Proteinases are isoform-specific regulators of the binding of transforming growth factor β to α_2-macroglobulin

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α_2-Macroglobulin (α_2M) regulates growth and gene expression in many cell types by binding and neutralizing transforming growth factor β (TGF-β). In this study we characterized the effects of the serine proteinase, plasmin, on the interaction of α_2M with TGF-β1 and TGF-β2. Binding of both TGF-β isoforms to purified α_2M–plasmin complex was primarily non-covalent and reversible. The binding affinity of α_2M for TGF-β1 was increased by plasmin; the K_d values were 320 and 84 nM for native α_2M and α_2M–plasmin respectively. In contrast the affinity of α_2M for TGF-β2 was decreased by plasmin; the K_d values were 14 and 80 nM for native α_2M and α_2M–plasmin respectively. Thrombin decreased the affinity of α_2M for TGF-β2 in a similar manner to plasmin. In assays of DNA synthesis in fetal bovine heart endothelial cells, native α_2M neutralized the activity of exogenously added TGF-β2, whereas α_2M–plasmin, at equivalent concentrations, had almost no effect. Native α_2M and methylamine-modified α_2M increased platelet-derived growth factor α-receptor expression in vascular smooth-muscle cells, an activity attributed to the neutralization of autocrine TGF-β activity, whereas α_2M–plasmin was less effective at the same concentration. These studies demonstrate that the effects of proteinases on the cytokine-binding and cytokine-neutralizing activities of α_2M are cytokine-dependent. By reacting with α_2M, proteinases might regulate not only the availability of cytokines in the extracellular spaces but also the composition of the cytokine milieu.

INTRODUCTION

α_2-Macroglobulin (α_2M) is a homotetrameric glycoprotein (M_r approx. 718000) that functions as an extracellular proteinase inhibitor and cytokine carrier [1–3]. Reaction with proteinases induces a major conformational change in α_2M, which traps the proteinase in a non-dissociable complex [4,5]. Conformational change also exposes the single β-cysteinyl-γ-glutamyl thiol-ester bond in each α_2M subunit; the thiol-esters react readily with nucleophilic entering groups or are hydrolysed by water [6–8]. Methylamine reacts directly with α_2M thiol-ester bonds, initiating a conformational change that is essentially identical with that caused by proteinases [9,10]. Methylamine-modified α_2M (α_2M-MA) and α_2M–proteinase complexes lack further proteinase-inhibitory activity and are both recognized equivalently by cellular receptors [2,3]. Thus α_2M-MA is frequently studied as a model of conformationally transformed α_2M.

Specific growth factors, including transforming growth factor β1 (TGF-β1) and TGF-β2, bind reversibly and non-covalently to native α_2M and α_2M-MA [11]. Once formed, TGF-β–α_2M-MA complexes can become covalently stabilized by thiol/disulphide exchange [12,13]. α_2M–proteinase complexes also bind cytokines [14–16]; however, the effects of proteinases on cytokine-binding affinity are not well characterized. α_2M can bind 2 mols/mol of certain proteinases (to form ternary complexes) and only 1 mol/mol of others (binary complexes). In early studies we demonstrated that binary α_2M–proteinase complexes are similar to α_2M-MA in that they bind increased amounts of TGF-β1 and platelet-derived growth factor BB (PDGF-BB) compared with native α_2M, whereas ternary α_2M–proteinase complexes bind greatly decreased amounts of the same cytokines [14–16]. Thus we hypothesized that α_2M-MA is an adequate model of binary α_2M–proteinase complexes in cytokine-binding studies.

More recently we used a rapid cross-linking method to determine equilibrium dissociation constants for the interaction of a number of cytokines with native α_2M and α_2M-MA [11,17–19]. All of the cytokines studied bound to α_2M-MA with higher affinity than to native α_2M, with the exception of TGF-β2, which bound to native α_2M and α_2M-MA with equivalent affinity. The K_d values determined for TGF-β isoforms and α_2M predicted the ability of the α_2M to neutralize TGF-β in cell culture systems [19,20].

Here we present the first equilibrium analysis of the binding of TGF-β1 and TGF-β2 to physiologically significant human α_2M–proteinase complexes. Our results demonstrate that plasmin increases the binding affinity of α_2M for TGF-β1, whereas both plasmin and thrombin decrease the binding affinity of α_2M for TGF-β2. Proteinase modification affected the activity of α_2M in assays of fetal bovine heart endothelial (FBHE) cell proliferation and studies of vascular smooth-muscle cell (VSMC) PDGF α-receptor expression. We conclude that proteinases that react with α_2M might alter not only the concentrations of cytokines in the extracellular spaces but also the balance between factors with supporting and/or opposing activities.

MATERIALS AND METHODS

Reagents and proteins

TGF-β1 and PDGF-AA were purchased from R & D Systems (Minneapolis, MN, U.S.A.). *p*-Nitrophenyl-*p'*-guanidinobenzoate (PNPGB) and BSA were from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium and trypsin/EDTA were from Life Technologies, Inc. Fetal bovine serum (FBS) was from Hyclone Laboratories

Abbreviations used: α_2M, α_2-macroglobulin; α_2M-MA, methylamine-modified α_2M; BS^3, bis(sulphosuccinimidyl)suberate; DMEM, Dulbecco's modified Eagle's medium; FBHE, fetal bovine heart endothelial; FBS, fetal bovine serum; lmw-uPA, low-molecular-mass urokinase plasminogen activator; PDGF, platelet-derived growth factor; PNPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; TGF-β, transforming growth factor β; VSMC, vascular smooth-muscle cell.

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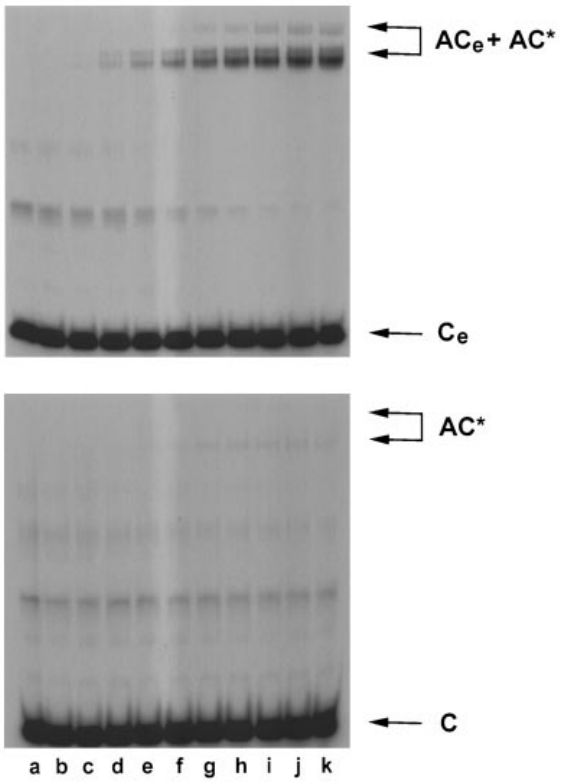

Figure 1 Autoradiographs showing BS^3-stabilized binding of ^{125}I-labelled TGF-β2 to α_2M–plasmin

^{125}I-labelled TGF-β2 was incubated with α_2M–plasmin for 1 h at 37 °C. The samples shown in the upper panel were pulse-exposed to 5 mM BS^3. The samples shown in the lower panel were not exposed to BS^3. All samples were subjected to SDS/PAGE and autoradiography. The concentrations of α_2M–plasmin were 0 nM (lane a), 3 nM (lane b), 6 nM (lane c), 12 nM (lane d), 24 nM (lane e), 48 nM (lane f), 96 nM (lane g), 0.19 μM (lane h), 0.38 μM (lane i), 0.77 μM (lane j) and 1.5 μM (lane k). The terms AC_e and C_e are defined in the text. AC* is a covalent α_2M-cytokine complex formed by thiol/disulphide exchange.

(Logan, UT, U.S.A.). Bis(sulphosuccinimidyl)suberate (BS^3) was from Pierce (Rockford, IL, U.S.A.). Na^{125}I was from Amersham (Arlington Heights, IL, U.S.A.). Low-molecular-mass urokinase plasminogen activator (lmw-uPA) was from Calbiochem (La Jolla, CA, U.S.A.). D-Valyl-L-leucyl-L-lysine nitroaniline dichloride (S-2251) was from Kabi Vitrum (Stockholm, Sweden). Plasminogen was purified from human plasma by lysine-Sepharose chromatography [21] and activated with lmw-uPA. The extent of activation was determined by the rate of S-2251 hydrolysis at 22 °C, with the kinetic parameters $k_{cat} = 11.7\ s^{-1}$ and $K_m = 0.18$ mM [22]. Human α-thrombin was kindly provided by Dr. J. Fenton (Wadsworth Laboratories, Albany, NY, U.S.A.), and active-site-titrated with PNPGB [23].

α_2M and related derivatives

α_2M was purified from human plasma by the method of Imber and Pizzo [24]. α_2M-MA was prepared by dialysing α_2M against 200 mM methylamine/HCl in 50 mM Tris/HCl, pH 8.2, for 12 h at 22 °C followed by exhaustive dialysis against 20 mM sodium phosphate/150 mM NaCl (pH 7.4) (PBS). α_2M–plasmin was prepared by incubating native α_2M with a 2.5-fold molar excess of active plasmin for 1 h at 22 °C and purified by chromatography on Superose-6 (flow rate 0.4 ml/min). The stoichiometry of plasmin binding to α_2M was 1.1 ± 0.2 mol of plasmin/mol of α_2M (determined by performing the same protocol with ^{125}I-labelled plasmin; result given $\pm$S.D., $n = 3$).

Radioiodination

Plasminogen was radioiodinated with Iodobeads, in accordance with the method suggested by the manufacturer (Pierce Chemical Co.). Specific activities were 1.0–1.5 μCi/μg. TGF-β1 and TGF-β2 were radioiodinated by the method of Ruff and Rizzino [25]. Specific activities were 100–200 μCi/μg. ^{125}I-labelled PDGF-AA (150–170 μCi/μg) was obtained from Biomedical Technologies (Stoughton, MA, U.S.A.).

Determination of apparent equilibrium dissociation constants

Equilibrium dissociation (K_d) constants were determined by the BS^3/rapid cross-linking method, as previously described [11,19]. Various concentrations of native α_2M, α_2M-MA, α_2M–plasmin complex or α_2M–thrombin complex were incubated with ^{125}I-labelled TGF-β1 or ^{125}I-labelled TGF-β2 (1.0 nM) in PBS with 75 μM BSA, for 1 h at 37 °C. BS^3 (in water) was then added to a final concentration of 5 mM and incubated for 1 min. Water alone was added to identical control incubations. Cross-linking was terminated by acidification. Samples were then denatured in 2.0 % (w/v) SDS and subjected to SDS/PAGE. Dried gels were subjected to autoradiography. The amount of free ^{125}I-labelled TGF-β and the amount of covalent α_2M–TGF-β complex (BS^3-stabilized plus covalent complex formed by thiol/disulphide exchange) were quantified by slicing the gels into sections and measuring the radioactivity in a γ-counter. Thiol/disulphide exchange products were quantified independently by analysing samples that had not been treated with BS^3. Apparent K_d values were determined from the following equation:

$$[C_e]/[AC_e] = (K_d/z)[A]^{-1} + (z^{-1} - 1) \quad (1)$$

where A is unbound α_2M, C is free (unbound) cytokine, AC is reversibly associated (non-covalent) α_2M–cytokine complex. The subscript e is used to denote that these are experimentally determined values, derived by analysis of the gels. The experimental values are related to the actual levels of free and reversibly bound TGF-β by the efficiency (z) of AC cross-linking by BS^3 [11]. K_d values determined by this method assume a single TGF-β-binding site per α_2M and that all of the α_2M in a given preparation binds TGF-β with equal affinity.

Inhibition of endothelial cell growth

FBHE cells were cultured in DMEM supplemented with 10 % (v/v) FBS, 20 ng/ml acidic fibroblast growth factor and 80 ng/ml basic fibroblast growth factor. Proliferation assays were performed in 0.2 % (v/v) serum as previously described [19,20]. Briefly, FBHE cells were treated with TGF-β2, in the presence or absence of various forms of α_2M, in DMEM with 0.2 % (v/v) FBS. After incubation for 30 h, [^{3}H]thymidine was added for an additional 18 h. The cells were harvested and [^{3}H]thymidine incorporation was determined [19,20].

Binding of ^{125}I-labelled PDGF-AA to VSMCs

VSMCs were isolated from Sprague–Dawley rat aortas by enzymic digestion [26] and cultured in a 1:1 formulation of DMEM and Ham's F-12 medium supplemented with 10 % (v/v) FBS, L-glutamine (0.68 mM), penicillin (100 i.u./ml) and streptomycin (100 μg/ml). To arrest the growth of the cells, cultures

were incubated in defined, serum-free medium for 4 days [27]. The quiescent VSMCs were then incubated in serum-free medium supplemented with native α_2M, α_2M-MA or α_2M–plasmin for 10 h at 37 °C. Cultures were chilled to 4 °C and washed with ice-cold binding medium (Ham's F-12 medium supplemented with 25 mM Hepes, 0.25% BSA and 1 mM $CaCl_2$, pH 7.4). ^{125}I-labelled PDGF-AA (200 pM) was added to the cultures, in the presence or absence of a 50-fold molar excess of non-radiolabelled PDGF-AA, and incubated for 4 h at 4 °C. The cells were washed twice with binding medium and twice with PBS supplemented with 1 mM $CaCl_2$ and 0.25% BSA. Cell-associated radioactivity was recovered by solubilizing the cells in 1% Triton X-100, 0.25% BSA and measured in a γ-counter. Cellular protein was determined in parallel cultures for each experiment with the use of the bicinchoninic acid assay.

RESULTS

Binding of TGF-β1 and TGF-β2 to α_2M–proteinase complexes

^{125}I-labelled TGF-β1 and ^{125}I-labelled TGF-β2 bound to purified α_2M–plasmin, as determined by the BS^3/rapid cross-linking method. In time-course experiments, the extent of non-covalent TGF-β binding maximized rapidly (results not shown), consistent with the results of previous studies on TGF-β binding to native α_2M and α_2M-MA [11,19]. Figure 1 shows a representative equilibrium analysis of ^{125}I-labelled TGF-β2 binding to α_2M–plasmin complex. In the experiment shown, ^{125}I-labelled TGF-β2 was incubated with increasing concentrations of α_2M–plasmin for 1 h before adding BS^3 for 1 min. The BS^3 stabilized a substantial fraction of the non-covalent α_2M–plasmin–TGF-β2 complex. Minimal covalent complex was detected in samples that had not been treated with BS^3 (less than 5% of total α_2M–plasmin–TGF-β2 complex).

Table 1 Equilbrium dissociation (K_d) constants for ^{125}I-labelled TGF-β binding to α_2M

Results were determined with the BS^3 stabilization method, and are presented as means ± S.E.M. ($n = 6$).

	K_d (nM)	
α_2M	TGF-β1	TGF-β2
α_2M–plasmin	84 ± 24	80 ± 18
Native α_2M	320 ± 65	14 ± 5
α_2M-MA	82 ± 6	15 ± 3

Binding isotherms and plots of $[C_e]/[AC_e]$ against $[A]^{-1}$ for the binding of TGF-β1 and TGF-β2 to native α_2M, α_2M–plasmin and α_2M-MA are shown in Figure 2. For each interaction, the results of six different studies were averaged to generate the presented graphs. Similar plots were also derived for each individual study. The separate K_d values were then averaged to obtain the mean constants presented in Table 1. Plasmin increased the binding affinity of α_2M for TGF-β1 (K_d 84 nM compared with 320 nM). The increase was equivalent to that observed when α_2M was modified by methylamine (K_d 82 nM). In contrast, plasmin decreased the affinity of α_2M for TGF-β2 (5-fold increase in K_d), whereas modification with methylamine had no effect. Thus the effects of plasmin on the binding affinity of α_2M for TGF-β were isoform-specific. To determine whether the decrease in TGF-β2-binding affinity was specific for α_2M–plasmin, the binding of TGF-β2 to α_2M-thrombin was studied. The K_d was 69 ± 11 nM ($n = 4$), a value that was 4.6-fold that of TGF-β2 binding to native α_2M.

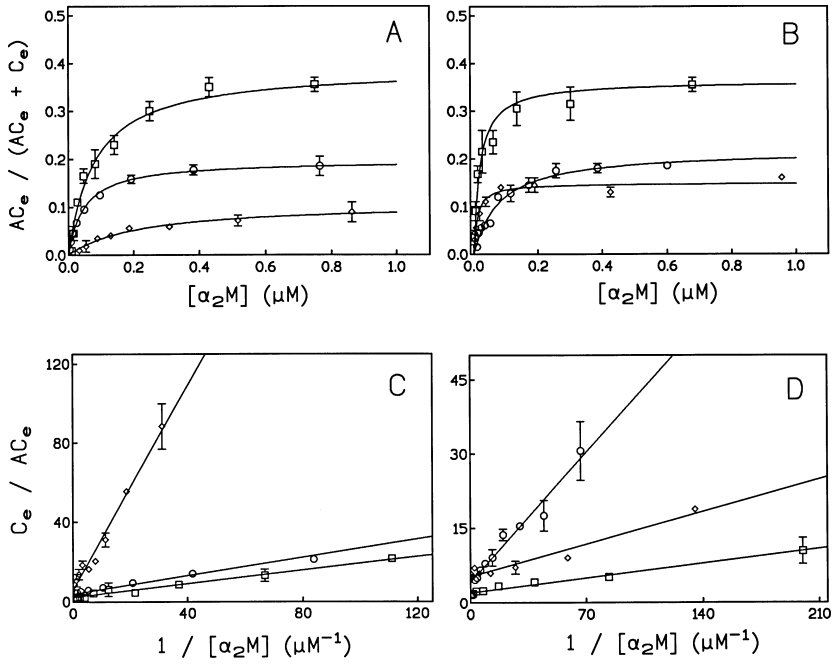

Figure 2 Determination of K_d values for ^{125}I-labelled TGF-β binding to native α_2M, α_2M–plasmin and α_2M-MA

^{125}I-labelled TGF-β1 and ^{125}I-labelled TGF-β2 were incubated with different concentrations of native α_2M (◇), α_2M–plasmin (○) or α_2M-MA (□). Binding was determined by the BS^3 stabilization method. The results of six separate experiments were averaged to generate the results, which are plotted in the form of standard binding isotherms (**A**, **B**) or in accordance with eqn. (1) (**C**, **D**). Binding of ^{125}I-labelled TGF-β1 is shown in (**A**) and (**C**); binding of ^{125}I-labelled TGF-β2 is shown in (**B**) and (**D**).

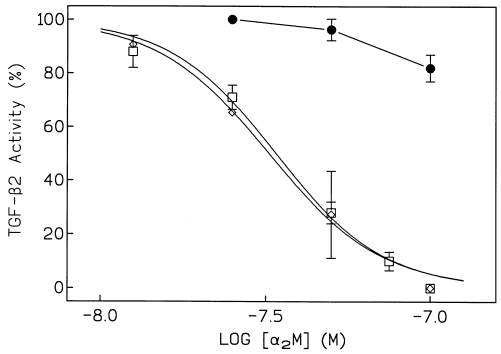

Figure 3 Effects of α_2M on TGF-β2 activity in an endothelial cell proliferation assay

FBHE cells were incubated with 10 pM TGF-β2 and different concentrations of native α_2M (◇), α_2M–plasmin (●) or α_2M-MA (□). The culture medium contained 0.2% (v/v) FBS. After 30 h, 1 μCi/ml [^{3}H]thymidine was added to the cultures for an additional 18 h. [^{3}H]Thymidine incorporation was then determined. TGF-β2 activity is expressed as a percentage of that observed in cultures that had not been treated with α_2M.

Table 2 Effects of α_2M–plasmin on PDGF α-receptor expression in VSMCs

VSMCs were incubated with serum-free medium (control), 280 nM α_2M–plasmin, 280 nM α_2M-MA or 280 nM native α_2M at 37 °C for 10 h. The cells were then chilled to 4 °C and specific ^{125}I-PDGF-AA binding was determined. Results are presented as means ± S.E.M. (n = 3).

α_2M	^{125}I-PDGF-AA bound (fmol/mg of cell protein)
Native	6.5 ± 0.9
α_2M-MA	7.4 ± 0.9
α_2M–plasmin	3.5 ± 0.6

Inhibition of endothelial cell growth by TGF-β

To determine whether the effects of plasmin on the TGF-β2-binding affinity of α_2M were significant in a biological system, FBHE proliferation assays were performed. Previous studies have shown that the ability of α_2M to neutralize TGF-β in this system correlates with the affinity of the α_2M/TGF-β interaction [19,20]. In the absence of α_2M, TGF-β2 (10 pM) inhibited FBHE [^{3}H]thymidine incorporation by 80 ± 2% (n = 8). Figure 3 shows that native α_2M and α_2M-MA were equally effective at counteracting the activity of TGF-β2, whereas purified α_2M–plasmin was significantly less effective, as would be predicted from the K_d values in Table 1.

Binding of ^{125}I-labelled PDGF-AA to VSMCs

In cultured VSMCs, α_2M up-regulates PDGF α-receptor expression by neutralizing autocrine cytokine activity [27]. The secreted cytokine is probably TGF-β; however, the TGF-β isoform(s) that are involved have not been identified. Because the TGF-β-neutralizing activities of α_2M–plasmin and native α_2M differ, the effects of α_2M–plasmin on PDGF α-receptor expression were studied. VSMCs that had been treated with native α_2M or with α_2M-MA bound increased amounts of ^{125}I-labelled PDGF-AA (Table 2), as shown previously [27]. α_2M–plasmin also increased the binding of ^{125}I-labelled PDGF-AA to the VSMCs; however, the extent of the increase was significantly less than that observed with native α_2M or α_2M-MA ($P < 0.05$ for α_2M–plasmin compared with native α_2M, and $P < 0.005$ for α_2M–plasmin compared with α_2M-MA; two-tailed t test). These studies demonstrate that native α_2M is more potent than α_2M–plasmin in regulating PDGF α-receptor expression, an activity previously attributed to TGF-β-carrier activity [27].

DISCUSSION

The proteinase inhibitory and cytokine-carrier activities of α_2M might allow this protein to function as a novel regulator of cellular growth and gene expression in extravascular spaces. Native α_2M binds specific cytokines and thereby alters the cytokine milieu. The concentration of native α_2M is regulated by cells that synthesize the protein, including macrophages and fibroblasts [28,29], and by changes in vascular permeability. The cytokine-carrier activity of α_2M is regulated by proteinases that alter cytokine-binding affinity. Once formed, α_2M–proteinase–cytokine complexes differ from native α_2M–cytokine complexes in that the former can be targeted, for clearance or as active species, to cells that express α_2M receptors [18].

The methylamine-induced conformational change in α_2M typically increases cytokine-binding affinity [11,18]. In contrast, plasmin increases the affinity of α_2M for one cytokine (TGF-β1) while decreasing the affinity for another (TGF-β2). Thus proteinases can indirectly, through α_2M, alter the relative proportions of various cytokines available to bind to signalling receptors. Other mechanisms by which proteinases alter cellular growth include the activation of latent growth factors [30,31], the release of growth factors from extracellular matrix-binding sites [32,33], and the proteolytic modification of cytokine receptors [34,35].

We previously demonstrated that rat α_2M–plasmin binds TGF-β1 with higher affinity than native rat α_2M [20]. Thus the effects of plasmin on the binding affinity for TGF-β1 are similar in rat α_2M and human α_2M. Binding of TGF-β2 to α_2M–plasmin has been studied previously only by non-denaturing PAGE [36]. In this system, human α_2M–plasmin seemed to bind more ^{125}I-labelled TGF-β2 than did native α_2M. The earlier results with non-denaturing PAGE are explained by the fact that this is a non-equilibrium method for studying binding interactions; the results obtained reflect not only binding affinity but also the extent of covalent binding and the rate of dissociation of the non-covalent complex [18].

In the FBHE system, the effects of α_2M on TGF-β activity are well defined [19,20]. Less is known about the mechanism(s) by which α_2M increases PDGF α-receptor expression in VSMCs. Weaver et al. [27] used a series of α_2M derivatives to demonstrate that α-receptor regulation results from the neutralization of autocrine cytokine activity. TGF-β-neutralizing antibody up-regulated PDGF α-receptor, in a similar manner to α_2M, implicating autocrine TGF-β in this process. In the present study the decreased activity of α_2M–plasmin, relative to native α_2M and α_2M-MA, suggests that TGF-β2 might be important in this system.

Plasmin and thrombin bind to α_2M primarily in binary (1:1) stoichiometry [37,38], eliminating the variability in cytokine-binding affinity expected for proteinases that react with α_2M to form both binary and ternary complexes. Even though plasmin and thrombin altered the strength of α_2M/TGF-β interactions, the proteinase-modified forms retained significant cytokine-carrier activity. Thus proteinases that form binary complexes by reacting with α_2M modify but do not eliminate cytokine-carrier function. Unless α_2M is present at a very low concentration in a

microenvironment that is particularly proteinase-rich, binary complexes should form almost exclusively *in vivo* [5,39]. Thus the results presented here are probably representative of diverse α_2M/proteinase interactions occurring *in vivo*.

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