Binding of platelet-derived growth factor-BB and transforming growth factor-β1 to α_2-macroglobulin *in vitro* and *in vivo*: comparison of receptor-recognized and non-recognized α_2-macroglobulin conformations

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α_2-Macroglobulin (α_2M) undergoes a major conformational change when reacting with proteinases or primary amines. This conformational change has been referred to as the 'slow' to 'fast' transformation based on the increase in α_2M mobility shown by non-denaturing PAGE. Previous studies demonstrated that many cytokines, including transforming growth factor β1 (TGF-β1) and interleukin-1β, bind preferentially or exclusively to α_2M which has undergone conformational change. In this study, we demonstrate that platelet-derived growth factor-BB (PDGF-BB) also binds preferentially to conformationally transformed α_2M (α_2M–methylamine, α_2M–trypsin) *in vitro*. Purified ^{125}I-PDGF-BB–α_2M–methylamine complex cleared rapidly from the circulation of mice via the α_2M receptor/low-density-lipoprotein-receptor-related protein (α_2M-R/LRP). In order to determine whether PDGF-BB or TGF-β1 binds to native α_2M, we defined the native conformation by lack of interaction with α_2M-R/LRP instead of electrophoretic mobility. ^{125}I-PDGF-BB was incubated with 4.3 μM native α_2M and 0.47 μM α_2M–methylamine. The ^{125}I-PDGF-BB distributed evenly between slow-form and fast-form α_2M without shifting the electrophoretic mobility of either species. When the mixed preparation was injected intravenously in mice, ^{125}I-PDGF-BB–fast-form-α_2M cleared rapidly and selectively from the circulation; ^{125}I-PDGF-BB which was bound to slow-form α_2M was stable in the blood (apparently not recognized by α_2M-R/LRP). Therefore, while conformationally transformed α_2M binds PDGF-BB preferentially *in vitro*, non-α_2M-R/LRP-recognized α_2M binds PDGF-BB as well. Binding of ^{125}I-PDGF-BB and ^{125}I-TGF-β1 to α_2M was demonstrated *in vivo* by injecting the free growth factors intravenously into mice. Plasma samples which were subjected to non-denaturing PAGE and autoradiography demonstrated binding of both growth factors exclusively to the slow-form of α_2M. Therefore, under normal physiological conditions, native α_2M (non-α_2M-R/LRP-recognized) is the primary form of the proteinase inhibitor functioning as a carrier of PDGF-BB and TGF-β1 in the blood.

INTRODUCTION

α_2-Macroglobulin (α_2M) is a large, homotetrameric glycoprotein (M_r approx. 718000) and an inhibitor of proteinases from all four major classes [1–3]. Upon reaction with proteinases, α_2M undergoes a major conformational change [4–6]. This conformational change results in irreversible proteinase trapping [4,7]. Each of the four α_2M subunits has a single β-cysteinyl-γ-glutamyl thiol ester bond [8,9]. Aminolysis of the thiol esters with small primary amines causes a conformational change in α_2M which is very similar, or equivalent, to that caused by proteinases [5,6]. The mobility of α_2M in non-denaturing PAGE systems is increased after conformational change [4]. This 'slow-form' to 'fast-form' mobility transition has become a commonly used indicator of α_2M conformation.

After reaction with proteinase or amine, α_2M is recognized by the cellular receptor, α_2M receptor/low-density-lipoprotein-receptor-related protein (α_2M-R/LRP) [10,11]. Cellular binding via α_2M-R/LRP is followed by ligand endocytosis. This process is responsible for the rapid clearance of α_2M–proteinase complexes and α_2M–methylamine from the circulation [12]. α_2M-R/LRP is expressed by a number of cell types including hepatocytes, macrophages, fibroblasts and neurons [12–14]. The native or unreacted conformation of α_2M (slow-form) demonstrates no affinity for α_2M-R/LRP [12]. Receptor recognition of transformed α_2M reflects the exposure or orientation of receptor-binding domains located near the C-terminus of each α_2M subunit [15,16].

α_2M is found in the plasma at high concentrations (2–3 mg/ml) and in the extracellular spaces; however, an essential role for α_2M as a regulator of proteinases has not been defined [17]. By mechanisms which are distinct from proteinase trapping, α_2M binds a number of growth factors and cytokines [17], including transforming growth factor-β1 (TGF-β1) [18–23], platelet-derived growth factor (PDGF) [24–27], interleukin-1β (IL-1β) [28–31], basic fibroblast growth factor (bFGF) [32], and tumour necrosis factor-α (TNF-α) [33,34]. In cell-culture systems, α_2M may inhibit [21] or promote cytokine activity [26]. In addition, cytokines that are bound to conformationally transformed α_2M may be targeted to cells expressing α_2M/LRP; this process results in the rapid clearance of fast-form-α_2M–TGF-β1 and fast-form-α_2M–TNF-α complexes from the circulation [20,23,33].

Conformational change of α_2M may be critical for the appropriate expression of cytokine regulatory activity [17,21]. Conformational change not only provides the signal for receptor targeting, but also increases the affinity of α_2M for many cytokines. In experiments with TGF-β1, the affinity is increased by at least an order of magnitude [22]. Apparently weak binding

Abbreviations used: α_2M, α_2-macroglobulin; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; α_2M-R/LRP, α_2-macroglobulin receptor/low-density-lipoprotein-receptor-related protein; IL-1β, interleukin 1β; bFGF, basic fibroblast growth factor; TNF-α, tumour necrosis factor-α; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PNPGB, *p*-nitrophenyl *p*′-guanidinobenzoate hydrochloride; DTT, 1,4-dithiothreitol; BS^3, bis(sulphosuccinimidyl) suberate; DSS, disuccinimidyl suberate; α_2M–methylamine-c, carboxamidomethylated α_2M–methylamine; *r*, molar ratio of active trypsin to α_2M.

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of TGF-β1 to native α_2M is also observed by non-denaturing PAGE and gel-filtration chromatography [20–23]; however, this result must be interpreted with caution for the following reasons: (1) All of the techniques used to examine TGF-β1 binding to α_2M typically include a large molar excess of α_2M. It is almost impossible to exclude trace levels of conformationally transformed α_2M from these preparations, especially when certain commercially available α_2M preparations are used. (2) Conformational change of α_2M is not a simple one-step process. Conformational intermediates have been characterized by electron microscopy, electrophoresis and spectroscopic techniques [35–38]. These intermediates may not always be resolved from native α_2M by non-denaturing PAGE.

In addition to TGF-β1, preferential or exclusive binding to conformationally transformed α_2M (fast-form) has been demonstrated with TNF-α [33,34], IL-1β [28–31], and bFGF [30,32]. PDGF may provide an exception to this rule. Bonner et al. [27] reported comparable binding of PDGF (AA, BB, AB isoforms) to native α_2M and α_2M–methylamine; however, the experimental procedures used in this study (non-denaturing PAGE, chromatography, incubations with large molar excesses of α_2M) were similar to those used by our laboratory with TGF-β1, and therefore subject to the same limitations (listed above). The possibility of significant cytokine binding to native α_2M is very important since PDGF, like TGF-β1, is associated with α_2M in the plasma [18,19,24,25]. The conformation of α_2M should determine whether PDGF (or TGF-β1) persists in the plasma or is rapidly cleared from the circulation. In addition, due to the function of α_2M-R/LRP, the concentration of native α_2M (slow-form) greatly exceeds the concentration of α_2M (fast-form) in the blood and probably in extravascular spaces. Finally, if native α_2M is a carrier of certain cytokines, such as PDGF, while the fast-form binds others, such as TGF-β1 and IL-1β, then the α_2M conformational change might be associated with cytokine uptake and release. Hypothetically, this shift could alter the cytokine milieu to which cells are exposed.

In the present investigation, we used a highly purified preparation of human α_2M to demonstrate that PDGF-BB binds preferentially to fast-form α_2M *in vitro*. Limited binding of PDGF-BB to native α_2M was also observed; however, the significance of this interaction was unclear due to the possible presence of trace levels of fast-form α_2M or conformational intermediates in our preparation. In order to determine whether cytokine binding to native α_2M is significant, it was necessary to redefine 'native α_2M' based on properties other than electrophoretic mobility alone. We defined native α_2M as the form (or forms) of the proteinase inhibitor that is not recognized by α_2M-R/LRP. This parameter was not only easily measured but also physiologically significant. Based on this new definition, we demonstrated that native α_2M binds PDGF-BB *in vitro* and that native α_2M is the primary form of the proteinase inhibitor responsible for carrying both PDGF-BB and TGF-β1 in the blood.

MATERIALS AND METHODS

Reagents

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), *p*-nitrophenyl *p*′-guanidinobenzoate hydrochloride (PNPGB), 1,4-dithiothreitol (DTT), methylamine hydrochloride, chloramine-T, iodoacetamide, trypsin and BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bis(sulphosuccinimidyl) suberate (BS^3) and disuccinimidyl suberate (DSS) were purchased from Pierce (Rockford, IL, U.S.A.). $Na^{125}I$ was purchased from Amersham (Arlington Heights, IL, U.S.A.).

α_2M and derivatives

α_2M was purified from human plasma by the method of Imber and Pizzo [39]. The concentration of α_2M was determined by absorbance at 280 nm, using an $A^{1.0\%}_{1.0\,cm}$ of 8.93 [3]. α_2M–methylamine was prepared by dialysing α_2M against 200 mM methylamine hydrochloride in 50 mM Tris/HCl (pH 8.2) for 12 h at 22 °C and then extensively against 20 mM sodium phosphate/150 mM NaCl, pH 7.4 (PBS) at 4 °C. Reaction of native α_2M with methylamine was confirmed by native PAGE (slow to fast transformation) and by loss of trypsin-binding activity (greater than 95%), as determined by the method of Ganrot [40]. Carboxamidomethylated α_2M–methylamine (α_2M–methylamine-c) was prepared by incubating α_2M–methylamine (6–8 μM) with 2.0 mM iodoacetamide for 1 h at 22 °C. α_2M–methylamine-c was dialysed into PBS to remove unreacted iodoacetamide. The thiol-group content was reduced from 3.5 mol/mol for α_2M–methylamine to less than 0.05 mol/mol for α_2M–methylamine-c, as determined by titration with DTNB.

Trypsin was active-site titrated with PNPGB by the method of Chase and Shaw [41]. α_2M–trypsin complexes were prepared by incubating 1.7 μM native α_2M with different molar ratios (r) of active trypsin, as described previously [7,21]. The trypsin was then inactivated with PNPGB before incubation with growth factors. PNPGB does not affect the TGF-β1-binding activity of α_2M–trypsin complex [21].

Growth factors

Radio-iodinated recombinant human PDGF-BB was purchased from New England Nuclear (specific radioactivity approx. 29–53 μCi/μg). Pig TGF-β1 (lyophilized without carrier proteins) was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Human TGF-β1 (identical in sequence to pig TGF-β1) was purified from acidic/ethanol extracts of platelets [42]. TGF-β1 was radio-iodinated by the chloramine-T method of Ruff and Rizzino [43]. The specific radioactivity was 100–200 μCi/μg. Human ^{125}I-TGF-β1 prepared in this laboratory and pig ^{125}I-TGF-β1 purchased commercially interact identically with α_2M [21,22]. The final preparations of ^{125}I-TGF-β1 and ^{125}I-PDGF-BB included 1.0 mg/ml and 10 mg/ml BSA respectively.

^{125}I-PDGF-BB binding to α_2M as determined by f.p.l.c.

Native α_2M, α_2M–methylamine, and α_2M–methylamine-c (1.4 μM) were incubated with 3.4 nM ^{125}I-PDGF-BB for 2 h at 37 °C in PBS with 8 μM BSA. The reaction mixtures were then subjected to chromatography on a Superose-6 column (Pharmacia) which had been equilibrated in PBS (0.4 ml/min). Binding of ^{125}I-PDGF-BB to α_2M was determined by the level of radioactivity co-eluting with α_2M (percentage of total radioactivity loaded) as described previously [20,21]. The minimum radioactivity co-eluting with the α_2M–methylamine peak in each experiment was 50000 c.p.m.

SDS/PAGE of PDGF-BB–α_2M complexes

^{125}I-PDGF-BB–α_2M complex was purified by f.p.l.c. on a Superose-6 column and subjected to SDS/PAGE on 5% slabs using a Hepes–imidazole buffer system, pH 7.4 [21]. Before electrophoresis, samples were denatured in 2.0% (w/v) SDS with or without 25 mM DTT. ^{125}I-PDGF-BB was detected in the gels by autoradiography. The gels were sliced and the radioactivity in each section was determined in a γ-counter. In control experiments, ^{125}I-PDGF-BB was subjected to electrophoresis in

the absence of α_2M. Significant radioactivity was not detected in the region of the gel typically occupied by α_2M.

Growth factor–α_2M cross-linking experiments

^{125}I-PDGF-BB (6 nM) and ^{125}I-TGF-β1 (1 nM) were incubated individually with α_2M or BSA for 2 h at 37 °C. BS^3 or DSS was added to a final concentration of 5.0 mM and incubated for 1 h at 22 °C. Tris/HCl, pH 7.4 (0.3 M, final concentration) was added to stop the cross-linking reactions. The samples were then subjected to SDS/PAGE on 5% slabs (without reductant).

Non-denaturing PAGE

Chromatography fractions, murine plasma, and purified α_2M that had been incubated with growth factors were studied by non-denaturing PAGE using the buffer system described by Van Leuven et al. [44]. Binding of ^{125}I-labelled growth factor to α_2M was assessed by autoradiography and by counting the radioactivity in gel slices.

Plasma clearance studies

^{125}I-PDGF-BB (16 nM) was incubated with α_2M–methylamine (1.5–3.5 μM) or with a combination of native α_2M (4.3 μM) and α_2M–methylamine (0.47 μM) for 2 h at 37 °C. ^{125}I-PDGF-BB–α_2M complex was resolved from unbound ^{125}I-PDGF-BB by chromatography on a Superose-6 column. Plasma clearance experiments were performed in 20–30 g female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA, U.S.A.). The method has been described in detail elsewhere [12,20]. Briefly, ^{125}I-PDGF-BB, ^{125}I-TGF-β1 or purified ^{125}I-PDGF-BB–α_2M complex was injected into the lateral tail veins of anaesthetized mice. At various times, blood samples (30 μl) were drawn from the retro-orbital venous plexus using calibrated, heparinized microhaematocrit tubes. The radioactivity in each blood sample was determined and expressed as a percentage of that present 5–10 s after injection. Unless otherwise noted, each experiment was performed in triplicate. Competition experiments were performed either by diluting purified ^{125}I-PDGF-BB–α_2M complex with a 20-fold molar excess of α_2M–methylamine (relative to α_2M present in radioligand preparation), or by first injecting excess α_2M–methylamine, followed by a second injection (into the opposite tail vein) containing the radioligand.

Blood samples to be analysed by non-denaturing PAGE and autoradiography were collected into sodium citrate (0.38%, w/v) at 4 °C instead of heparin and subjected to centrifugation in order to prepare platelet-deficient plasma. The mobilities of ^{125}I-PDGF-BB–α_2M complexes in the recovered plasma samples were calibrated using murine plasma which had been treated with methylamine *in vitro*. Slightly less than 50% of the methylamine-treated α_2M in murine plasma was converted into the fast-form, as expected based on a previous investigation [45].

Organ-distribution experiments

Radioligands were injected intravenously and allowed to clear from the plasma for 30 min. The mice were then killed by cervical dislocation under heavy anaesthesia. The major body organs were removed, rinsed briefly in water, blotted to remove surface moisture and weighed. The radioactivity in each organ was determined and expressed as a fraction of the total recovered radioactivity. These results were then normalized for organ size by dividing the percentage of recovered radioactivity in each organ by organ mass. More than 50% of injected radioactivity was recovered with the listed organs.

RESULTS

PDGF-BB binding to α_2M

PDGF-BB binding to α_2M was studied initially by f.p.l.c. ^{125}I-PDGF-BB (3.4 nM) was incubated with a 400-fold molar excess of α_2M for 2 h at 37 °C. Table 1 shows that α_2M–methylamine (fast-form) bound significantly more PDGF-BB than native α_2M. Since non-covalent α_2M–growth factor complexes may dissociate during chromatography, the results presented in Table 1 represent minimum estimates. Nevertheless, these results differ from those determined previously by Bonner et al. [27] using an equivalent chromatographic procedure. In the previous investigation, comparable binding of PDGF to commercially purchased native α_2M and α_2M–methylamine was demonstrated. In our experiments, α_2M–methylamine and α_2M–methylamine-c bound equivalent levels of PDGF-BB. These results suggest that modification of the cysteine residues generated by thiol ester aminolysis (the only free thiol groups in α_2M–methylamine) does not significantly alter total PDGF-BB binding (covalent and non-covalent).

In order to determine whether binding of PDGF-BB to α_2M–methylamine had reached apparent equilibrium by 2 h, 3.4 nM ^{125}I-PDGF-BB was incubated with 0.7 μM α_2M–methylamine at 37 °C for various periods of time. Binding was analysed by native PAGE. After incubation for 2 h, 24% of the ^{125}I-PDGF-BB was recovered in association with the α_2M–methylamine. Further incubation, for up to 8 h, increased binding by no more than 3% (results not shown).

To determine whether PDGF-BB binds covalently to α_2M, ^{125}I-PDGF-BB–α_2M complexes were purified by chromatography on a Superose-6 column and immediately subjected to SDS/PAGE. Under non-reducing conditions, only 19±1% (n = 5) of the ^{125}I-PDGF-BB migrated with the α_2M–methylamine, suggesting that most of the PDGF-BB–α_2M–methylamine complex was non-covalent in nature. After reduction with DTT, covalent binding was decreased to 3.0% or less. Covalent binding of ^{125}I-PDGF-BB to α_2M–methylamine-c was reduced by 75% compared with α_2M–methylamine (assessed under non-reducing conditions). These results indicate that disulphide bonds are responsible for the majority of covalent PDGF-BB–α_2M–methylamine complexes. Since total binding of PDGF-BB to α_2M–methylamine-c was unchanged compared with α_2M–methylamine, while covalent binding was decreased, covalent binding probably does not play an important role in stabilizing PDGF-BB–α_2M complexes during chromatography.

^{125}I-PDGF-BB–α_2M–methylamine was purified by chromatography on a Superose-6 column and stored at 4 °C. Covalent binding was determined by SDS/PAGE after 6 weeks. Approx. 30% of the PDGF-BB was recovered in SDS-stable complexes with α_2M–methylamine (50% greater than that observed when ^{125}I-PDGF-BB–α_2M–methylamine was analysed immediately after recovery from chromatography). Greater than 70% of the

Table 1 Analysis of ^{125}I-PDGF-BB binding to α_2M by Superose 6 chromatography

α_2M species	Percentage bound (mean±S.E.M.)
Native α_2M	3.9±0.6
α_2M–methylamine	18.0±3.2
α_2M–methylamine-c	19.4±2.3

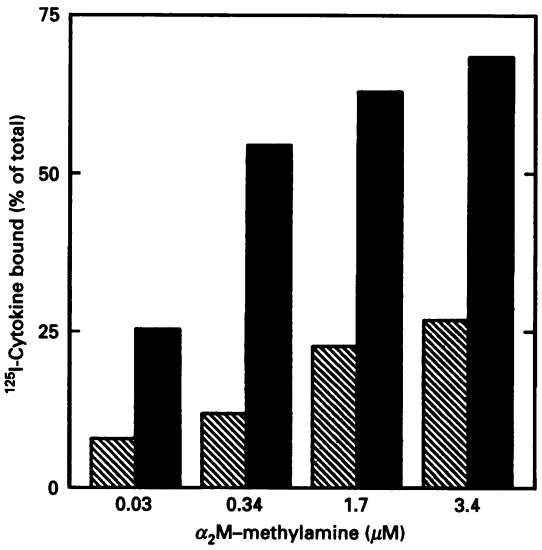

Figure 1 Comparison of ^{125}I-PDGF-BB and ^{125}I-TGF-β1 binding to α_2M–methylamine

^{125}I-PDGF-BB (hatched bars) or ^{125}I-TGF-β1 (solid bars) (3.4 nM) was incubated with the indicated concentrations of α_2M–methylamine. Binding was determined by non-denaturing PAGE.

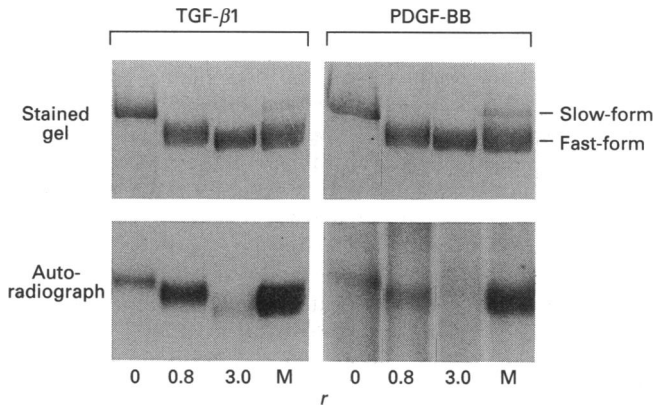

Figure 2 Binding of ^{125}I-PDGF-BB and ^{125}I-TGF-β1 to α_2M–trypsin

Native α_2M (1.7 μM) was reacted with trypsin. The ratio of active trypsin to α_2M is designated *r*. After inactivating the trypsin with PNPGB, samples were incubated with 13 nM ^{125}I-PDGF or 2 nM ^{125}I-TGF-β1. Binding was determined by non-denaturing PAGE and autoradiography. α_2M–methylamine (M) is shown for comparison.

covalent binding was dissociated by DTT, confirming the importance of disulphide bonds.

Comparison of PDGF-BB and TGF-β1 binding to α_2M

To compare binding of ^{125}I-TGF-β1 and ^{125}I-PDGF-BB to α_2M–methylamine, equivalent concentrations of each growth factor were incubated with the same α_2M–methylamine preparation. Binding was determined by non-denaturing PAGE (Figure 1). Complex formation with both growth factors increased as the α_2M–methylamine concentration was increased; however, TGF-β1 binding always exceeded PDGF-BB binding.

α_2M (1.7 μM) was incubated with different concentrations of trypsin for 10 min at 22 °C. Under the specified conditions,

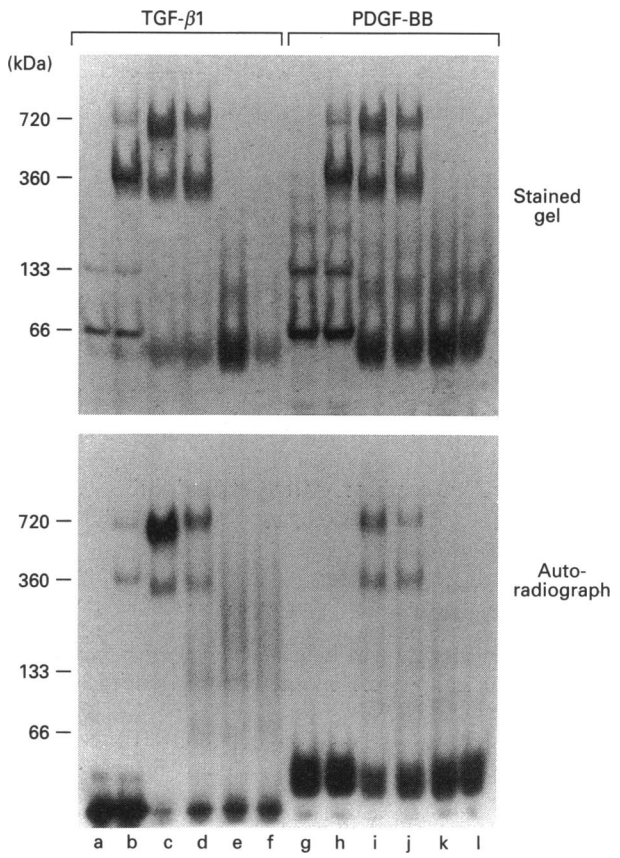

Figure 3 Cross-linking of α_2M to ^{125}I-TGF-β1 and ^{125}I-PDGF-BB

^{125}I-TGF-β1 (1 nM) or ^{125}I-PDGF-BB (6 nm) was incubated with α_2M or BSA. Some of the samples (c–f, i–l) were subsequently treated with 5 mM BS^3. SDS/PAGE was then performed (no reduction). The concentration of α_2M was 0.8 μM. The incubations included: a, TGF-β1 and 1.2 μM BSA (no BS^3); b, TGF-β1, α_2M–methylamine, and 1.2 μM BSA (no BS^3); c, TGF-β1, α_2M–methylamine and 1.2 μM BSA (+ BS^3); d, TGF-β1, native α_2M and 1.2 μM BSA (+ BS^3); e, TGF-β1 and 10 μM BSA (+ BS^3); f, TGF-β1 and 1.2 μM BSA (+ BS^3); g, PDGF-BB and 12 μM BSA (no BS^3); h, PDGF-BB, α_2M–methylamine and 12 μM BSA (no BS^3); i, PDGF-BB, α_2M–methylamine and 12 μM BSA (+ BS^3); j, PDGF-BB, native α_2M and 12 μM BSA (+ BS^3); k, PDGF-BB and 21 μM BSA (+ BS^3); l, PDGF-BB and 12 μM BSA (+ BS^3). The molecular-mass markers shown to the left include the α_2M tetramer (720 kDa), α_2M dimer (360 kDa), BSA dimer (133 kDa), and BSA monomer (66 kDa).

complex formation between α_2M and trypsin is quantitative when the ratio of active trypsin to α_2M (*r*) is less than two [7]. The trypsin distributes into the two proteinase-binding sites in each α_2M module, without evidence of co-operativity. In a previous study [21], we demonstrated that TGF-β1 binding to α_2M is increased (relative to native α_2M) in binary α_2M–trypsin complex (one mol of trypsin/mol of α_2M) and decreased in ternary α_2M–trypsin complex (two mol of trypsin/mol of α_2M). This dependence of TGF-β1-binding activity on *r* value is shown in Figure 2. When α_2M was incubated with trypsin at $r = 0.8$, mostly binary α_2M–trypsin formed [7] and TGF-β1 binding was increased. At $r = 3.0$, only ternary α_2M–trypsin was formed [7], and TGF-β1 binding was decreased.

The binding of ^{125}I-PDGF-BB to α_2M–trypsin demonstrated the equivalent dependence on *r* value. Increased PDGF-BB binding was observed (relative to the native α_2M preparation) when $r = 0.8$; decreased binding was observed when $r = 3.0$. These studies demonstrate that α_2M–proteinase complex binds increased levels of PDGF-BB compared with native α_2M. The dependence of PDGF-BB binding on the *r* value and the greater

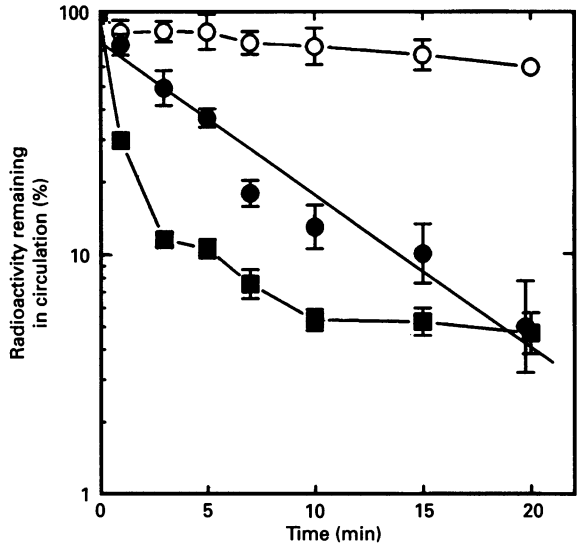

Figure 4 Plasma clearance of ^{125}I-PDGF-BB

Radioactivity remaining in the circulation after intravenous injection of free ^{125}I-PDGF-BB (■), purified ^{125}I-PDGF-BB–α_2M–methylamine complex (●) or ^{125}I-PDGF-BB–α_2M–methylamine complex and excess non-radiolabelled α_2M–methylamine (○). The error bars represent the S.E.M.

binding of PDGF-BB to α_2M–methylamine compared with α_2M–trypsin represent similarities with the α_2M/TGF-β1 interaction.

Cross-linker stabilization of PDGF-BB–α_2M complexes

Since most of the ^{125}I-PDGF-BB–α_2M–methylamine complex and ^{125}I-TGF-β1–α_2M–methylamine complex is non-covalent, a fraction of these complexes probably dissociates during chromatography or non-denaturing PAGE. We developed a system for analysing equilibrium binding of radio-iodinated cytokines to immobilized α_2M–methylamine [22]; however, the specific radioactivity of ^{125}I-PDGF-BB and the extent of binding of ^{125}I-PDGF-BB to immobilized α_2M–methylamine were insufficient for analysis in this system. As an alternative, BS^3 and DSS were used to stabilize complexes of α_2M with ^{125}I-PDGF-BB or ^{125}I-TGF-β1 covalently before SDS/PAGE. Figure 3 shows that BS^3 did not cross-link ^{125}I-PDGF-BB or ^{125}I-TGF-β1 to BSA, even when the concentration of BSA was quite high (21 μM and 10 μM in incubations with PDGF-BB and TGF-β1 respectively). Therefore, under the conditions used here, BS^3 does not cross-link proteins which are present together in solution, but not associated. Substantial binding of ^{125}I-TGF-β1 to α_2M–methylamine was observed. In four separate experiments with 0.8 μM α_2M–methylamine, 56–75% of the TGF-β1 was covalently stabilized in complex with α_2M–methylamine by BS^3. Under equivalent conditions, ^{125}I-TGF-β1 binding to native α_2M ranged from 22–33%. As expected, greatly decreased binding of TGF-β1 to native α_2M and α_2M–methylamine was observed when the BS^3 was omitted.

Complexes of ^{125}I-PDGF-BB and α_2M were also stabilized by the cross-linking agents. The amount of recovered PDGF-BB–α_2M–methylamine complex exceeded the level of PDGF-BB–native α_2M complex (37% versus 24% with BS^3 and 18% versus 10% with DSS). When the cross-linking agents were omitted, trace binding of PDGF-BB to α_2M–methylamine was detected; binding to native α_2M was below the detection limit. Stabilization of growth factor–native α_2M complexes by cross-linking agents suggests that these complexes exist in solution; however, as with other techniques, the problem of α_2M conformational heterogeneity cannot be fully resolved.

Table 2 Organ-distribution studies

Normalized radioactivities were determined by dividing the radioactivity recovered in each organ (percentage of total recovered radioactivity) by the organ mass (g). Values represent the mean ± S.E.M. ($n = 3$).

	PDGF		PDGF–α_2M–methylamine	
Organ	Radioactivity recovered (%)	Normalized radioactivity (%/g)	Radioactivity recovered (%)	Normalized radioactivity (%/g)
Heart	0±0	1±0	0±1	1±0
Kidney	10±1	18±5	3±1	5±2
Liver	86±1	52±6	95±1	63±6
Lungs	1±0	2±0	0±0	2±0
Spleen	4±0	27±1	2±0	16±2

Plasma clearance of ^{125}I-PDGF-BB in mice

Intravenously injected ^{125}I-PDGF-BB cleared rapidly from the circulation of mice; however, clearance was not a simple first-order process (Figure 4). Almost 90% of the ^{125}I-PDGF-BB was eliminated within 3 min. The rate of clearance then decreased and the level of intravascular radioligand stabilized at about 5% within 10 min. A similar clearance pattern was observed previously with ^{125}I-TGF-β1; however, the plateau level in the curve was about 15–20% [20]. Table 2 shows that large amounts of the injected ^{125}I-PDGF-BB were recovered in the liver, spleen and kidneys.

Part of the plateau effect seen in the plasma clearance of free ^{125}I-PDGF-BB was explained by the presence of minor heterogeneity in the injected radioligand preparation. When ^{125}I-PDGF-BB was subjected to chromatography on a Superose-6 column in 13 separate experiments (with and without α_2M), a small but constant percentage of the radioactivity eluted at a column volume typical of BSA. The protein in this peak was subjected to SDS/PAGE and autoradiography. The mobility of the autoradiography band was equivalent to that of BSA in the Coomassie-Blue-stained gel before and after reduction (results not shown). DTT did not significantly affect the recovery of radioactivity in the BSA band. Based on this analysis, we estimated that 2.08±0.18% of the ^{125}I-PDGF-BB preparation consisted of ^{125}I-BSA or covalent (non-reducible) ^{125}I-PDGF-BB–BSA complex. The presence of these contaminants was not unexpected because of the high concentration of BSA (0.15 mM) in the ^{125}I-PDGF-BB preparation.

Plasma clearance of ^{125}I-PDGF-BB–α_2M–methylamine in mice

^{125}I-PDGF-BB–α_2M–methylamine was purified by chromatography on a Superose-6 column and immediately studied in plasma clearance experiments. The complex was eliminated from the circulation relatively quickly (Figure 4). The first 50% of the radioligand was cleared within 3 min; nearly 90% was eliminated in 10 min. In organ-distribution studies, almost all of the radioactivity was recovered in the liver, as would be expected for

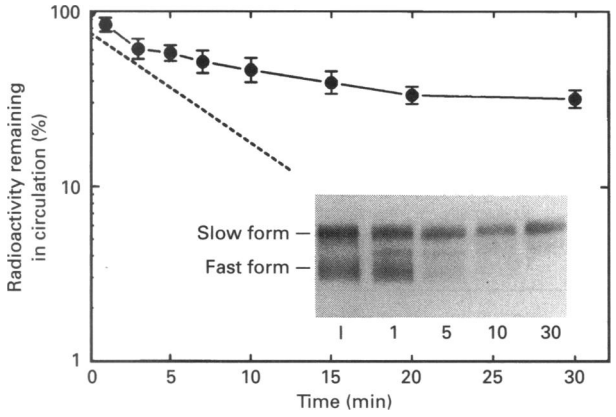

Figure 5 Plasma clearance of a mixed preparation of purified ^{125}I-PDGF-BB–α_2M complexes formed by incubating ^{125}I-PDGF-BB with native human α_2M and α_2M–methylamine

The mixed preparation is shown (●) together with the reproduced clearance curve for purified ^{125}I-PDGF-BB–α_2M–methylamine from Figure 4 for comparison (broken line). The insert is an autoradiograph showing plasma samples drawn at the indicated times (in min) and subjected to non-denaturing PAGE. Equal amounts of plasma were loaded in each lane of the gel. The migration of slow-form and fast-form human α_2M (based on the Coomassie-Blue-stained gel) is shown for reference.

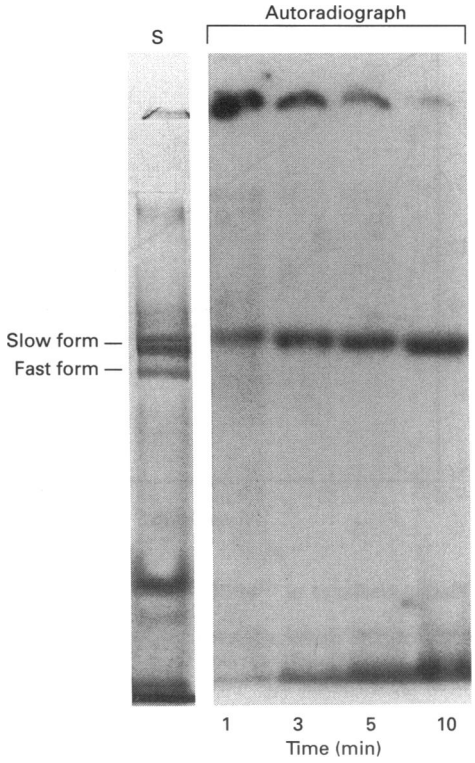

Figure 6 Binding of ^{125}I-PDGF-BB to murine α_2M *in vivo*

Free ^{125}I-PDGF-BB was injected intravenously in mice. Blood drawn at the indicated times was processed to form plasma and subjected to non-denaturing PAGE and autoradiography. Equal amounts of radioactivity were loaded in each well. The lane labelled 'S' is the Coomassie-Blue-stained control showing murine plasma treated with methylamine.

an α_2M-R/LRP-mediated process [12]. When excess non-radiolabelled α_2M–methylamine was co-injected with purified ^{125}I-PDGF-BB–α_2M–methylamine in order to block the α_2M-R/LRP pathways, the rate of radioligand clearance was significantly decreased. These studies indicate that PDGF-BB binding to the fast-form of α_2M does not interfere with α_2M-R/LRP binding. Similar results have been reported for complexes of fast-form α_2M with TGF-β1 [20] and TNF-α [33]. In control experiments, the clearance of free ^{125}I-PDGF-BB was not significantly affected by excess α_2M–methylamine (results not shown).

A plasma clearance model for identifying growth factor binding to native α_2M

^{125}I-PDGF-BB was incubated simultaneously with 4.3 μM native α_2M (human) and 0.47 μM α_2M–methylamine. The PDGF-BB which was bound to α_2M was purified by chromatography and studied by SDS/PAGE and autoradiography. As shown in the autoradiograph (inset, Figure 5), the ^{125}I-PDGF-BB distributed fairly evenly into two bands. These autoradiography bands superimposed precisely over the major bands of the slow-form and fast-form α_2M in the Coomassie-blue-stained gel. The nearly even distribution of ^{125}I-PDGF-BB between slow-form and fast-form α_2M was expected due to the excess of native α_2M present during the incubation.

Since human and murine α_2M are recognized comparably by murine α_2M-R/LRP [45], the mixture of purified ^{125}I-PDGF-BB–α_2M complexes was studied in plasma clearance experiments. Compared with pure ^{125}I-PDGF-BB–α_2M–methylamine complex, the ^{125}I-PDGF-BB which was bound to the mixed α_2M preparation cleared at a decreased rate (Figure 5). This result suggested that some of the ^{125}I-PDGF-BB bound to α_2M which was not receptor-recognized. Plasma samples obtained at various times were subjected to non-denaturing PAGE and autoradiography. The ^{125}I-PDGF-BB which was bound to fast-form α_2M cleared rapidly from the plasma. ^{125}I-PDGF-BB which was bound to slow-migrating α_2M was not cleared. These studies demonstrate that PDGF-BB binds to native α_2M, as defined by lack of receptor recognition and by electrophoretic mobility. PDGF-BB–native α_2M complexes are not readily cleared from the plasma.

Binding of PDGF-BB to α_2M *in vivo*

Since ^{125}I-PDGF-BB binds to native α_2M *in vitro* without altering the mobility of the α_2M, experiments were designed to determine whether growth factor–native α_2M complexes form *in vivo*. Free ^{125}I-PDGF-BB was injected intravenously and blood was sampled after different time periods. Equal amounts of radioactivity from each sample were subjected to non-denaturing PAGE and autoradiography. As shown in Figure 6, ^{125}I-PDGF-BB–α_2M complex was observed within one min. It is possible that some of the ^{125}I-PDGF-BB–α_2M complex detected at this early stage formed after the blood was drawn from the mice; however, the amount of ^{125}I-PDGF-BB–α_2M complex recovered increased significantly as the clearance experiment progressed. This result can only be attributed to complex formation *in vivo*.

The ^{125}I-PDGF-BB which bound to α_2M *in vivo* was entirely associated with native α_2M, demonstrating slow-form mobility. Radioactivity was also recovered in increasing amounts with time near the dye-front (the location of albumin in this PAGE system). This result probably reflects the presence of ^{125}I-BSA or ^{125}I-PDGF-BB–BSA complex in the ^{125}I-PDGF-BB preparation, as described above. These contaminants would be expected to comprise an increasingly large percentage of the total radio-

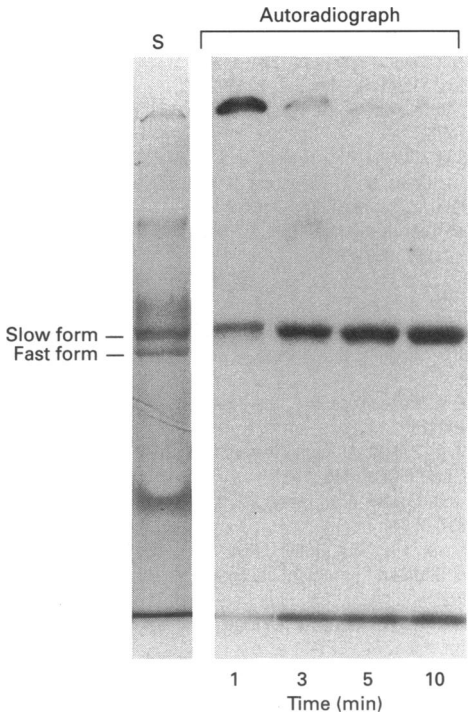

Figure 7 Binding of ^{125}I-TGF-β1 to murine α_2M *in vivo*

Free ^{125}I-TGF-β1 was injected intravenously in mice. Blood drawn at the indicated times was processed to form plasma and subjected to non-denaturing PAGE and autoradiography. Equal amounts of radioactivity were loaded in each well. The lane labelled 'S' is the Coomassie-Blue-stained control showing murine plasma treated with methylamine.

activity in the blood as the experiment progresses and the majority of the ^{125}I-PDGF-BB is cleared.

Binding of TGF-β1 to α_2M *in vivo*

^{125}I-TGF-β1 binding to α_2M was also studied within the context of a plasma clearance experiment. Free ^{125}I-TGF-β1 was injected intravenously and allowed to clear from the circulation. Plasma samples were obtained at various times and analysed by non-denaturing PAGE and autoradiography. As shown in Figure 7, binding of TGF-β1 to α_2M was demonstrated *in vivo*. The TGF-β1 associated exclusively with the slow-form of α_2M. Therefore, while conformationally transformed α_2M binds TGF-β1 with increased affinity *in vitro*, the primary form of α_2M responsible for PDGF-BB- and TGF-β1-carrier activity in plasma is non-α_2M-R/LRP-recognized, native, slow-form α_2M.

DISCUSSION

Many of the original investigations implicating α_2M as a major carrier of cytokines were based on analyses of human plasma. It was recognized that PDGF [24,25] and TGF-β1 [18,19] associate with a high-molecular-mass plasma component which was identified as α_2M. Subsequent studies with purified proteins demonstrated that TGF-β1-binding to α_2M *in vitro* is highly dependent on α_2M conformation. Analyses performed in our laboratory demonstrated that TGF-β1 binds to the fast-form of α_2M [20–22]. A similar conformation-dependency was demonstrated with bFGF [32], IL-1β [28–31], and TNF-α [33,34]. While lower affinity or less efficient cytokine binding to native α_2M has been observed, these results are difficult to interpret since, in most experiments, in order to detect binding, the concentration of α_2M greatly exceeds the concentration of cytokine. When the α_2M is present in great excess, low levels of contaminating fast-form or α_2M conformational intermediates may incorrectly suggest cytokine binding to native α_2M.

In this investigation, we show for the first time that PDGF-BB binds primarily to the transformed conformation of α_2M. Occupancy of both proteinase-binding sites in α_2M effectively precludes PDGF-BB binding, as has been demonstrated with TGF-β1 [21]. The extent of binding of ^{125}I-PDGF-BB to α_2M–methylamine reported here is about the same as that reported previously [27]. By contrast, our native α_2M, purified in the laboratory from fresh plasma, bound significantly less PDGF-BB. These studies demonstrate that the properties of the PDGF-BB/α_2M interaction are similar to those of the TGF-β1/α_2M interaction. Although the specific radioactivity of ^{125}I-PDGF-BB and the level of binding to α_2M–methylamine precluded the determination of an equilibrium dissociation constant, studies presented in Figure 1 suggest that the PDGF-BB/α_2M–methylamine interaction may be weaker than the TGF-β1/α_2M–methylamine interaction studied previously (K_D 10–100 nM, [22]).

PDGF-BB which was bound to the receptor-recognized conformation of α_2M (α_2M–methylamine) was rapidly cleared from the plasma. The organ distribution data and the inhibition of clearance by excess α_2M–methylamine strongly suggest that α_2M-R/LRP is responsible for the hepatic uptake of ^{125}I-PDGF-BB–α_2M–methylamine complex (excess α_2M–methylamine did not affect the clearance of free PDGF-BB). Similar results have been demonstrated with TGF-β1–α_2M–methylamine [20] and TNF-α–α_2M–methylamine complexes [30]. These studies demonstrate that the fate of a cytokine bound to α_2M in the plasma will depend on the conformation of α_2M.

Since studies examining direct binding of cytokines to native α_2M *in vitro* cannot be independently interpreted, we studied this interaction in experiments which incorporated *in vitro* and *in vivo* techniques. In the plasma clearance system, the ability of purified cytokine–α_2M complexes to interact with α_2M-R/LRP (and thereby clear from the circulation) provided an index of α_2M conformation and an alternative to using mobility in non-denaturing PAGE alone. Defining 'native α_2M', based on interaction with α_2M-R/LRP, is appropriate in experiments with cytokines, since receptor binding is an important factor that may determine how α_2M affects cytokine function. In the plasma clearance system, we conclusively determined that PDGF-BB binds to native α_2M *in vitro* when native α_2M was defined by the lack of binding to α_2M-R/LRP.

We then studied the interaction of cytokines with α_2M *in vivo*. Based on the non-linear clearance profile of free TGF-β1, we previously suggested that some TGF-β1 may quickly bind to α_2M after intravenous injection [20]. By subjecting plasma samples to non-denaturing PAGE and autoradiography, we demonstrated that both ^{125}I-TGF-β1 and ^{125}I-PDGF-BB bind α_2M in the circulation. Since the mobilities of the bound cytokines (when measured by autoradiography) were identical to the mobility of slow-form murine α_2M, we propose that native α_2M (non-α_2M-R/LRP-recognized) is the major carrier of TGF-β1 and PDGF-BB in the blood. Philip and O'Connor-McCourt [23] drew the same conclusion for TGF-β1 based on Western-blot analyses of plasma samples subjected to non-denaturing PAGE. In their studies, TGF-β1 which was endogenous to plasma co-migrated with slow-form α_2M.

The binding of cytokines to native α_2M *in vivo* probably reflects interactions which are weak but favoured because of the very high concentration of native α_2M. Hypothetically, any process which generates significant levels of intravascular proteinase may shift the balance so that PDGF-BB and TGF-β1 bind to receptor-recognized α_2M. In patients with sepsis, α_2M–kallikrein complexes can be detected by immunological methods in the blood [46]. Other pathophysiological processes which might result in the formation of intravascular fast-form α_2M–cytokine complexes were reviewed recently by Borth [47]. The major question to be answered is whether α_2M–proteinase complexes can survive in the plasma long enough to alter intravascular levels of cytokines. Some α_2M–proteinase complexes such as α_2M–thrombin clear at a decreased rate compared with α_2M–methylamine and α_2M–trypsin [38], but still bind increased levels of TGF-β1 compared with native α_2M [48]. These complexes would be expected to compete most favourably with native α_2M for cytokine binding in the blood. Finally, the ratio of native α_2M to α_2M–proteinase complexes may be quite different in extravascular spaces compared with the blood. Therefore, the predominant conformation of α_2M responsible for cytokine binding and the result of this interaction on cytokine function may depend on the tissue compartment under consideration.

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