Purification of PDGF-AB and PDGF-BB from Human Platelet Extracts and Identification of All Three PDGF Dimers in Human Platelets†

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ABSTRACT: We have developed a panel of monoclonal antibodies to platelet-derived growth factor (PDGF) which have variable specificities for the three dimeric forms of the molecule (AA, AB, and BB). We have used these antibodies to detect and immunoaffinity purify the individual dimers from human platelet rich plasma. Extracts of outdated platelet preparations were initially chromatographed over CM-Sepharose and then passed over the Sepharose-coupled monoclonal antibodies in series in selectively isolate the three dimeric forms of PDGF. The PDGF eluted from the affinity columns was subsequently further purified by reversed-phase HPLC. From 300 units of outdated platelet preparations, we purified 58 μg of PDGF-BB and 140 μg of PDGF-AB. Using the monoclonal antibodies to develop PDGF dimer-specific ELISAs, it was observed that all three PDGF dimer forms are present in fresh human platelet extracts and that the ratios of the three dimer forms vary depending upon the extraction conditions used. The identification of all three PDGF dimer forms in human platelets points to the need to view PDGF isolated from human platelets by conventional techniques as a mixture of all three forms and not solely as PDGF-AB.

Platelet-derived growth factor (PDGF)[1] has been identified as the principal mitogen in serum for cultured cells of mesenchymal origin, including fibroblasts, smooth muscle cells, and glial cells [reviewed in Ross et al. (1986)]. PDGF was first identified (Ross et al., 1974; Kohler & Lipton, 1974) and purified (Antoniades, 1981; Deuel et al., 1981; Heldin et al., 1981; Raines & Ross, 1982) from human platelet sources as a disulfide-linked dimer. Amino acid sequence data demonstrated two primary peptide sequences and suggested that PDGF is a heterodimer of two distinct but homologous polypeptide chains termed A and B (Johnsson et al., 1984). The two chains share 60% amino acid sequence identity and can form dimers of PDGF-AA, PDGF-BB, and PDGF-AB. All three of the potential dimers of PDGF have been identified from natural sources and shown to be biologically active (Stroobant & Waterfield, 1984; Heldin et al., 1986; Hart et al., 1988; Hammacher et al., 1988). PDGF isolated from human platelets was thought to be composed predominantly of the AB heterodimer, but it was not until recently that PDGF-AB was selectively purified (Hart et al., 1988; Hammacher et al., 1988). The finding of all three dimer forms of PDGF in human platelets, described in this paper, points to the need to view preparations of PDGF isolated from platelets by previously described methods (Antoniades, 1981; Deuel et al., 1981; Heldin et al., 1981; Raines & Ross, 1982) as a potential mixture of all three dimer forms, and not solely as PDGF-AB.

Initial studies of the biological effects of PDGF were based upon the assumption that all three dimer forms of PDGF would bind to a single cell-surface receptor. However, it has recently been demonstrated that there are multiple receptors for PDGF that exhibit different specificities for the three dimer forms (Hart et al., 1988; Heldin et al., 1988; Nister et al., 1988; Claesson-Welsh et al., 1989; Matsui et al., 1989; Seifert et al., 1989). The mitogenic potency of the different dimers of PDGF depends on the cell type studied and parallels the ability of the cells to bind the different dimers (Seifert et al., 1989). Thus, when studying the biological and biochemical effects stimulated by PDGF, it is critical to have both pure preparations of the individual dimer forms and knowledge of the PDGF receptor makeup of the cells being studied.

In this paper, we use a panel of anti-PDGF monoclonal antibodies to selectively purify PDGF-AB and PDGF-BB from human platelet rich plasma and demonstrate that human platelets contain all three dimer forms of PDGF.

Experimental Procedures

Generation of Anti-PDGF Monoclonal Antibodies. (A) Immunization and Fusion. Eight-week-old female Balb/c mice were immunized with either purified recombinant PDGF-AA or PDGF-BB homodimer. A total of 500 ng of pure ligand was blotted onto nitrocellulose paper (Schleicher & Schuell, Keene, NH) and dried at 37 °C for 1 h. The nitrocellulose was then solubilized with DMSO, mixed with an equal volume of Freund's adjuvant, and injected into the peritoneal cavity of the mice. This procedure was repeated 4 times at 2-week intervals. The 3 days following the third booster immunization the mice received intravenous injections of 100 ng of pure PDGF-AA or PDGF-BB. On the fourth day, the spleen was removed, splenocytes were isolated, and the cells were fused with the NS-1 mouse myeloma cell line using poly(ethylene glycol) as described by Brown et al. (1980). The fused cells were grown in 96-well culture dishes in RPMI 1640 growth medium (GIBCO, Grand Island, NY) supplemented with 15% fetal calf serum (Hyclone Labs, Logan, UT)

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[1] Abbreviations: PDGF, platelet-derived growth factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography.

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using HAT selection (10 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Ten days following the fusion, conditioned medium from the culture wells was harvested and assayed for the presence of anti-PDGF antibody as described below.

(*B*) *Detection of Anti-PDGF Antibodies.* Hybridomas generated were screened for the production of anti-PDGF antibody by immunoprecipitation of either ^{125}I-PDGF-AA or ^{125}I-PDGF-BB$_{Tyr}$. Aliquots of conditioned culture media were incubated for 2 h at 22 °C with 100 000 cpm of ^{125}I-labeled PDGF-AA or PDGF-BB$_{Tyr}$, which had been diluted in RIP buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 1% bovine serum albumin). Rabbit anti-mouse IgG (cappel Labs, Melvern, PA) and *Staphylococcus aureus* (Calbiochem-Behring, San Diego, CA) were then added to precipitate the immune complexes. The samples were washed 2 times with RIP buffer and then counted in a γ counter to determine the amount of ^{125}I-labeled ligand precipitated.

Cell lines producing antibody of interest were twice cloned by limiting dilution and grown in ascites fluid, and the antibody was purified by protein A–Sepharose chromatography. Antibody subclass was determined by ELISA using subclass-specific rabbit anti-mouse antisera (Miles Scientific, Naperville, IL).

PDGF Ligands. Recombinant PDGF-AA and PDGF-BB homodimers were produced in recombinant expression systems in the yeast *Saccharomyces cerevisiae* (Kelly et al., 1985). The A-chain and B-chain genes were constructed from synthetic oligonucleotides and encode 110 and 109 amino acid polypeptides, respectively. The A-chain sequence was based upon the "endothelial cell" sequence (Tong et al., 1987; Collins et al., 1987). The homodimers were purified from yeast culture media to greater than 95% purity as determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and N-terminal amino acid sequence. A mutant form of B-chain termed BB$_{Tyr}$, which has a tyrosine residue at position 23 of the mature coding sequence in place of phenylalanine, was used for iodination of PDGF-BB. A purified preparation of PDGF was isolated from human platelet rich plasma as previously described (Raines & Ross, 1982) and contained an unknown composition of the three dimer forms (PDGF-AA, -BB, and -AB). Recombinant PDGF-AA and PDGF-BB$_{Tyr}$, platelet-isolated PDGF-AB, and the anti-PDGF receptor monoclonal antibody PR7212 (Hart et al., 1987) were labeled with ^{125}I by use of Iodobeads (Pierce Chemical Co., Rockford, IL) to approximate specific activities of 15×10^6, 30×10^6, 100×10^6, and 20×10^6 cpm/μg, respectively. The platelet-isolated PDGF sample which contained an unknown composition of the three dimer forms was labeled with ^{125}I using iodomonochloride as previously described (Bowen-Pope & Ross, 1985) to a specific activity of approximately 80×10^6 cmp/μg.

Purification of PDGF-AB and PDGF-BB. PDGF was partially purified from 300 units of outdated platelet-rich plasma preparations which had been obtained from regional blood banks as previously described (Raines & Ross, 1982). The platelet preparations were subjected to three rounds of freeze/thaw, heated to 55–57 °C for 8 min, and then left overnight at 4 °C. The platelet debris and precipitated fibrinogen were removed from the sample by centrifugation at 27000*g* for 30 min at 4 °C. The supernatant was removed and adjusted to pH 7.4 with 1 M Tris base. The sample was then loaded onto a CM-Sepharose Fast-Flow column, the column was washed with 10 mM Tris, pH 7.4, and 0.19 M NaCl, and then the PDGF was eluted with 10 mM Tris, pH 7.4, and 0.5 M NaCl. The peak eluate fractions containing the PDGF activity, as determined by radioreceptor assay, were pooled, concentrated on an Amicon concentrator using a YM-10 membrane Amicon, Danvers, MA), and then stored frozen at −20 °C.

To selectively purify the individual dimer forms of PDGF, partially purified platelet preparations were passed over a series of anti-PDGF monoclonal antibody affinity columns. Monoclonal antibodies 120.1.2.1 (BB specificity), 121.6.1.1 (BB/AB), 127.5.7.3 (AA/AB), and 127.1.1.5 (AA) were coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) under conditions specified by the manufacturer, at a ratio of 10 mg of antibody to 1 g of dry gel. The coupling efficiency was greater than 90% for each antibody. Five milliliters of antibody–Sepharose gel was used for each affinity column.

The partially purified sample was thawed and any precipitate removed by centrifugation at 35000*g* for 1 h at 4 °C. The sample was initially cycled over the 120.1.2.1–Sepharose column to selectively isolate PDGF-BB; the column was washed with 100 mL of phosphate-buffered saline (PBS) containing 0.5 M NaCl and then eluted with 0.1 M glycine, pH 2.5, and 0.5 M NaCl. The peak eluate fractions containing PDGF activity, as determined by ELISA using antibody 120.1.2.1, were pooled and then chromatographed by reversed-phase HPLC on a Micro Pak SP C_{18} column (Varian, Sunnyvale, CA) using a 0–100% acetonitrile gradient containing 0.1% trifluoroacetic acid, collecting 0.5-mL fractions. The peak fractions containing PDGF, as determined by ELISA and silver stain, were pooled and lyophilized. The flow through from the 120.1.2.1 column was then cycled over the 121.6.1.1–Sepharose column to bind PDGF-AB. The column was washed and eluted the same as the 120.2.1.2 column. The peak fractions containing PDGF, as determined by ELISA using antibody 121.6.1.1, were pooled, adjusted to pH 7.2 with Tris base, and then cycled over the 127.5.7.3 column to selectively bind PDGF-AB, eliminating any potential contaminating PDGF-BB. This column was then washed and eluted, and the peak eluate fractions, as determined by ELISA and silver stain, were chromatographed by HPLC as described above. The effluent from the 121.6.1.1 column was cycled over the 127.1.1.5 column to isolate PDGF-AA; the column was washed and eluted as described above.

Amino Acid Composition and N-Terminal Sequence Analysis. Amino acid composition analysis was done for both the purified PDGF-AB and PDGF-BB preparations to determine protein concentration. Composition analysis was done by using the Pico-Tag method of Waters Associates (Milford, MA). Briefly, protein samples were hydrolyzed in the vapors of constant-boiling HCl containing 1% (v/v) phenol for 22–24 h, in vacuo, at 110 °C. The amino acids were then derivatized with phenyl isothiocyanate (PITC) and the resulting phenylthiocarbamyl amino acids separated by reversed-phase HPLC using a Varian 5500 and recorded on a Varian Vista 400 data system.

N-Terminal amino acid sequence of the preparations was determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase protein sequencer equipped with a 120A on-line phenylthiohydantion amino acid analyzer (Applied Biosystems, Foster City, CA).

Development of PDGF Dimer-Specific ELISAs. Enzyme-linked immunoadsorption assays (ELISAs) were developed to detect and quantitate the individual dimer forms of PDGF. These assays utilize the ligand recognition specificity provided by the anti-PDGF monoclonal antibodies. The primary monoclonal antibody is coated onto 96-well

microtiter plates (5 μg/mL in 0.1 M Na_2HCO_3, pH 9.6) for 16 h at 4 °C. The wells are blocked with phosphate-buffered saline, pH 7.2, containing 0.25% bovine serum albumin and 0.5% Tween-80; then either the test samples or known PDGF standards are added to the wells for 1 h at 37 °C. The samples are removed, the wells are washed, and then rabbit anti-PDGF polyclonal antisera are added for an additional hour at 37 °C. The wells are washed, and biotin-conjugated goat anti-rabbit IgG is added (Vector Labs, Burlingame, CA) followed by streptavidin coupled to horseradish peroxidase (Amersham, Arlington Heights, IL) and *o*-phenylenediamine (Sigma Chemical Co, St Louis, MO) as the color reagent. The concentration of the PDGF dimers is determined by comparison to a standard curve generated by using known amounts of purified ligand.

Generation of Rabbit Anti-PDGF Antisera. Rabbits were immunized with either recombinant PDGF-AA or PDGF-BB. Five micrograms of purified protein was dried onto nitrocellulose; the nitrocellulose was dissolved into DMSO and then mixed with Freund's adjuvant. Rabbits were immunized at monthy intervals for at least 6 months to obtain high-titer antisera. Antisera from the PDGF-AA-immunized rabbits were mixed with anti-PDGF-BB antisera to obtain a polyclonal antibody that recognizes all three dimer forms of PDGF.

Mitogenic and Receptor Binding Assays. Mitogenic assays were performed essentially as described by Raines and Ross (1985) monitoring stimulation of [^{3}H]thymidine incorporation. Radioreceptor assays were done essentially as described by Bowen-Pope and Ross (1985). PDGF receptor down-regulation assays were done as described by Hart et al. (1988), using both ^{125}I-PDGF-AB and ^{125}I-labeled antibody PR7212 (Hart et al., 1987) as probes, and human dermal fibroblasts (SK-5 cells) as the target cell line. Briefly, the culture media were removed from the cells, and the test samples, diluted into binding medium (Ham's F12 medium, pH 7.4, 25 mM Hepes, and 0.25% bovine serum albumin), were added to the cells for 2 h at 37 °C. The samples were removed and the cells incubated with either ^{125}I-PDGF-AB (0.5 ng/mL) or ^{125}I-labeled antibody PR7212 (20 ng/mL) for an additional 2 h at 4 °C with constant shaking. The cells are washed 3 times with phosphate-buffered saline to remove unbound probe and harvested with solubilization buffer (phosphate-buffered saline, pH 7.2 and 1% Nonidet P-40), and the cell-associated ^{125}I was determined.

Results

Generation and Characterization of Anti-PDGF Monoclonal and Polyclonal Antibodies. A panel of anti-PDGF monoclonal antibodies has been developed which have different specificities for each of the three dimeric forms of PDGF. The mice used to generate hybridomas were immunized with either recombinant PDGF-AA or PDGF-BB. A series of fusions were performed with lymphocytes from both the PDGF-AA- and PDGF-BB-immunized mice. Antibodies from the hybridomas that were generated were initially examined for their ability to immunoprecipitate ^{125}I-PDGF-AA or ^{125}I-PDGF-BB_{Tyr}. Two cell lines were detected that secreted antibodies that immunoprecipitated PDGF-AA, 127.1.1.5 and 127.5.7.3, and two that immunoprecipitated PDGF-BB, 120.1.2.1 and 121.6.1.1 (Table I). These four antibodies were then examined for their ability to immunoprecipitate a ^{125}I-labeled preparation of PDGF isolated from platelet-rich plasma and which was composed of an unknown mixture of the three dimeric forms of PDGF. The labeling procedure used to iodinate the PDGF mixture specifically labels tyrosine residues. Since only the A chain of PDGF contains tyrosine, only PDGF-AA and PDGF-AB should be labeled. Antibodies 127.5.7.3 and 121.6.1.1 were both able to immunoprecipitate the iodinated mixture of PDGF (data not shown). The fact that 121.6.1.1 was developed against PDGF-BB and does not recognize PDGF-AA suggests that it must be recognizing PDGF-AB in the labeled mixture. The ability of 127.5.7.3 to immunoprecipitate the labeled mixture of PDGF, and the inability to 127.1.1.5 do so, suggests that 127.5.7.3 also detects PDGF-AB. Immunoprecipitation of subsequently purified PDGF-AB has verified these conclusions (Table I). The binding specificity for each of the antibodies, shown in Table I, is further supported by the cross-reactivities observed in the dimer-specific ELISA assays (Figure 5).

Table I. Immunoprecipitation of ^{125}I-Labeled PDGF by Anti-PDGF Monoclonal and Polyclonal Antibodies[a]

antibody	subclass	^{125}I-labeled ligand (cpm precipitated) AA	AB	BB	specificity
monoclonal Ab					
120.1.2.1	G_1	941	1201	28784	BB
121.6.1.1	G_1	900	16065	8629	BB AB
127.1.1.5	G_{2b}	4089	984	2720	AA
127.5.7.3	G_1	32529	13802	2820	AA AB
neg cont	G_1	857	1089	2847	
rabbit antisera					
anti-PDGF-AA		43357	32275	5166	
anti-PDGF-BB		2937	10890	30132	
anti-PDGF-AA + anti-PDGF-BB		42373	39248	34272	
nonimmune sera		3327	3470	3770	

[a] Two microgrms of purified monoclonal antibody along with rabbit anti-mouse IgG and *S. aureus* was used to immunoprecipitate the ^{125}I-labeled PDGF preparations. An anti-human GM-CSF monoclonal antibody was used for the negative control. One microliter of rabbit antisera was used for the immunoprecipitations. 100000 counts per minute (cpm) of ^{125}I-labeled ligand was added for each immunoprecipitation.

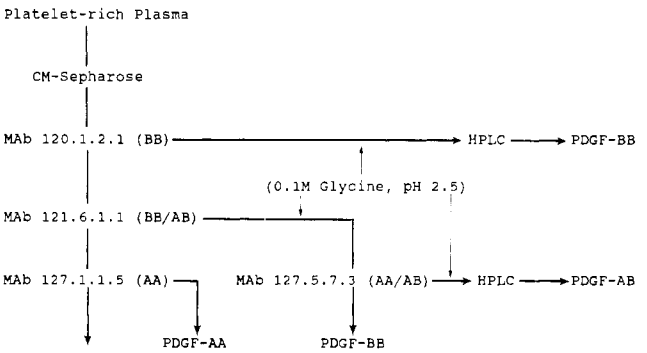

FIGURE 1: Schematic of purification steps to isolate PDGF-AB and PDGF-BB. A series of anti-PDGF immunoaffinity columns were used to selectively isolate the different dimer forms of PDGF (ligand specificity).

The rabbit anti-PDGF antisera were found to show selectivity for the immunizing homodimer of PDGF along with the heterodimer but had little cross-reaction with the opposite homodimer (Table I). When the rabbit anti-PDGF-AA and rabbit anti-PDGF-BB antisera were pooled, all three dimer forms of PDGF were detected.

Purification of PDGF Isoforms from Platelet-Rich Plasma. A series of immunoaffinity columns composed of the anti-PDGF monoclonal antibodies were used to selectively purify PDGF-AB, PDGF-BB, and PDGF-AA from human platelet rich plasma. This purification scheme (Figure 1) relies upon the different specificities of the antibodies for the three dimer forms of PDGF. Freeze/thaw lysates of outdated platelet-rich plasma were utilized for this study. The lysates were initially

chromatographed over CM-Sepharose as described by Raines and Ross (1982). The peak eluate fractions from this column were pooled, concentrated, and then passed over the immunoaffinity columns in series so as to selectively purify each of the three dimer forms of PDGF. The first column, antibody 120.1.2.1 (BB) coupled to Sepharose, was used to selectively isolate PDGF-BB from the mixture. PDGF-BB eluted from this column was subsequently further purified by reversed-phase HPLC. The effluent from the 120.1.2.1 column, which still contains both PDGF-AA and PDGF-AB, was then passed over Sepharose-Coupled antibody 121.6.1.1 (BB/AB) to isolate PDGF-AB. The PDGF eluted from this column was subsequently neutralized and then passed over Sepharose-coupled antibody 127.5.7.3 (AA/AB) to selectively bind the PDGF-AB in the mixture, allowing any contaminating PDGF-BB eluted from the 121.6.1.1 column to be eliminated. PDGF-AB eluted from the 127.5.7.3 column was then further purified by HPLC. The effluent from the 121.6.1.1 column, which should contain PDGF-AA, was passed over antibody 127.1.1.5 coupled to Sepharose. Upon elution of this column, no substantial levels of PDGF-AA were detected for further purification, probably due to the low levels of PDGF-AA in the starting preparation.

Physical Characterization of Purified PDGF-AB and PDGF-BB. A total of 58 μg of PDGF-BB and 140 μg of PDGF-AB were purified from 300 units of outdated platelet-rich plasma, as determined by amino acid composition analysis. The total yield of 198 μg (PDGF-AB + PDGF-BB) following HPLC purification represents a 2274-fold purification from the CM-Sepharose eluate and corresponds to an overall yield of approximately 80% of the PDGF-competing activity, as determined from the initial levels of PDGF receptor competing activity in the CM-Sepharose eluate. Recoveries varied in subsequent purifications from 40 to 90% and averaged 66% (n = 7). The percentages of PDGF-BB and PDGF-AB purified in these preparations were (mean ± standard deviation) 13.5% ± 7.8% and 85.0% ± 5.6%, respectively.

The PDGF-BB and PDGF-AB samples were subjected to N-terminal amino acid sequence analysis. Sequences beginning with serine at position 1 of the mature coding sequence, threonine at position 6, and threonine at position 33 were detected for the PDGF-BB preparation. The determination of the relative amounts of B-chain cleavage products was accomplished with nine cycles of Edman degredation. The amino acids detected at each cycle were aligned with the known PDGF B-chain sequence, allowing for the detection of multiple N-terminal in the preparation. The percentages of the three sequences were quantitated by using amino acids unique to each of the observed cleavage products, and were approximately 20% Ser_1, 45% Thr_6, and 35% Thr_{33}. No A-chain sequence was detected in the PDGF-BB preparation. It is important to note that no tyrosine residues were detected in the amino acid composition analysis of the final PDGF-BB preparation, since only the A chain of PDGF contains tyrosine residues. This further verifies the purity of the PDGF-BB sample.

When PDGF-AB was analyzed for the N-terminal sequence, the three B-chain sequences were also detected in approximately the same ratios as in the PDGF-BB preparation. In contrast, only the sequence beginning with N-terminal serine was detected for the A-chain sequence. In addition, the sums of the A-chain and B-chain sequences were present in equal molar ratios. When the PDGF-AB preparation was analyzed by ELISA using antibody 120.1.2.1 to check for contaminating PDGF-BB, no ELISA-positive material was detected.

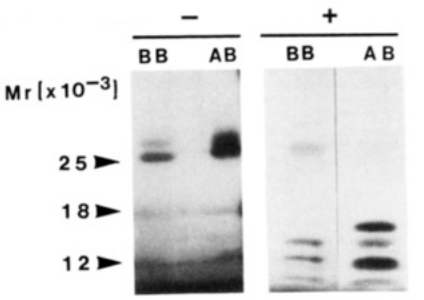

FIGURE 2: Silver stain of purified PDGF-BB and PDGF-AB HPLC-purified PDGF-BB (500 ng) and PDGF-AB (1 μg) were separated by SDS–PAGE on a 10–20% gradient gel in the presence (+) and absence (−) of reducing agent. Molecular weight standards are shown on the left.

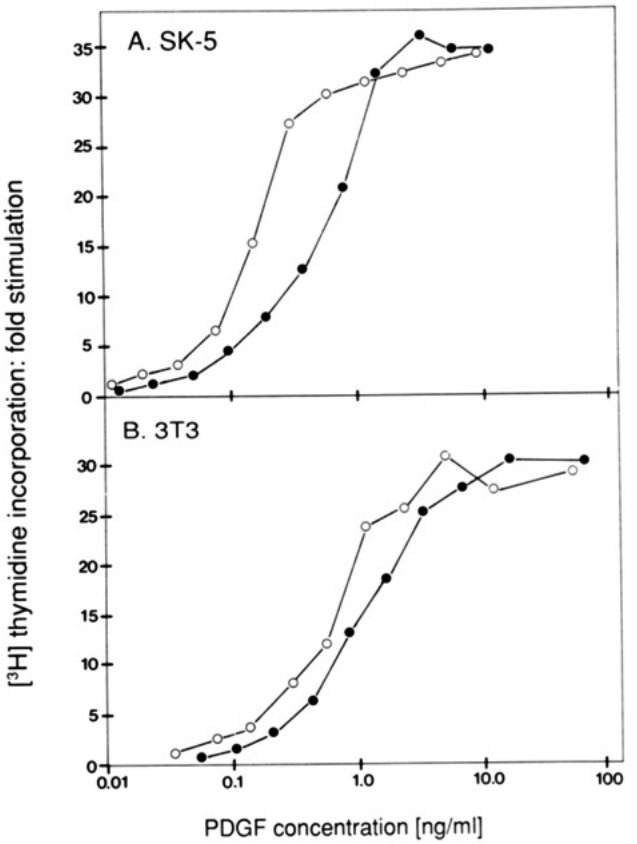

FIGURE 3: Analysis of mitogenic activity for PDGF-AB and PDGF-BB. PDGF-AB (open circles) and PDGF-BB (closed circles) were analyzed for mitogenic activity on (A) human dermal fibroblasts, SK-5 cells, and (B) Swiss 3T3 cells. The results are expressed as x-fold stimulation of [^{3}H]thymidine incorporation over basal levels and represent the mean of triplicate determinations from a representative experiment.

Silver stains of the final HPLC-purified product for PDGF-BB showed two major bands under nonreducing conditions of 28 and 30 kDa (Figure 2). Two bands of 28 and 30 kDa were also observed for PDGF-AB separated under nonreducing conditions (Figure 2). When PDGF-BB and -AB were separated under reducing conditions, multiple bands ranging from 10 to 14 kDa and from 10 to 16 kDa were observed for PDGF-BB and PDGF-AB, respectively.

Analysis of Biological Properties of Purified PDGF-AB and PDGF-BB. PDGF-AB and PDGF-BB were compared for mitogenic activity in a standard [^{3}H]thymidine incorporation assay as described by Raines and Ross (1985). The mitogenic activity of these preparations was measured on both human dermal fibroblasts (SK-5 cells) and Swiss 3T3 cells (Figure

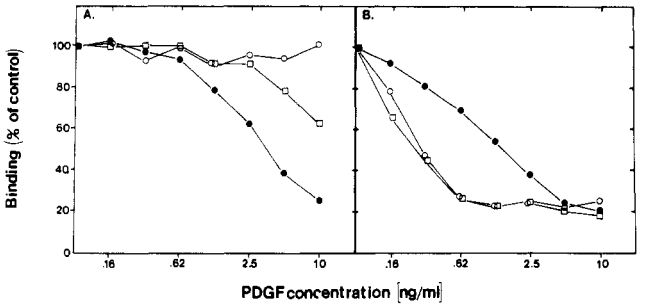

FIGURE 4: Ligand-induced PDGF receptor down-regulation. Monolayers of human dermal fibroblasts grown in 24-well culture dishes were incubated at 37 °C with increasing concentrations of PDGF-AB (open circles), PDGF-BB (closed circles), or PDGF-AB contaminated with 20% PDGF-BB (open boxes). The cells were subsequently incubated with either (A) ^{125}I-labeled antibody PR7212 or (B) ^{125}I-labeled PDGF-AB. The cells were washed and harvested with solubilization buffer, and the total cell-associated ^{125}I was determined. The data are plotted as the mean of triplicate determinations from a representative experiment. Nonspecific binding for each probe has not been subtracted.

3). PDGF-AB was found to stimulate a half-maximal response on human dermal fibroblasts at approximately a 3-fold lower concentration than PDGF-BB, 0.18 and 0.55 ng/mL, respectively (Figure 3A). On Swiss 3T3 cells, the concentration of PDGF-AB required to induce a half-maximal response was only 20% lower than for PDGF-BB, 0.74 and 0.96 ng/mL, respectively (Figure 3B).

Purified PDGF-AB and PDGF-BB were also compared for the ability to bind to cell-surface receptors at 37 °C and stimulate receptor internalization, a phenomenon known as down-regulation (Heldin et al., 1982). The depletion of PDGF receptors from the cell surface was then monitored by incubation at 4 °C with either ^{125}I-labeled PDGF-AB or ^{125}I-labeled anti-PDGF receptor monoclonal antibody PR7212, which sees a subset of the PDGF receptors (specific for the PDGF receptor β-subunit) and that we have described elsewhere (Seifert et al., 1989). Human dermal fibroblasts incubated with PDGF-BB showed a dose-dependent decrease in antibody binding (Figure 4A) due to down-regulation of the PDGF receptor β-subunit. In contrast to this, no significant decrease in antibody binding was observed following the addition of PDGF-AB to the cells at 37 °C (Figure 4A). This latter finding is similar to the results we previously described using recombinant PDGF-AA (Hart et al., 1988). Using a PDGF-AB preparation which was found to be 20% contaminated with PDGF-BB, we observed a dose-dependent decrease in antibody PR7212 binding (Figure 4A). The dose response observed correlates with the concentration of contaminating PDGF-BB in the PDGF-AB preparation. When the two PDGF-AB preparations and PDGF-BB were assayed in a standard radioreceptor assay format using ^{125}I-labeled PDGF-AB as the probe, all three samples stimulated equal maximal decrease in ^{125}I-PDGF-AB binding, although a larger concentration of PDGF-BB was required (Figure 4B). This is due to the presence of approximately 5-fold more PDGF-BB than PDGF-AB binding sites on SK-5 cells (Seifert et al., 1989). It is important to note that the binding of ^{125}I-PR7212 is dependent only upon the presence of the PDGF receptor β-subunit while ^{125}I-PDGF-AB binding depends upon both the presence and the ratio of the α- and β-subunits of the PDGF receptor. For the SK-5 cells used in these studies, the ratio of β- to α-subunit is approximately 10:1 (Seifert et al., 1989).

PDGF Dimer-Specific ELISA. The ligand binding specificities of the anti-PDGF monoclonal antibodies have allowed us to develop ELISAs for detecting and quantitating each of

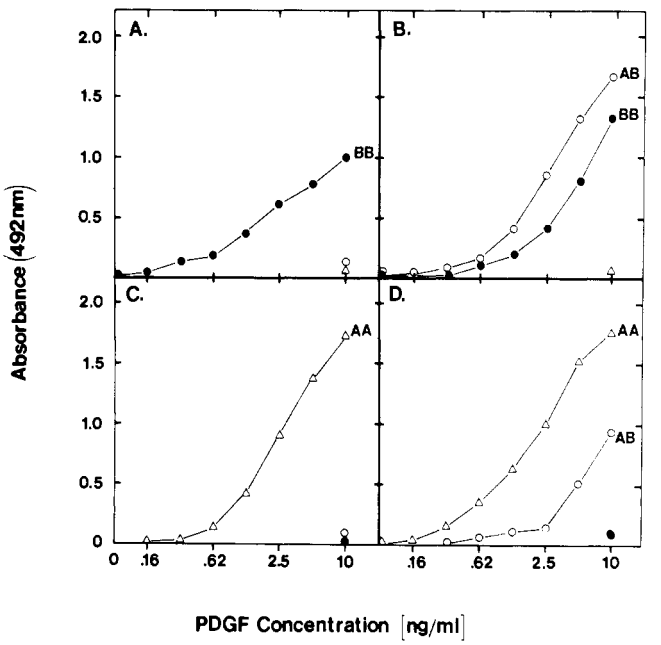

FIGURE 5: PDGF-specific ELISA assays. Sandwich ELISA assays were developed to detect and quantitate each of the three forms of PDGF utilizing the PDGF ligand binding specificity of a panel of anti-PDGF monoclonal antibodies. The individual monoclonal antibodies are coated onto 96-well microtiter plates, followed by the addition of either test samples or PDGF standards: PDGF-AA (open triangles), -AB (open circles), or -BB (closed circles). Rabbit anti-PDGF is then added, followed by biotin-conjugated goat anti-rabbit IgG, streptavidin–horseradish peroxidase, and substrate color reagent. Sample standard curves are ploted to demonstrate assay sensitivity and antibody cross-reactivity to the three PDGF dimers. (A) Antibody 120.1.2.1; (B) antibody 121.6.1.1; (C) antibody 127.1.1.5; (D) antibody 127.5.7.3.

the three dimer forms of PDGF in complex mixtures. Standard curves generated for each of the monoclonal antibodies are shown in Figure 5. The ELISA using antibody 120.1.2.1 (Figure 5A) can be used to specifically detect PDGF-BB. In contrast, antibody 121.6.1.1 (Figure 5B) preferentially detects PDGF-AB over PDGF-BB with less than 1% cross-reaction to PDGF-AA. Antibody 127.1.1.5 (Figure 5C) can specifically detect PDGF-AA with less than 3% cross-reaction with either PDGF-BB or PDGF-AB. Antibody 127.5.7.3 (Figure 2D) can also be used to detect PDGF-AA but has a 50% cross-reaction with PDGF-AB. Having determined homodimer concentrations using PDGF-AA- and PDGF-BB-specific assays, the influence of the homodimers on the PDGF-AB assays using antibodies 121.6.1.1 and 127.5.7.3 was calculated from the standard curves shown in Figure 5. PDGF-AB heterodimer concentrations were calculated by subtraction of the PDGF-AA and PDGF-BB influences on the PDGF values obtained with antibodies 127.5.7.3 and 121.6.1.1, respectively.

This analysis was done on fresh platelets extracted with three different conditions (Table II). For platelets extracted by freeze/thaw in a neutral pH buffer (pH 7.4), PDGF-AB was the predominant form of PDGF found (68% of total), followed by PDGF-BB (23%) and PDGF-AA (9%). In contrast, for platelets extracted by freeze/thaw in 100 mM acetic acid, all three PDGF forms were found at similar levels: PDGF-BB (41%), PDGF-AB (32%), and PDGF-AA (27%). When the platelets were incubated with thrombin to stimulate platelet activation and degranulation, PDGF-AB was the predominant form detected (60%). The increase in the relative levels of PDGF-AA and PDGF-BB in the acetic acid extract was a result of increased levels of the homodimers, increasing by 5-

Table II: PDGF Dimer Forms in Extracts of Fresh Human Platelets[a]

extraction condition	ng of PDGF (% of total) AA	BB	AB	total (ng)
primary extract				
(A) neutral pH	68 (9)	167 (23)	496 (68)	731
(B) 100 mM HOAc	350 (27)	540 (41)	418 (32)	1308
(C) thrombin	96 (18)	118 (22)	311 (60)	525
reextraction				
(A) neutral pH	69	144	85	298
(B) 100 mM HOAc	28	30	42	100
(C) thrombin	109	148	121	378

[a] Three hundred milliliters of freshly drawn blood was split into three equal volumes, and the platelets were isolated by differential centrifugation. Platelet pellets A and C were resuspended in 4.0 mL of a neutral pH extraction buffer (12 mM Na_2HCO_3, pH 7.4, 130 mM NaCl, 2 mM KCl, 19 mM sodium citrate, and 0.09% dextrose). Pellet B was resuspended in 4.0 mL of 100 mM acetic acid (HOAc). Pellets A and B were frozen and thawed 3 times, and the platelet debris were removed by centrifugation at 10000*g* for 1 h at 4 °C. The supernatant was removed and stored at −20 °C until analyzed by ELISA. To pellet C were added 5 mM $CaCl_2$ and 10 units/mL bovine thrombin. The mixture was incubated 15 min at 22 °C and then centrifugated as described above and the supernatant stored at −20 °C. The subsequent three platelet debris pellets were resuspended with 1.0 mL of 100 mM acetic acid, frozen, thawed twice, and centrifuged at 10000*g* for 1 h at 4 °C. The reextracted platelet supernatants were then stored at −20 °C until analyzed. The values given are total amounts of PDGF in the extracts.

and 3-fold, respectively. In contrast, there was no increase in PDGF-AB concentration in the acid extract compared to neutral pH extraction or thrombin stimulation.

Platelet debris obtained by centrifugation of the above extracts were reextracted with 100 mM acetic acid and the extracts analyzed by ELISA (Table II). Approximately equal amounts of PDGF-AA and PDGF-BB were detected in the acid reextractions of the neutral pH and the thrombin pellets, as compared to the levels detected in the initial extracts. In contrast, only 20–40% as much PDGF-AB was present in the second extracts. The PDGF levels detected in the reextraction of the first acetic acid extract were less then 10% of the amount measured in the initial extract.

The overall yield of total PDGFs in the three initial extracts was greatest for the acetic acid extract, releasing 1.8- and 2.5-fold more PDGF than the neutral pH and thrombin extracts, respectively. When extracts of freshly isolated platelets from four individual donors were analyzed by ELISA to look for variability in PDGF dimer composition, differences were detected in the total levels of PDGF extracted; however, there was little variability between donors in the percentages of the three dimers (data not shown).

Discussion

Initial studies examining the biological actions of PDGF were based upon the assumption that there existed a single cell-surface receptor which bound all three forms of the molecule (PDGF-AA, -AB, and -BB). However, it has recently been demonstrated that there are two receptor subunits for PDGF which dimerize to form three receptor phenotypes (Seifert et al., 1989) and that these three phenotypes have different binding specificities for the three dimeric forms of PDGF. The presence of three different receptor phenotypes determines the capacity of the cells to respond to each of the three dimers of PDGF.

The demonstration that all three dimers of PDGF can be found in platelets points out the necessity to develop purification procedures to selectively isolate and purify each of the three forms free of contamination from the other forms. Analysis of PDGF purified by using previously described procedures (Raines & Ross, 1982) demonstrated a wide variation in the dimer composition of these preparations. The importance of having pure preparations of each dimer free from contamination with the other dimeric forms of PDGF is demonstrated in Figure 4. When PDGF-AB was analyzed for its ability to down-regulate the β-subunit of the PDGF receptor, detected by antibody PR7212, it was observed that PDGF-AB had only minimal effect. In contrast, when a PDGF-AB preparation containing 20% PDGF-BB was analyzed, this preparation stimulated receptor down-regulation and subsequent loss of antibody binding. Thus, the presence of 20% PDGF-BB in a preparation of PDGF-AB can markedly affect receptor down-regulation and the subsequent interpretation of the results.

When platelet-isolated PDGF-BB was compared to PDGF-AB for stimulation of a mitogenic response, it was found that PDGF-AB was approximately 3-fold more potent on human dermal fibroblasts, but only 20% more potent on Swiss 3T3 cells (Figure 3). The reason for the differences in potency detected is unclear, but probably resides in the receptor subunit composition on the two cell types.

Amino acid sequence analysis of both PDGF-BB and PDGF-AB preparations demonstrated the presence of three N-terminal sequences for the B-chain. Further analysis of additional preparations has shown variation in the ratios of the three N-terminal sequences as well as the presence of a fourth B-chain amino-terminal sequence starting at lysine in position 80. It is unclear whether the cleavages observed in the B-chain have any biological significance or consequence, i.e., inactivation, or have simply occurred during purification. Preliminary results suggest that the various B-chain cleavages reported here do not affect mitogenic potency. The finding of only a single N-terminal sequence for the A chain suggests that the B chain is more sensitive to proteolytic attack. The reason for the difference in proteolytic sensitivity between A chain and B chain is unclear. The size heterogeneity detected for PDGF-AB and PDGF-BB following separation by SDS–PAGE under both nonreducing and reducing conditions (Figure 2) is probably due to the presence of the different proteolytically cleaved B chains. Consequently, these data show that PDGF dimer identity cannot be determined solely by apparent size following SDS–PAGE separation.

Hammacher et al. (1988) recently reported the purification of PDGF-AB from human platelets and proposed that the heterodimer constitutes the majority of PDGF in platelets. Since their purification method only provided a 10% yield, they were unable to assess the levels of the three dimeric forms of PDGF in fresh platelet lysates. They also reported the purification of PDGF-BB from platelets. There are no reports of PDGF-AA purification from platelets. In the studies described here, we demonstrate that all three dimeric forms of PDGF exist in human platelets and that the ratios detected for the three dimeric forms varies depending upon the extraction conditions used (Table II). It is important to note that the ELISA data presented in this paper was on extracts of freshly isolated platelets. When outdated platelet-rich plasma preparations obtained from regional blood banks are analyzed by ELISA for PDGF dimer content, PDGF-AB is the predominant form detected, 60–90%, followed by PDGF-BB, 10–40% (data not shown). Only trace amounts of PDGF-AA are detected. The range in percentages of PDGF-BB and PDGF-AB purified in multiple preparations agrees with the ELISA data of the starting material. The lack of detection of substantial levels of PDGF-AA in extracts of

platelet-rich plasma correlates with the inability to purify PDGF-AA from the outdated platelet preparations. The low levels of detectable PDGF-AA are probably partially due to the neutral pH freeze/thaw extraction conditions used to generate the platelet extracts. However, when platelet-rich plasma preparations have been freeze/thawed in the presence of 100 mM acetic acid, there is only a small increase in the amount of PDGF-AA detected (data not shown). The finding that PDGF-AA can constitute up to 27% of the total PDGF extracted from freshly isolated platelets, and the inability to detect substantial levels of PDGF-AA in platelet-rich plasma preparations even under acid conditions, suggests either that PDGF-AA is preferentially degraded in the platelet-rich plasma or that PDGF-AA may be associated with binding proteins in the plasma.

It has become clear that when investigating the actions of PDGF it is important to know both the exact dimer composition of the PDGF preparation and the receptor subunit composition of the target cells being examined. This is particularly critical in the assignment of specific actions to each of the three dimers since differences in the number and ratio of the two PDGF receptor subunits can affect the observed activities of the dimers. The detection of all three dimeric forms of PDGF in human platelets emphasizes the need to view preparations of PDGF isolated from human platelet sources as a mixture of all three PDGF dimers and not solely as PDGF-AB.

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