M. In the present investigation, the binding of 125 I-TGF- β 1 to α_2 M-trypsin was re-examined with preparations formed at different r values (ratio of active trypsin to α_2 M). Under the conditions applied here, trypsin binding to α_2 M is quantitative for r values of 2 or less [8]. Since there is no positive cooperativity for the binding of trypsin to the two distinct proteinase-binding sites in a single α_2 M molecule, different r values yielded different amounts of binary α_2 M-trypsin (1 mol of trypsin/mol of α_2 M) and ternary α_2 M-trypsin (2 mol/mol) [8,50]. Fig. 1 shows that the 125 I-TGF- β 1-binding activity of α_2 M-trypsin was clearly a function of the r value. For r values greater than 2, exclusively ternary α_2 M-trypsin was present and TGF- β 1 binding was decreased compared with native α_2 M. r values greater than 0 but less than 2 generated some binary α_2 M-trypsin; the fraction of binary α_2 M-trypsin was greatest for r approx. 1.0 [8]. These α_2 M-trypsin preparations demonstrated enhanced 125 I-TGF- β 1-binding activity compared with native α_2 M. Binding

Fig. 1. Binding of 125 I-TGF- β 1 to α_2 M-trypsin as determined by native PAGE and autoradiography

Native α_2 M was treated with increasing amounts of trypsin for 10 min at 22 °C. The ratio of active trypsin to α_2 M is shown by r . The active trypsin was inhibited with PNPGB, and 125 I-TGF- β 1 was added for 1.5 h at 37 °C. Samples were analysed by PAGE. Panel (a) shows the Coomassie Blue-stained gel; panel (b) is the autoradiograph. Migration of native α_2 M ('slow') and α_2 M-methylamine ('fast') is designated by s and f respectively. For comparison, the binding of 125 I-TGF- β 1 to α_2 M-methylamine is also shown.

was greatest for the α_2 M-trypsin preparation formed at an r value of 1.0. These studies strongly suggest that 125 I-TGF- β 1-binding activity in α_2 M-trypsin preparations is a function of the binary complex.

Comparison of different α_2 M-proteinase complexes

α_2 M-chymotrypsin and α_2 M-elastase preparations were formed at different r values, as described for α_2 M-trypsin. 125 I-TGF- β 1-binding activity was determined by native PAGE, autoradiography and gel slicing (Fig. 2). Once again, TGF- β 1 binding varied as a function of r value. Optimal binding was observed with α_2 M-proteinase preparations formed at r values near 1.0. As the r value was increased above 1.0, TGF- β 1 binding to α_2 M-chymotrypsin and α_2 M-elastase decreased. With both α_2 M-proteinase complexes, TGF- β 1 binding was unchanged or decreased relative to native α_2 M when the α_2 M was apparently saturated with proteinase.

Compared with α_2 M-methylamine, 125 I-TGF- β 1 binding to the α_2 M-proteinase complexes ($r = 1.0$) was decreased by about 50 %. In order to determine whether the intact bait regions of α_2 M-methylamine are necessary for increased TGF- β 1 binding, α_2 M-methylamine was treated with a 20-fold molar excess of trypsin for 8 h. SDS/PAGE under reducing conditions demonstrated complete bait-region cleavage (conversion of the 180 kDa subunit into the 90 kDa cleavage products). 125 I-TGF- β 1 binding to this preparation, as determined by native PAGE, was unchanged compared with α_2 M-methylamine before proteolysis.

Binding of TGF- β 1 to native α_2 M and α_2 M-methylamine

In order to examine TGF- β 1 binding, α_2 M-methylamine was prepared as described in the Materials and methods section and studied within 24 h (α_2 M-M). DTNB titration revealed 3.5 mol of thiol groups/mol of α_2 M-M. A second α_2 M-methylamine preparation (α_2 M-M*) was stored at 4 °C for 2 months. DTNB titration detected 2.0 mol of free thiol groups/mol of α_2 M-M*. Table 1 shows that α_2 M-M and α_2 M-M* bound equivalent amounts of 125 I-TGF- β 1, as determined by chromatography on Superose-6. α_2 M-M that was modified with iodoacetamide demonstrated no free thiol groups by DTNB titration (sensitivity

Fig. 2. Binding of ^{125}I -TGF- β 1 to $\alpha_2\text{M}$ complexed with trypsin, chymotrypsin and elastase

Native $\alpha_2\text{M}$ was treated with increasing amounts of trypsin (panel a), elastase (panel b) or chymotrypsin (panel c) for 10 min at 22 °C. The ratio of proteinase to $\alpha_2\text{M}$ (r) was varied from 0.1 to 5.0. Proteinases were inhibited by the addition of specific low-molecular-mass inhibitors (listed in the Materials and methods section). ^{125}I -TGF- β 1 was then incubated with each preparation for 1.5 h at 37 °C. Samples were analysed by native PAGE followed by gel slicing to quantify the amount of ^{125}I -TGF- β 1 bound. Each experiment was performed three times with duplicate determinations for each point ($\pm$ S.E.M.).

0.05 mol of thiol groups/mol of $\alpha_2\text{M}$). Iodoacetamide-treated $\alpha_2\text{M}$ -M and $\alpha_2\text{M}$ -M modified with DTNB demonstrated unchanged ^{125}I -TGF- β 1-binding activity compared with unmodified $\alpha_2\text{M}$ -M.

Covalent binding of ^{125}I -TGF- β 1 to $\alpha_2\text{M}$ -methylamine was determined by SDS/PAGE with chromatography-purified complexes. As shown in Table 1, 10–20 % of the ^{125}I -TGF- β 1 bound covalently to $\alpha_2\text{M}$ -methylamine. After modification of the free thiol groups with either iodoacetamide or DTNB, covalent binding was decreased to less than 5 %. These results suggest that the low level of covalent binding is the result of thiol/disulphide interchange.

In the experiments presented in Table 1, the $\alpha_2\text{M}$ preparations were present at 350-fold molar excess to the ^{125}I -TGF- β 1. In order to determine if a low concentration of unmodified $\alpha_2\text{M}$ -methylamine in the iodoacetamide-treated preparations affected our results, the molar ratio of $\alpha_2\text{M}$ to ^{125}I -TGF- β 1 was varied. Fig. 3 shows that the TGF- β 1-binding activity of iodoacetamide-treated $\alpha_2\text{M}$ -methylamine and untreated $\alpha_2\text{M}$ -methylamine

Table 1. Analysis of TGF- β 1 binding to $\alpha_2\text{M}$ by Superose-6 chromatography

Various $\alpha_2\text{M}$ preparations were incubated with ^{125}I -TGF- β 1 for 1.5 h at 37 °C and subjected to f.p.l.c. on Superose-6. Total ^{125}I -TGF- β 1 binding (covalent and non-covalent) was determined by the percentage of radioactivity eluted with the $\alpha_2\text{M}$. Covalent binding was determined by SDS/PAGE with f.p.l.c.-purified ^{125}I -TGF- β 1- $\alpha_2\text{M}$ complexes. The ^{125}I -TGF- β 1 covalently bound is expressed as a percentage of the total (covalent and non-covalent) binding.

$\alpha_2\text{M}$ species	^{125}I -TGF- β 1 bound (%)	^{125}I -TGF- β 1 covalently bound (% of total)
Native $\alpha_2\text{M}$	6.5	1.0
$\alpha_2\text{M}$ -methylamine ($\alpha_2\text{M}$ -M)	52.2	21.2
$\alpha_2\text{M}$ -methylamine ($\alpha_2\text{M}$ -M*)	50.0	10.9
Iodoacetamide-treated $\alpha_2\text{M}$ -M	48.8	4.9
DTNB-treated $\alpha_2\text{M}$ -M	43.5	5.2

Fig. 3. Binding of ^{125}I -TGF- β 1 to different concentrations of native $\alpha_2\text{M}$, $\alpha_2\text{M}$ -methylamine and iodoacetamide-treated $\alpha_2\text{M}$ -methylamine

Different concentrations of native $\alpha_2\text{M}$ ($\square$), $\alpha_2\text{M}$ -methylamine ($\circ$) or iodoacetamide-treated $\alpha_2\text{M}$ ($\bullet$) were incubated with 0.7 nM ^{125}I -TGF- β 1 for 1.5 h at 37 °C. Binding was determined by native PAGE.

remained equivalent as the $\alpha_2\text{M}$ /TGF- β 1 molar ratio was decreased from 1000:1 to 3.0:1.

When different preparations of native $\alpha_2\text{M}$ were studied, the binding of ^{125}I -TGF- β 1 was somewhat variable and sometimes undetectable. As shown in Fig. 1, the mobility of ^{125}I -TGF- β 1 bound to native $\alpha_2\text{M}$ (by autoradiography) was actually somewhat faster than the Coomassie Blue-stained slow form, confirming the results of a previous study [38]. On the basis of a standard curve plotting TGF- β 1-binding activity against $\alpha_2\text{M}$ -methylamine concentration (as shown in Fig. 3), we determined that contamination of our native $\alpha_2\text{M}$ preparations with 0.4 % fast form was sufficient to account for the TGF- β 1-binding activity detected.

Neuraminidase treatment of $\alpha_2\text{M}$ -methylamine

The effect of neuraminidase on the TGF- β 1-binding activity of $\alpha_2\text{M}$ was investigated. After treatment with 0.01 unit of neuraminidase for 15 or 105 min, native $\alpha_2\text{M}$ and $\alpha_2\text{M}$ -methylamine demonstrated decreased mobility by native PAGE, most probably reflecting loss of negative charge (Fig. 4). Similar results were presented by Barrett *et al.* [9]. $\alpha_2\text{M}$ -M* (stored for 2 months at 4 °C) already demonstrated decreased mobility and was less affected by the neuraminidase. The ^{125}I -TGF- β 1-binding

Fig. 4. Effect of neuraminidase on the ^{125}I -TGF- β 1-binding activity of $\alpha_2\text{M}$

Freshly prepared native $\alpha_2\text{M}$ (lanes a), native $\alpha_2\text{M}$ stored at 4 °C for 2 months (lanes c), freshly prepared $\alpha_2\text{M}$ -methylamine (lanes b) and $\alpha_2\text{M}$ -methylamine stored at 4 °C for 2 months (lanes d) were incubated with or without 0.01 unit of neuraminidase for 15 min at 37 °C. ^{125}I -TGF- β 1 was then added and incubated for 1.5 h. Samples were analysed by native PAGE. Panel (a) shows the Coomassie Blue-stained gel; panel (b) shows the autoradiograph.

Table 2. Effect of $\alpha_2\text{M}$ on TGF- β 1-induced mitoinhibition in mouse keratinocytes

Various $\alpha_2\text{M}$ species were present with or without TGF- β 1 before the addition of [^3H]thymidine. The r value refers to the preparation of $\alpha_2\text{M}$ -proteinase complex, as described in the text. Incorporation (means $\pm$ S.E.M., $n = 6$) is expressed as the percentage increase under each set of conditions relative to the control (TGF- β 1 present/no $\alpha_2\text{M}$).

TGF- β 1 present (+/-)	$\alpha_2\text{M}$ variant	r value	Increase in incorporation (%)
+	None	-	0 $\pm$ 1
-	None	-	173 $\pm$ 34
+	Native $\alpha_2\text{M}$	-	1 $\pm$ 2
+	$\alpha_2\text{M}$ -methylamine	-	122 $\pm$ 9
+	$\alpha_2\text{M}$ -trypsin	1.0	21 $\pm$ 6
+	$\alpha_2\text{M}$ -trypsin	3.0	6 $\pm$ 4
+	$\alpha_2\text{M}$ -chymotrypsin	1.0	49 $\pm$ 5
+	$\alpha_2\text{M}$ -chymotrypsin	3.0	-3 $\pm$ 4

activity of $\alpha_2\text{M}$ -methylamine (and $\alpha_2\text{M}$ -M*) was unchanged by neuraminidase. The TGF- β 1-binding activity of native $\alpha_2\text{M}$ was slightly increased or unchanged.

Inhibition of TGF- β 1 activity in cell culture

TGF- β 1 inhibited [^3H]thymidine incorporation by mouse keratinocytes *in vitro* (Table 2), confirming the work of other investigators [51]. The keratinocyte cell-culture medium included 8.0 % fetal bovine serum, a necessary component. Therefore it is not clear whether the bovine $\alpha_2\text{M}$ present in the fetal bovine serum affected the level of inhibition observed with TGF- β 1.

[^3H]Thymidine-incorporation experiments were performed with cell-culture medium that was supplemented with various $\alpha_2\text{M}$ preparations. $\alpha_2\text{M}$ -methylamine significantly increased [^3H]thymidine incorporation in the presence of TGF- β 1, whereas native $\alpha_2\text{M}$ did not. $\alpha_2\text{M}$ -trypsin and $\alpha_2\text{M}$ -chymotrypsin prepared at an r value of 3.0 had no effect on incorporation. By

contrast, the same $\alpha_2\text{M}$ -proteinase complexes prepared at an r value of 1.0 significantly counteracted the activity of TGF- β 1 ($P < 0.08$ for $\alpha_2\text{M}$ -trypsin and $P < 0.001$ for $\alpha_2\text{M}$ -chymotrypsin by unpaired two-tailed t test).

DISCUSSION

The results of other investigations have suggested that TGF- β 1 binds primarily, or even exclusively, to the fast form of $\alpha_2\text{M}$ [26,31,38]. This conclusion was based on comparisons of native $\alpha_2\text{M}$ and $\alpha_2\text{M}$ -methylamine. Most spectroscopic, hydrodynamic and functional studies suggest that $\alpha_2\text{M}$ -methylamine is an appropriate model for the $\alpha_2\text{M}$ fast form, which includes $\alpha_2\text{M}$ -proteinase complexes [5-7]. With regard to TGF- β 1 binding, however, the apparent difference between $\alpha_2\text{M}$ -methylamine and $\alpha_2\text{M}$ -trypsin represented a significant inconsistency [26,31].

LaMarre *et al.* [31] demonstrated that $\alpha_2\text{M}$ -plasmin binds increased amounts of TGF- β 1 relative to native $\alpha_2\text{M}$. This was the first report of an $\alpha_2\text{M}$ -proteinase complex with enhanced TGF- β 1-binding activity. In the present investigation the TGF- β 1-binding activity of $\alpha_2\text{M}$ -trypsin was re-evaluated together with other $\alpha_2\text{M}$ -proteinase complexes. Saturating concentrations of trypsin, elastase or chymotrypsin yielded $\alpha_2\text{M}$ -proteinase complexes with minimal TGF- β 1-binding activity. By contrast, non-saturating concentrations of the same proteinases consistently yielded preparations with increased TGF- β 1-binding activity. On the basis of these studies, we proposed that all proteinases that react with $\alpha_2\text{M}$ are capable of enhancing TGF- β 1-binding activity. The critical event resulting in increased TGF- β 1 binding is probably $\alpha_2\text{M}$ conformational change. Since $\alpha_2\text{M}$ -proteinase complexes, like $\alpha_2\text{M}$ -methylamine, are recognized by $\alpha_2\text{M}$ receptors [13], the TGF- β 1-binding activity of $\alpha_2\text{M}$ resides with variants capable of mediating clearance of the cytokine from the circulation.

The formation of binary $\alpha_2\text{M}$ -proteinase and ternary $\alpha_2\text{M}$ -proteinase after incubation at various r values has been studied for chymotrypsin [10] and trypsin [8]. On the basis of the distributions observed in our experiments, it may be postulated that binary $\alpha_2\text{M}$ -proteinase complexes demonstrate enhanced TGF- β 1-binding activity whereas ternary complexes do not. Ternary $\alpha_2\text{M}$ -plasmin complexes have been reported [52,53]; however, $\alpha_2\text{M}$ -plasmin complexes formed under most conditions are binary. Therefore the previously reported TGF- β 1-binding activity of $\alpha_2\text{M}$ -plasmin [31] is consistent with the present study.

The structure of $\alpha_2\text{M}$ fast form probably resembles an incomplete hollow cylinder [53,54]. The two proteinase-binding sites or pockets are located within the cylinder on opposite sides of the inhibitor central plate. The hollow-cylinder model explains why $\alpha_2\text{M}$ -proteinase complexes are non-dissociable, even in the absence of a covalent linkage. The model also explains why most anti-proteinase antibodies do not recognize $\alpha_2\text{M}$ -proteinase complexes. Wakefield *et al.* [55] suggested that the TGF- β 1-binding site may be analogous with the proteinase-binding site. In support of this hypothesis, data were presented suggesting that anti-TGF- β 1 antibodies do not recognize $\alpha_2\text{M}$ -TGF- β 1 complex [25]. The inability of ternary $\alpha_2\text{M}$ -proteinase complexes to bind TGF- β 1 might also be explained by the model proposed by Wakefield *et al.* [55]. Accordingly, the two bound proteinases in a single $\alpha_2\text{M}$ complex would block entry of TGF- β 1 into the interior of the cylinder structure.

Previous studies regarding the interaction of $\alpha_2\text{M}$ with proteins other than TGF- β 1 suggest that the model proposed by Wakefield *et al.* [55] may not be correct. Once the $\alpha_2\text{M}$ 'trap' is shut and the hollow-cylinder structure is fully adopted, movement of proteinases such as trypsin (approx. 23.8 kDa) in or out of the trap is highly restricted [5,8,54]. Therefore it is unlikely, although

not inconceivable, that TGF- β 1, which is larger than trypsin, might have easy access to these internal binding sites.

As an alternative explanation for the decreased TGF- β 1-binding activity of ternary α_2 M-proteinase complexes, we propose that the bound proteinases may induce a localized but functionally significant change in the secondary or tertiary structure of the TGF- β 1-binding site in α_2 M fast form. At the present time the information necessary to distinguish between this hypothesis and the model proposed by Wakefield *et al.* [55] is not available.

Miyazono & Heldin [36] demonstrated that the latent-TGF- β 1 secretion complex can be dissociated by removing sialic acid residues from the *N*-terminal latency or pro-region [36]. By contrast, Wakefield *et al.* [56] demonstrated that the purified pro-region still associates with active TGF- β 1 after desialylation with neuraminidase. Results obtained in the present investigation demonstrated that α_2 M sialic acid residues are not critical for TGF- β 1-binding activity. This result was confirmed by using neuraminidase and by studying preparations with decreased sialic acid content due to prolonged storage. Therefore our studies suggest that TGF- β 1-binding activity is not a general property of glycoproteins with properly oriented sialic acid residues.

In the mouse keratinocyte system, TGF- β 1 activity was inhibited by α_2 M derivatives that bound 125 I-TGF- β 1 (as determined by native PAGE), whereas TGF- β 1 activity was unaffected by α_2 M derivatives that did not bind 125 I-TGF- β 1. Therefore the results of the cell-culture experiments supported the 125 I-TGF- β 1-binding analyses. The absolute activity of each α_2 M derivative was not always identical in both experimental systems. For example, α_2 M-trypsin ($r = 1.0$) increased cellular [3 H]thymidine incorporation by less than 20% compared with α_2 M-methylamine and the 125 I-TGF- β 1-binding activity of the same α_2 M-trypsin preparation was 50% of that demonstrated by α_2 M-methylamine. In the cell-culture system, incubations were conducted for 24 h and other macromolecules (including TGF- β cellular receptors) competed with α_2 M for TGF- β 1 binding. Therefore the relative activities of each α_2 M derivative in cell culture may have reflected TGF- β 1-binding properties (association rate, dissociation rate, equilibrium binding constants etc.) that were not characterized independently in the present investigation.

The binding of TGF- β 1 to α_2 M-methylamine is unique relative to many other α_2 M-cytokine and α_2 M-polypeptide interactions. Like interleukin-1 β [22,23], TGF- β 1 may undergo a thiol/disulphide interchange to form a covalent linkage with α_2 M-methylamine; however, this type of binding occurs in a minority of the complexes and is not critical for a high-affinity interaction. TGF- β 1 may also covalently bind to α_2 M by nucleophilic attack on emerging thioesters during conformational change, as has been reported for insulin [30]; the experiments presented here were not designed to address this question. However, reaction with thioesters is clearly not essential for TGF- β 1 binding to α_2 M, since TGF- β 1 associates with preformed purified α_2 M fast forms. All three basic mechanisms for the interaction of α_2 M with cytokines (as described for TGF- β 1, interleukin-1 β and insulin) may represent important arms of the α_2 M growth-regulatory system. The differences in binding mechanism for different cytokines may translate into important α_2 M functional properties *in vivo*. In addition, the mouse keratinocyte experiments presented here suggest that various forms of α_2 M in serum may influence growth-regulation and cell-physiology experiments performed *in vitro*.

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