Reversible Binding of Platelet-derived Growth Factor-AA, -AB, and -BB Isoforms to a Similar Site on the "Slow" and "Fast" Conformations of α2-Macroglobulin*

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The mechanism by which the platelet-derived growth factor (PDGF)-binding protein, α2-macroglobulin (α2M), modulates PDGF bioactivity is unknown, but could involve reversible PDGF-α2M binding. Herein we report that >70% of 125I-PDGF-BB or -AB complexed to α2M was dissociated by SDS-denaturation followed by SDS-polyacrylamide gel electrophoresis, i.e., most of the binding was noncovalent. Reduction of the PDGF-α2M complex following denaturation dissociated the cytokine from α2M by >90%, suggesting covalent disulfide bond formation. Approximately 50% of the dissociated growth factor was dissociated by lowering the pH from 7.5 to 4.0. 125I-PDGF-BB bound α2M in a time-dependent manner (t1/2 = ~1 h), reaching equilibrium after 4 h. The 125I-PDGF-BB/α2M complex dissociated more slowly (t1/2 = ~2.5 h). "Slow" and "fast" α2M bound nearly equal amounts of PDGF-AB or -BB. Trypsin treatment converted PDGF-BB/α2M complex to the fast conformation but did not release bound 125I-PDGF-BB. All PDGF-isoforms (AA, -AB, and -BB) competed for binding with 125I-PDGF-BB binding to slow α2M and fast α2M-methylene by 65–80%. Other cytokines that bind α2M (transforming growth factor β1 and β2, tumor necrosis factor-α, basic fibroblast growth factor, interleukin-1β, and -6) did not compete for 125I-PDGF-BB binding slow α2M, but transforming growth factor-β and basic fibroblast growth factor inhibited 125I-PDGF-BB binding α2M-methylene by 30–50%. The reversible nature of the PDGF-α2M complex could allow for targeted PDGF release near mesenchymal cells which possess PDGF receptors.

Platelet-derived growth factor (PDGF)1 and PDGF-like factors secreted by smooth muscle cells (1), endothelial cells (2), monocytes (3, 4), and macrophages (4–6) are potent mitogens and chemotactants for cells of mesenchymal origin. Numerous studies that implicate PDGF as a key mediator in the normal processes of development, tissue maintenance, and wound healing have been reviewed (7). PDGF has also been proposed as a link in the progression of diseases such as atherosclerosis (8) and pulmonary fibrosis (9, 10). Two different monomeric chains of PDGF (A and B) give rise to three possible dimers (AA, AB, BB), and these dimeric isoforms recognize dimeric cell-surface receptors composed of α and/or β chains (11). PDGF isoforms recognize their receptors according to a receptor subunit model, i.e., AA, AB, BB dimers bind to αα receptors; BB, AB dimers to αβ; and the BB dimer to ββ receptors (12). The different subtypes of PDGF and receptors could allow for a fine tuning of cell responsiveness, since different cell types can vary greatly in the ratio of isoforms secreted and in the receptor composition which the target cell possesses (13). Cell responsiveness to PDGF in vitro can be further modulated by other growth factors such as TGF-β (14) and by PDGF-binding proteins (15).

The major PDGF-binding protein is α2-macroglobulin (α2M) and PDGF-α2M complexes have been isolated from plasma and from macrophage supernatants (16–19). This 725-kDa protein apparently serves multiple functions as a cytokine-binding protein (20), wide spectrum proteinase inhibitor (21–25), and immune regulator (26, 27). α2M was first described as a proteinase inhibitor and the mechanism whereby native or electrophoretically "slow" α2M covalently entraps proteinases has been extensively studied (see Ref. 25 for review). A proteinase cleaves α2M in its "bait region," and this cleavage induces a conformational change in the α2M molecule which entraps the proteinase. The conformational change makes the α2M more compact and hence has greater mobility on nondenaturing gel electrophoresis than the native or slow form of α2M. The irreversible triggering of the proteinase trap is mimicked by primary amines (28), and the electrophoretically "fast" α2M-proteinase or α2M-amine complex is receptor-recognized by fibroblasts (29, 30) and macrophages (31–34). PDGF binds both slow and fast forms of α2M (15), and PDGF-stimulated fibroblast proliferation (15) and chemotaxis (35) are inhibited by slow α2M. α2M inhibits the binding of PDGF to its cell-surface receptor and thus has been suggested to limit the amount of PDGF that is available to bind to these receptors (16). On the other hand, methyamine-modified, fast, α2M synergistically enhances the growth promoting activity of human PDGF purified from platelets (15). Thus, α2M modulates the biological activities of PDGF in vitro. It has been speculated that α2M could serve multiple functions as a PDGF-binding protein in vivo (16), including (1) modulation of PDGF biological activity as discussed above, (2) protection of PDGF against proteolytic degradation, and

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1 The abbreviations used are: PDGF, platelet-derived growth factor; α1M, α1-macroglobulin; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IL, interleukin; FPLC, fast protein liquid chromatography; BAPNA, Nα-benzoyl-DL-arginine-p-nitroanilide; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
3) clearance of PDGF from the circulation.

α2M also binds and modulates the biological activities of several other growth promoting cytokines, including transforming growth factor-β (TGF-β) (36-32), tumor necrosis factor-α (TNF-α) (43), basic fibroblast growth factor (bFGF) (44), interleukin-1β (IL-1β) (45), interleukin-6 (IL-6) (46), nerve growth factor (47) and human growth hormone (48). The biological activity of some of these cytokines is inhibited when bound to α2M, as is the case with TGF-β (42). Others such as IL-6 (46) and PDGF (15, 18) retain biological activity when complexed to this binding protein. While it is becoming increasingly apparent that a number of different cytokines utilize α2M as a binding protein, the sites on the α2M molecule to which these factors bind could differ. Determining whether or not these different cytokines compete for the same binding site(s) on this protein could be important in discerning the in vivo effects of α2M as a potential modulator of cytokine activity. This is likely since many cell types (e.g. macrophages) secrete mixtures of these cytokines and α2M (49). Also, other cytokines bind α2M, it is of major importance to determine the possible competitive nature of these factors for PDGF binding to α2M. Herein, we report that the majority of PDGF binding to slow or fast α2M is reversible or noncovalent, and that all PDGF isoforms compete for a similar site on α2M.

MATERIALS AND METHODS

Growth Factors and α2M—Human PDGF purified from platelets, TGF-β1, and TGF-β2 were purchased from R & D Systems (Minneapolis, MN). PDGF-AA, -AB, and -BB isoforms, bFGF, TGF-α, IL-1β, and IL-6 were obtained from UpState Biotechnology (Lake Placid, NY). Human α2M was purchased from Calbiochem (San Diego, CA) and bovine plasma α2M was obtained from Boehringer Mannheim.

Conversion of α2M from Slow to Fast—All α2M preparations were subjected to dialysis against 100 volumes of distilled water to precipitate fast α2M, which was present to some extent in all preparations. The native α2M was tested for trypsin binding activity as described below. Slow α2M was converted to fast α2M by incubation with 25 mM methylamine (Tris-HCl, 50 mM, pH 8.0) overnight at 25 °C or by incubation with a 4:1 molar excess of trypsin or plasmin for 30 min at room temperature. Excess methylamine was removed from α2M-methylamine complexes by dialysis against 100 volumes of 50 mM Tris-HCl, pH 8.2, at 4 °C. Excess trypsin or plasmin was removed from α2M-trypsin or α2M-plasmin complexes by gel filtration chromatography (Superose 6 FPLC). Fast α2M preparations were stored at 4 °C in 50 mM Tris, pH 8.2, to prevent precipitation. Slow α2M was stored at 4 °C in 20 mM sodium citrate buffer, pH 6.5. α2M preparations were tested for PDGF contamination as described previously (15).

Trypsin Binding Assay for α2M—Native α2M was tested for trypsin binding activity by a modification of a previously described method (50). Increasing concentrations of α2M were added to 96-well microtiter plates to a final volume of 50 μl/well in 25 mM Tris-HCl, 150 mM NaCl, pH 7.4. 3 μl/well of 1 mg/ml trypsin (Sigma) was then added for 10 min to bind available native α2M, followed by the addition of 6 μl/well of 1 mg/ml soybean trypsin inhibitor (Sigma), which inhibited all trypsin activity not bound to α2M. After 10 min, 80 μl/well of 0.1 M Tris-HCl, 10 mM CaCl2 buffer, pH 8.0, was added, followed immediately by 100 μl/well 3 mM Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA) hydrochloride (Sigma). The colorimetric reaction was stopped by the addition of 10 μl of glacial acetic acid. The increase in the optical density read at 405 nm is proportional to the quantity of active trypsin (covalently trapped within α2M) available to convert the BAPNA substrate to its product.

Gel Filtration Chromatography—PDGF-α2M complexes were routinely prepared by incubating 1 ng of human 152I-PDGF-AB or human 125I-PDGF (100 pg/ml of 125I-PDGF-AB) with 100 pg/ml of α2M fast or slow form for 24 h at 37 °C. These mixtures were isolated by loading onto a gel filtration, molecular weight exclusion column (Superose 6 FPLC, Pharmacia LKB Biotechnology Inc.) equilibrated in phosphate-buffered saline, pH 7.5, operating at a flow rate of 0.5 ml/min. The column was standardized with the following molecular mass markers: aprotinin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), β-amylose (200 kDa), apoferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (V₀). Fractions (1 ml) were counted on a γ-counter to measure radiolabeled PDGF or assayed for α2M by ELISA as described below. Protein was routinely measured by absorbance (280 nm) to ensure that identical amounts of PDGF/α2M were loaded on the column.

Gel Electrophoresis—Electrophoresis of the PDGF/α2M mixtures in a nondenaturing (5% Tris-borate) gel was performed as described previously (51). α2M that was incubated with 152I-PDGF-AB or -BB as desorbed above for gel filtration chromatography was mixed with Triton X-100 buffer (10 μg of α2M in 40 μl added to 4 μl of 10 × buffer with 10 μl of glycerol) and electrophoresed on a native 5% gel. Native gels were either stained with Coomassie Blue and dried for autoradiography or transferred to nitrocellulose, blocked with 5% BSA for 2 h, then shaken with 1:2000 sheep anti-human α2M-horseradish peroxidase (Immuno-technie, The Netherlands) for 1 h, washed, and treated with 6 μl of 1 mg/ml soybean trypsin inhibitor (Sigma), which inhibited all trypsin activity not bound to α2M. After 100 μl/well of 1% BSA in PBS was added, the plate was washed five times with PBS containing 0.05% Tween-20 (PBST) and then 200 μl/well of 1% BSA in PBS was added. After a 5-h incubation at 4 °C, the wells were washed five times with PBST, and 100 μl/well of standard α2M or unknown sample diluted in 1% BSA-PBS were added and incubated overnight at 4 °C. Zero antibody activity was considered if 25% of the quantity of active trypsin (covalently trapped within a2M) available to convert the BAPNA substrate to its product. The following day, the plate was washed five times with PBST and 100 μl/well of horseradish peroxidase-conjugated, sheep anti-human α2M (Serotec, Kidlington, Oxford, United Kingdom) diluted in 1% BSA-PBS (1:5000) was added and incubated at room temperature for 5 h. After washing five times with PBST, the wells were developed for 15–30 min with the diaminonium salt of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) containing 0.003% hydrogen peroxide. The absorbance (405 nm) was measured on a Titertek Multiscan (Flow Laboratories, McLean, VA). Doubling dilutions of α2M (2–1000 ng/ml, which were coated 100 pl/well with a 1:1000 dilution of anti-α2M, which inhibited all trypsin activity not bound to α2M. The increase in the optical density read at 405 nm is proportional to the quantity of active trypsin (covalently trapped within α2M) available to convert the BAPNA substrate to its product.

RESULTS

Isolation of PDGF-α2M Complexes by Gel Filtration Chromatography—To establish the elution profile of the PDGF-α2M complex, human 152I-PDGF-AB was incubated with human α2M in Ham’s F-12 buffer with HEPES, CaCl2, and 0.25% BSA (binding buffer) at pH 7.4 and the mixture was loaded onto a Superose 6 FPLC column equilibrated in PBS at the same pH. Later experiments were designed to study the formation of this complex on the same column and the
subsequent dissociation of human PDGF-AB or recombinant PDGF-BB from human or bovine α2M following a decrease in the pH of the binding buffer. A separate series of experiments addressed the formation of the PDGF-α2M complex on this column in the absence or presence of the three recombinant human PDGF isoforms or several other cytokines (TGF-β, bFGF, IL-1β, IL-6, TNF-α) that have been reported to bind to α2M. Human α2M eluted as single peak as measured by ELISA and the major peak of human 125I-PDGF-AB coeluted with this immunoreactive α2M (Fig. 1).

Binding of PDGF to Slow and Fast α2M—Isolation of the 125I-PDGF-AB-α2M complex by nondenaturing gel electrophoresis was performed to demonstrate the purity of slow and fast α2M preparations and to establish the amount of human plasma-derived 125I-PDGF-AB bound to these forms of α2M. Both purified forms of slow human α2M and fast methylamine-modified α2M bound human 125I-PDGF-AB (Fig. 2A). A quantitative analysis of the 125I-PDGF-AB bound to these α2M forms showed that approximately equivalent amounts of PDGF bound either the fast or the slow form (Fig. 2B).

Preferential Noncovalent Binding of PDGF to α2M—To measure the extent of covalent or noncovalent binding of 125I-PDGF-AB to native α2M, the 125I-PDGF-AB-α2M complex was subjected to SDS denaturation or 2-mercaptoethanol reduction prior to SDS-polyacrylamide gel electrophoresis. As expected, SDS treatment dissociated the α2M molecule into its ~180-kDa subunits as determined by protein staining and autoradiography of 125I-α2M-methylamine (Fig. 3). No 125I-PDGF-AB was initially detected by autoradiography at the molecular weight of this α2M subunit (Fig. 3A). Instead, the 125I-PDGF-AB was detected at the molecular mass of the ~30-kDa PDGF dimer (SDS treatment) or the ~15-kDa PDGF monomer following reduction. However, a longer exposure of the SDS-denatured 125I-PDGF-AB-α2M complex revealed a minor autoradiographic signal, indicating that some PDGF remained covalently bound to the α2M (Fig. 3B). The same experiments were performed with recombinant 125I-PDGF-BB. γ-Counting the intact 125I-PDGF-AB-α2M complex from the nondenaturing gels and the α2M subunits from denatured or reduced SDS gels (Fig. 4) demonstrated that 20–30% of the 125I-PDGF-BB remained bound to the denatured α2M subunit and less than 10% of the 125I-PDGF-BB remained bound to the binding protein subunits after reduction (Fig. 4). Similar results were obtained for bovine 125I-PDGF-BB-α2M complex.

![Fig. 1. Binding of human slow α2M to human 125I-PDGF purified from platelets.](image1)

100 µg of α2M was mixed with 1 ng of 125I-PDGF-AB in 500 µl of binding buffer and incubated for 1 h at 37 °C prior to loading on a Superose 6 FPLC column. 125I-PDGF-AB (open circles) coeluted with a single peak of α2M (closed circles) that was measured by ELISA. The α2M was quantified based on a standard curve (inset) generated with the same human α2M that was present in the PDGF-AB-α2M complex. Arrows indicate molecular mass markers, 1, blue dextran (440 kDa); 2, thyroglobulin (668 kDa); 3, apoferritin (440 kDa); 4, β-amylase (200 kDa); 5, BSA (66 kDa); 6, carbonic anhydrase (29 kDa); and 7, cytochrome c (12.4 kDa).

![Fig. 2. Binding of methylamine-treated and untreated slow human α2M to human 125I-PDGF-AB purified from platelets.](image2)

Slow α2M was modified by methylamine as described under "Materials and Methods." 125I-PDGF-AB (1 ng) was mixed 100 µg of human slow α2M or α2M-methylamine in 500 µl of binding buffer. 10 µg of the 125I-PDGF-AB/α2M mixture was loaded onto a nondenaturing 5% Tris-borate gel and electrophoresed to demonstrate fast (F) α2M-methylamine and slow (S) α2M migration. Complexes of 125I-PDGF-α2M were transferred to nitrocellulose. Panel A, Western blot of slow α2M (lane 1) and methylamine-modified α2M (lane 2) using a rabbit anti-human α2M horseradish peroxidase-conjugated antibody and the corresponding autoradiograph of 125I-PDGF bound to slow α2M (lane 3) and α2M-methylamine (lane 4). Panel B, quantification of bound 125I-PDGF-AB in slices of nitrocellulose from the same experiment shown in panel A as determined by γ-counting. S and F represent slow α2M (open circles) and α2M-methylamine (closed circles), respectively.

![Fig. 3. Effect of denaturation and reduction on the human 125I-PDGF-AB-α2M complex and 125I-α2M-methylamine.](image3)

Human slow α2M (100 µg) was incubated with 125I-PDGF-AB (1 ng) in 500 µl of binding medium, pH 7.4, and incubated for 1 h at 37 °C and then treated with either 7.5% SDS or 10% 2-mercaptoethanol in SDS prior to electrophoresis in a 7.5% SDS gel. Panel A, Coomassie Blue-stained protein of 125I-PDGF-AB/α2M that was denatured (lane 1) or reduced (lane 2) arrow (a) indicates α2M subunit in lane 1. Autoradiography of lane 1 shows that human 125I-PDGF-AB was dissociated from the α2M subunit upon denaturation by SDS and all visible radioactivity appears at ~30 kDa (lane 3). Autoradiography of lane 2 demonstrates that reduction results in the appearance of a ~15-kDa monomer of 125I-PDGF-AB (lane 5). Autoradiography of standard human 125I-α2M-methylamine that contained no PDGF following denaturation (lane 4) or reduction (lane 6) was useful in visualizing the subunits of α2M. Panel B, overexposure of lane 1 from Fig. 3 allowed visualization of some 125I-PDGF-AB bound to the denatured α2M subunit.
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**Fig. 4.** Quantitation of $^{125}$I-PDGF-BB bound to slow and methyamine or plasmin-treated fast $\alpha_2$M under nondenaturating, denaturing, and reducing conditions. 100 $\mu$g of human (panel A) or bovine (panel B) $\alpha_2$M, $\alpha_2$M-methyamine, or $\alpha_2$M-plasmin was incubated with 1 ng $^{125}$I-PDGF-BB in 500 $\mu$l of binding buffer for 24 h at 37 °C, and then equivalent aliquots containing 10 $\mu$g of $\alpha_2$M were electrophoresed in a nondenaturing (Tris-borate) gel or were pretreated with either SDS or SDS-containing 2-mercaptoethanol prior to electrophoresis in a SDS gel. The amount of $^{125}$I-PDGF-BB bound to either the intact (720 kDa) $\alpha_2$M on a nondenaturing gel (hatched bars) or bound to ~180-kDa $\alpha_2$M subunits on a denatured gel (open bars) or a reduced gel (solid bars) was measured on a y-counter. See Fig. 3A, lanes 4 and 6 and for visualization of the $^{125}$I-$\alpha_2$M-methyamine subunits by autoradiography. The data are expressed as the percentage of $\alpha_2$M-bound radioactivity relative to the total amount of radioactivity excised from the gel (i.e. $\alpha_2$M-bound and free $^{125}$I-PDGF-BB).

**pH-dependent Release of PDGF from $\alpha_2$M—Denaturation of the PDGF-$\alpha_2$M complex by treatment with SDS or 1 M acetic acid was shown to release the majority of PDGF bound to $\alpha_2$M, and these harsh conditions are known to dissociate the $\alpha_2$M molecule into its subunits. Thus, we studied the possible release of PDGF from $\alpha_2$M over a range of "physiological" pH (7.5-4.0) where the $\alpha_2$M molecule remains intact. Recombinant human $^{125}$I-PDGF-BB incubated with bovine slow $\alpha_2$M at pH 7.5 at 37 °C for 24 h formed a high molecular weight complex that eluted on a Superose 6 FPLC column at the same position as the plasma-derived human $^{125}$I-PDGF-$\alpha_2$M (see Fig. 1). By lowering the pH of solutions containing PDGF $\alpha_2$M complexes with acetic acid for 1 h prior to loading onto the Superose 6 column, a pH-dependent decrease in $^{125}$I-PDGF-$\alpha_2$M bound to bovine $\alpha_2$M was demonstrated (Fig. 5). The $\alpha_2$M retained its tetrameric (~725 kDa) structure across this pH range, and the amounts of $\alpha_2$M that eluted at this high molecular mass were equivalent as determined by protein absorbance (280 nm). The quantity of $^{125}$I-PDGF-BB bound to $\alpha_2$M, i.e. isolated from the PDGF-$\alpha_2$M complex on the gel filtration column, decreased ~50% as the pH was decreased from 7.5 to 4.0 (Fig. 5). At pH less than 4.0, the $\alpha_2$M was denatured and migrated as subunits with molecular mass >440 kDa.

**Time Course of $^{125}$I-PDGF-BB-$\alpha_2$M Association and Dissociation—**The time course of association of $^{125}$I-PDGF-BB with $\alpha_2$M and the subsequent dissociation of $^{125}$I-PDGF-BB from the purified $^{125}$I-PDGF-BB-$\alpha_2$M complex was investigated at pH 7.4. Identical aliquots of slow $\alpha_2$M (200 $\mu$g/ml) and $^{125}$I-PDGF-BB (2 ng/ml) were incubated at various time points (30 min to 24 h) at 37 °C in 0.5 ml of binding buffer prior to loading onto a Superose 6 FPLC column. The $\alpha_2$M peak zone (20–28 min) was pooled and bound $^{125}$I-PDGF-BB quantitated by y-counting as demonstrated in Fig. 1. The association time course showed that PDGF-BB bound $\alpha_2$M with a $t_{1/2} = 1$ h, and equilibrium was reached at ~4 h (Fig. 6A). Of the total amount of free $^{125}$I-PDGF-BB added to the binding buffer (1 ng = 25,000-27,000 cpm), approximately half of this radiolabeled PDGF was bound to $\alpha_2$M under these conditions. Panel A, to measure association of radiolabeled PDGF with its binding protein, slow bovine $\alpha_2$M (100 $\mu$g) was incubated at increasing time points (30 min to 24 h) at 37 °C in 0.5 ml of binding buffer, pH 7.4, with $^{125}$I-PDGF-BB (1 ng = ~26,000 cpm) prior to isolating $^{125}$I-PDGF-BB-$\alpha_2$M complex by FPLC gel filtration chromatography as described under "Materials and Methods." The amount of radioactivity bound to $\alpha_2$M in the 20–28 min elution zone of the gel filtration column (see Fig. 1) was pooled for each time point. At equilibrium binding (>4 h), approximately half of the radiolabeled PDGF was bound to $\alpha_2$M under these conditions. Panel B, dissociation of $^{125}$I-PDGF-BB from $^{125}$I-PDGF-BB-$\alpha_2$M complex was determined by incubating $^{125}$I-PDGF-BB-$\alpha_2$M for 24 h as shown in panel A, then incubating the isolated $^{125}$I-PDGF-BB-$\alpha_2$M complex for increasing time prior to FPLC separation. Again, the 20–28 min elution zone was pooled and radioactivity quantitated by y-counting. An equilibrium of bound to free $^{125}$I-PDGF-BB was reached by 6 h; i.e. approximately half of the PDGF dissociated by this time point.
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BB dissociation at pH 7.4. Under these conditions, 50–60% of the bound 125I-PDGF-BB dissociated from α2M (t1/2 = ~2.5 h) and an equilibrium was reached by ~6 h (Fig. 6B).

**PDGF Remains Bound to α2M Following Proteolytic Conversion to the Fast Form**—In order to determine whether or not PDGF would be released from slow α2M during the proteolytic conversion of the α2M from slow to fast by trypsin, recombinant human 125I-PDGF-BB complexed to bovine slow α2M at pH 7.5 was treated with a 4:1 molar excess (trypsin/α2M) for 20 min prior to non-denaturing gel electrophoresis. Autoradiography of the 125I-PDGF-BB-α2M complex before and after trypsin exposure showed that this proteolytic treatment converted the α2M to the electrophoretically fast form, but the 125I-PDGF-BB remained bound to the trypsin-activated α2M (Fig. 7).

**Competition of PDGF Isoforms and Other Cytokines for 125I-PDGF-BB Binding to α2M**—The three different isoforms of PDGF were tested for competitive binding to α2M to evaluate the capacity of this binding protein to potentially modulate the activity of all of the PDGF dimers. Human recombinant 125I-PDGF-BB (2 ng/ml) and bovine α2M (200 μg/ml) were incubated in the absence or presence of an excess of non-radioactive PDGF-AA, -AB, or -BB (8 μg/ml) for 24 h at 37°C. All three non-radioactive isoforms inhibited the majority of 125I-PDGF-BB binding as determined by a decrease in the radioactivity associated with the slow bovine α2M peak on the Superose 6 FPLC column (Fig. 8).

**Superose 6 FPLC chromatography demonstrating competition of human recombinant PDGF-AA, -AB, and -BB dimers for 125I-PDGF-BB binding to slow bovine α2M.** 125I-PDGF-BB (1 ng) was mixed with 100 μg of α2M in 500 μl of Ham’s F-12 binding buffer with HEPES, CaCl2, and 0.25% BSA, pH 7.4, in the absence (open circles) or presence of 5 μg of non-radioactive human recombinant PDGF-AA (open triangles), -AB (closed triangles), or -BB (closed circles) for 24 h at 37°C prior to loading chromatography in phosphate-buffered saline, pH 7.4. α2M eluted between 20–28 min (see Fig. 1 for immunoreactive profile). All three nonradioactive PDGF isoforms inhibited the formation of the 125I-PDGF-BB-α2M complex by ~70% (see Fig. 5). Arrows indicate molecular mass markers: 1, blue dextran (V,); 2, thyroglobulin (669 kDa); 3, apoferritin (440 kDa); 4, β-amylose (200 kDa); 5, BSA (66 kDa); 6, carbonic anhydrase (29 kDa); and 7, cytochrome c (12.4 kDa).

**Fig. 9. Competition of the known α2M-binding cytokines for recombinant 125I-PDGF-BB binding to slow bovine α2M and fast α2M-methylamine.** 125I-PDGF-BB (1 ng) was mixed with 100 μg of either slow bovine α2M (hatched bars) or α2M-methylamine (hatched bars) in binding buffer in the absence or presence of 5 μg of nonradioactive cytokine. The amount of radioactivity bound to α2M in the 10–30 min elution zone of the gel filtration column (see Fig. 4) was pooled for each column run. None of the cytokines tested, other than the PDGF isoforms, competed for slow α2M, while bFGF, IL-6, and TGF-β1 prevented complex formation between PDGF-BB/α2M and α2M-methylamine by as much as 50%, while TGF-β2 inhibited this interaction by only 10%. IL-6 and bFGF inhibited 125I-PDGF-BB binding to α2M-methylamine 30–35%, while IL-1β and TNF-α had negligible inhibitory effects on this complex formation. Bovine α2M and α2M-methylamine bound approximately equivalent amounts of 125I-PDGF-BB on the gel filtration column, a result similar to that obtained with human 125I-PDGF-AB binding to human α2M and α2M-methylamine on a non-denaturating gel (Fig. 2). Bovine α2M and α2M-methylamine were not run on a non-denaturating gel to test for differences in electrophoretic migration because, unlike human α2M-methylamine, bovine α2M-methylamine does not migrate as a fast form in a nondenaturing gel.**
turing gel (27). For this reason, the complete conversion of bovine azM to azM-methylamine was determined by the inability of the methylamine-treated azM to bind and thus inactivate trypsin (Fig. 9). Trypsin that is trapped within the azM molecule is still able to react with small molecules such as the BAPNA reagent (50), producing a yellow product that increases in proportion to the amount of slow azM in the reaction mixture (Fig. 9). These data demonstrated that methylamine treatment inhibited the trypsin binding capacity of bovine slow azM.

DISCUSSION

Earlier studies on plasma-derived PDGF introduced the concept that this growth factor forms covalent bonds with its binding protein, azM (17, 18). In the process of isolating and purifying PDGF from plasma and macrophage supernatants, we and others observed that it was necessary to acidify these biological fluids in order to separate the PDGF from its higher molecular weight-binding proteins before the growth factor could be detected by immunoassay or receptor assay (16, 19). The principal PDGF-binding proteins in these fluids were identified as azM macroglobulins and they were found to inhibit the binding of PDGF to either its cell-surface receptor or anti-PDGF antibodies, presumably by masking the receptor and antibody recognition site on the growth factor (19). The observation that PDGF could be detected after acidification suggested that at least a portion of the PDGF complexed to azM was bound noncovalently. The issue of covalent versus noncovalent binding of PDGF to azM is key to understanding the biological role(s) that this binding protein could serve in affecting the growth promoting activity of this cytokine. For example, azM has been proposed as a clearance protein for PDGF released into the circulation following platelet degranulation and the PDGF-azM complex could be cleared in the liver via azM receptors on hepatocytes (52). In extravascular tissues, macrophages, among other cell types, produce PDGF-like molecules and azM (19, 53, 54), and the proliferative response of fibroblasts to PDGF may be inhibited or enhanced by azM, depending on whether it is in the slow form or the fast receptor-recognized conformation (15). Thus, azM could serve as a clearance pathway for PDGF in the circulation and in extravascular tissues, but also as a positive or negative regulator of growth factor activity. We postulated that the control of PDGF-stimulated growth by azM likely involves the release of the growth factor from this binding protein, allowing PDGF to bind to its own receptor and trigger a mitogenic response (15). For this reason, the observation that the majority of the PDGF is bound to azM noncovalently (Figs. 3 and 4) and the demonstration that approximately 50% this growth factor can be released from its binding protein by lowering the pH of the incubation medium from 7.5 and 4.0 (Fig. 5) or in a time-dependent manner at pH 7.4 (Fig. 6) are consistent with our hypothesis that PDGF binding to azM is reversible. Such information is basic to our understanding of the mechanism(s) by which the binding protein influences cytokine activity.

Human 125I-PDGF-AB and recombinant human 125I-PDGF-BB both bound human or bovine slow azM, as well as fast azM that was prepared by reaction with either methylamine, plasmin, or trypsin. All three recombinant PDGF isofoms were observed to compete for 125I-PDGFB binding to either azM or azM-methylamine, suggesting that these isofoms all bind to a similar site on the azM molecule and that conversion from the slow to fast conformation by methylamine does not alter PDGF isoform binding. Indeed, azM and azM-methylamine that had been incubated with radiolabeled PDGF and then isolated by nondenaturing gel electrophoresis were found to contain approximately equivalent amounts of human 125I-PDGF-AB or human recombinant 125I-PDGF-BB. It will be of interest to determine whether or not the biological activities of PDGF-AA, -AB, and -BB are modulated by azM in a similar manner. Interestingly, the 125I-PDGF-BB remained bound to bovine azM after the 125I-PDGF-BB-azM complex was treated with an excess of trypsin to convert the azM to fast form (Fig. 7). Thus, proteinases apparently do not displace PDGF bound to azM, and these data suggest that azM could serve to protect PDGF from proteolytic degradation. Such a role for azM has been suggested for IL-6, which is inactivated by trypsin, but retains IL-6-like activity in the presence of trypsin when complexed to azM (46). These observations also suggest that PDGF and IL-6 interact with azM by a mechanism different from that of proteinases and primary amines.

The PDGF-azM complex could provide a readily obtainable source of PDGF in vivo since the growth factor can be removed under physiological conditions in vitro. Kinetic studies showed that under conditions which are similar to those found in plasma, i.e. high [azM] and relatively low [PDGF], half of the PDGF bound to azM in a time-dependent manner reaching equilibrium after about 4 h at 37 °C and subsequently half of this azM-bound PDGF dissociated from isolated PDGF/azM complex more slowly and reached a new equilibrium after 6 h at 37 °C (Fig. 6). A calculation of the rate constants for association (k1) and dissociation (k2), which must assume an excess of radioligand over receptor (or binding protein), were not performed on these kinetic data due to the low molarity of 125I-PDGF-BB (6.7 × 10^-11 M) relative to azM (3 × 10^-7 M); i.e. there was likely an excess of binding sites for PDGF. Further studies using saturation binding and kinetic studies are in progress to address the relative affinities of PDGF for azM versus the PDGF cell-surface receptor. The time course data shown in Fig. 6 for association of 125I-PDGF-BB with azM are closely similar to the time course of 125I-bFGF binding azM (44); i.e. low concentrations of both bFGF and PDGF binding to 200 μg/ml azM reached an equilibrium state at about 4 h. 125I-PDGF-BB was released rapidly from the 125I-PDGF-BB-azM complex by lowering the pH over a physiological range of 7.4-4.0 (Fig. 5). Such low pH could be encountered within lysosomes following internalization of the PDGF-azM complex via the azM receptor. In this case, it would be of interest to learn whether or not released PDGF (which is acid stable) could then be recycled to the cell-surface and remain bioactive. Thus, it is conceivable that PDGF could be dissociated from the PDGF-azM complex by either deprotonation of unbound PDGF via internalization by PDGF cell-surface receptors (which would favor the release of azM-bound PDGF to establish a new extracellular equilibrium) or PDGF could be released under conditions where pH is reduced.

Since several other cytokines bind to azM, we sought to establish if these growth factors compete for PDGF binding to azM. Excess concentrations of TGF-β1, TGF-β2, TNF-α, bFGF, IL-1β, and IL-6 were tested for their inhibitory potency in preventing complex formation between 125I-PDGF-BB and azM or azM-methylamine. None of these cytokines inhibited the binding of 125I-PDGF-BB to the slow form of azM (Fig. 9). These data suggest that PDGF isomers could bind to slow azM unhindered in the presence of cytokine mixtures in vivo. In contrast, some of these cytokines competed for PDGF binding to fast azM. TGF-β1, but not TGF-β2, inhibited complex formation between 125I-PDGF-BB and azM-methylamine by ~50%. While both TGF-β1 and TGF-β2 bind to fast azM (42), it is conceivable that these two factors have
differing affinities for αM or bind to different sites on the α2M molecule. Thus, the TGF-β1-binding site on the fast α2M could overlap or allosterically modulate the PDGF-binding site. Similarly, IL-6 and bFGF inhibited complex formation between 125I-PDGF-BB and α2M-methylamine by ~20–30%, which could suggest some overlap or allosteric hinderance by PDGF and these cytokines binding simultaneously to fast α2M. TGF-β1 has been reported to inhibit the binding of 125I-bFGF binding to α2M, while PDGF does not compete for this interaction (44). It is conceivable that certain αM-binding cytokines, such as TGF-β1 and bFGF, could mediate the release of PDGF from the fast form of α2M, but not the slow form. Other cytokines apparently do not interfere with PDGF binding to α2M. For example, TNF-α binds fast α2M-methylamine (43) but did not inhibit the formation of the PDGF-BB-αM-methylamine complex (Fig. 9). Such interactions between these cytokines and the two forms of αM are likely to be complex and require further study.

A variety of growth-promoting cytokines, including PDGF, are likely to be involved in the processes of normal tissue maintenance and repair, and their abberant expression may well be linked to pathogenic disorders such as pulmonary fibrosis and atherogenesis that are characterized by an increase in cell proliferation and extracellular matrix production (7). A growing number of studies suggest that αM could play a role in modulating the biological activity, clearance, and degradation of these cytokines in either the circulation or in extravascular tissues (36–48). Because αM also serves as a proteinase inhibitor and since proteinases irreversibly convert αM to a fast or receptor-recognized form, this adds another layer of complexity to the problem of understanding the mechanisms by which a number of cytokines interact with a common binding protein. Proteinases apparently are capable of “turning on” the modulatory effects that αM has for some cytokines in at least two different ways: 1) cytokines such as TNF-α (43) and IL-1β (45) preferentially bind fast αM (42) and thus the activity of these cytokines would not be expected to be directly regulated by slow form αM, and 2) PDGF binds both native and proteinase-reacted forms and α2M converted to the fast form by methylamine synergistically enhances the growth promoting activity of PDGF, while the slow form inhibits PDGF-stimulated growth (15). It is presently unclear whether or not αM-proteinase complexes potentiate or inhibit the growth promoting activity of the different PDGF isoforms, and this is the subject of ongoing studies. Understanding the interactions between the network of proteinases, αM, and growth factors which bind αM will be fundamental to our knowledge of cytokine function in vivo.

In summary, the majority of the PDGF-αM association is noncovalent and all three PDGF isoforms (AA, AB, and BB) bind similarly to αM. PDGF binds to slow and fast αM and the slow to fast conversion by trypsin does not dissociate bound PDGF. PDGF-BB dissociates slowly from isolated PDGF-αM complex (t1/2 = 2–3 h) at pH 7.4 and reaches an equilibrium state after 6 h. Furthermore, a decrease in pH from 7.4 to 4.0 causes a rapid, progressive release of PDGF from αM. Because PDGF binding to αM is reversible, αM could release PDGF near the cell surface in close proximity to its own receptor.

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REFERENCES

1. Sjolund, M., Hedin, U., Sejersen, T., Heldin, C., and Thyberg, J. (1988) J. Cell Biol. 106, 403–413
2. Paulson, Y., Hammersch, A., Heldin, C-H., and Westermark, B. (1987) Nature 328, 715–717
3. DiCorleto, P. E., and Bowen-Pope, D. F. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1919–1923
4. Martinet, Y., Bitterman, P. B., Mornex, J-F., Grotendorst, G. R., Martin, G. R., and Crystal, R. G. (1986) Nature 319, 158–160
5. Shimokado, K., Raines, E. W., Madtes, D. K., Barrett, T. B., Benditt, E. P., and Ross, R. (1985) Cell 43, 277–286
6. Bonner, J. C., Osorno-Vargas, A. R., Badgett, A., and Brody, A. R. (1991) Am. J. Respir. Cell Mol. Biol. 5, 539–547
7. Raines, E. W., Bowen-Pope, D. F., and Ross, R. (1990) in Peptide Growth Factors and Their Receptors I. (Sporn, M. B., and Roberts, A. B., eds) pp. 173–265, Springer-Verlag, New York
8. Ross, R. (1981) Atherosclerosis 1, 293–311
9. Antoniades, H. N., Bravo, M. A., Avila, R. E., Galanopoulos T., Neville-Golden J., Maxwell, M., and Selman, M. (1990) J. Clin. Invest. 86, 1055–1064
10. Nagaoa, J., Trapnell, B. C., and Crystal, R. G. (1990) J. Clin. Invest. 85, 2023–2027
11. Heldin, C.-H., and Westermark, B. (1990) J. Cell. Biol. 96, 193–196
12. Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, P. A., Ross, R., Murray, M. J., and Bowen-Pope, D. F. (1988) Science 240, 1529–1531
13. Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. J., and Bowen-Pope, D. F. (1989) J. Biol. Chem. 264, 8771–8778
14. Battegay, E. J., Raines, E. W., Seifert, R. A., Bowen-Pope, D. F., and Ross, R. (1990) Cell 63, 515–524
15. Bonner, J. C., Badgett, A., Osorno-Vargas, A. R., Hoffman, M., and Brody, A. R. (1990) J. Cell. Physiol. 145, 1–8
16. Raines, E. W., Bowen-Pope, D. F., and Ross, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3424–3428
17. Huang, J. S., Huang, S. S., and Deuel, T. S. (1983) J. Cell Biol. 93, 383–388
18. Huang, J. S., Huang, S. S., and Deuel, T. S. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 342–346
19. Bonner, J. C., Hoffman, M., and Brody, A. R. (1989) Am. J. Respir. Cell Mol. Biol. 1, 171–179
20. LaMarre, J., Wollenberg, G. K., Gonias, S. L., and Hayes, M. A. (1991) J. Cell. Biol. 115, 3–14
21. Barrett, A. J. (1981) Methods Enzymol. 80, 737–754
22. Barrett, A. J., and Starkey, P. M. (1973) Biochem. J. 133, 709–724
23. Sottrop-Jensen, L. (1987) in The Plasma Proteins (Putnam, F. W., ed.) pp. 192–291, Academic Press, Orlando, FL
24. Sottrop-Jensen, L., and Birkedal-Hansen, H. (1989) J. Biol. Chem. 264, 393–401
25. Sottrop-Jensen, L. (1988) J. Biol. Chem. 263, 11539–11542
26. Hoffman, M. R., Pizzo, S. V., and Brice, J. B. (1987) J. Immunol. 139, 1885–1890
27. James, K. (1980) Trends Biochem. Sci. 5, 43
28. Van Leuven, F., Cassiman, J. J., and Van den Berghe, H. (1981) J. Biol. Chem. 256, 9016–9022
29. Willingham, M. C., Maxfield, F. R., and Pastan, I. H. (1979) J. Cell Biol. 82, 614–625
30. Marynen, P., Van Leuven, F., and Cassiman, J. J. (1983) Ann. N.Y. Acad. Sci. 421, 401–403
31. Ney, K. A., Gitditz, S., and Pizzo, S. V. (1985) Biochemistry 24, 4586–4592
32. Kaplan, J., and Nielsen, M. L. (1979) J. Biol. Chem. 254, 7323–7328
33. Kaplan, J., Ray, F. A., and Keogh, E. A. (1981) J. Biol. Chem. 256, 7705–7707
34. Imber, M. J., and Pizzo, S. V. (1981) J. Biol. Chem. 256, 8134–8139
35. Osorno-Vargas, A. R., Bonner, J. C., Badgett, A., and Brody, A. R. (1990) Am. J. Respir. Cell Mol. Biol. 3, 595–602
36. Huang, S. S., O’Grady, P., and Huang, J. S. (1988) J. Biol. Chem. 263, 1535–1541
37. LaMarre, J., Wollenberg, G. K., Gauldie, J., and Hayes, M. A. (1990) Lab Invest 62, 545–551
38. LaMarre, J., Hayes, M. A., Wollenberg, G. K., Hussaini, I., Hall, S. W., and Gonias, S. (1991) J. Clin. Invest. 87, 39–44
39. LaMarre, J., Wollenberg, G. K., Gonias, S. L., and Hayes, M. A. (1991) Biochem. Biophys. Acta 1091, 197–204
40. McCaffrey, T. A., Falcone, D. J., Brayton, C. F., Agarwal, L. A.,
PDGF Isoforms Binding α2-Macroglobulin

41. O’Connor-McCourt, M., and Wakefield, L. (1987) J. Biol. Chem. 262, 14090-14099
42. Danielpour, D., and Sporn, M. B. (1990) J. Biol. Chem. 265, 6973-6977
43. Wollenberg, G. K., LaMarre, J., Rosendal, S., Gonias, S. L., and Hayes, M. A. (1991) Am. J. Pathol. 138, 265-272
44. Dennis, P. A., Saksela, O., Harpel, P., and Rifkin, D. B. (1989) J. Biol. Chem. 264, 7210-7216
45. Borth, W., and Luger, T. A. (1989) J. Biol. Chem. 264, 5818-5825
46. Matsuda, T., Hirano, T., Nagasawa, S., and Kishimoto, T. (1989) J. Immunol. 142, 148-152
47. Koo, P. H., and Stach, R. W. (1989) J. Neurosci. Res. 22, 247-261
48. Adham, N. F., Chakmakjian, Z. H., Wehl, J. W., and Bethune, J. E. (1969) Arch. Biochem. Biophys. 132, 175-183
49. Nathan, C. F. (1987) J. Clin. Invest. 79, 319-326
50. Ganrot, P. O. (1966) Clin. Chim. Acta 14, 493-501
51. Nelles, L. P., Hall, P. K., and Roberts, R. C. (1980) Biochem. Biophys. Acta 623, 46-56
52. Davidson, O., Christensen, E. I., and Glieman, J. (1985) Biochem. Biophys. Acta 846, 85-92
53. Moshier, D., and Wing, D. (1976) J. Exp. Med. 143, 462-467
54. White, R., Janoff, A., and Godfrey, H. P. (1980) Lung 158, 9-14