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Purification of human platelet-derived growth factor

(platelets/polypeptide hormones/fibroblast growth factor)

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ABSTRACT **Human platelets contain a polypeptide growth factor that stimulates the proliferation of connective tissue cells. Purification of this platelet-derived growth factor (PDGF) was accomplished by heat (100°C) treatment of washed platelets and subsequent ion-exchange chromatography, gel filtration in 1 M acetic acid, isoelectric focusing, and preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis. PDGF has an isoelectric point of 9.8 and a molecular weight ranging from 13,000 to 16,000 as judged by gel filtration in 1 M acetic acid or analytical sodium dodecyl sulfate gel electrophoresis under reducing conditions. The specific activity of the purified PDGF is 20 million times greater than that found in unfractionated human serum. Purified PDGF stimulates replicative DNA synthesis and cell proliferation in quiescent density-arrested cultures of BALB/c 3T3 cells at concentrations of 1 ng/ml (0.1 nM).**

Normal animal fibroblasts cultured *in vitro* require the presence of blood serum factors for growth. In the absence of serum the cells become growth arrested in the G_0/G_1 phase of the cell cycle. The readdition of serum to quiescent fibroblasts stimulates DNA synthesis and cell proliferation (1–10). Recently, we purified a heat-stable (100°C) cationic polypeptide from human serum that stimulates DNA synthesis and proliferation of mouse and human fibroblasts (11, 12). Nanomolar concentrations of this polypeptide were shown to stimulate the replication of about 10^4 confluent BALB/c 3T3 cells.

Biological assays with cell cultures and a specific radioimmunoassay for the human serum-derived growth factor (13) have demonstrated that the human serum growth factor polypeptide is largely derived from platelets (12, 13). The growth factor was present in clotted blood serum, but its concentration was greatly diminished in platelet-poor plasma; growth factor activity was recovered from platelet suspensions heated at 100°C for 2–10 min (13, 14). Thus, the growth factor that we had purified from serum was similar, if not identical, to the platelet-derived growth factor (PDGF) that was described by Ross *et al.* (15) and by Kohler and Lipton (16).

Platelets constitute a better starting material for the preparation of homogeneous PDGF than serum, because PDGF is concentrated in platelets and serum contains a high concentration of protein. The present studies outline a protocol for large scale purification of PDGF from clinically outdated human platelets.

EXPERIMENTAL PROCEDURES

Separation of Platelets from Plasma. Clinically outdated human platelets (3–5 days old) were pooled, concentrated by centrifugation at 3200 × *g* for 30 min, and washed twice in 9 vol of 17 mM Tris·HCl, pH 7.5/0.15 M NaCl/0.1% glucose and 1 vol of acid/citrate/dextrose buffer.§ The washed platelets were concentrated by centrifugation, transferred into plastic containers with a minimal volume of 0.01 M sodium phosphate/0.08 M NaCl at pH 7.4, and stored at −15°C. All further manipulations with platelets were conducted at 4°C in plastic containers unless otherwise stated.

Extraction of Growth Factor from Platelets. Approximately 500–1000 units of washed platelets were thawed, and sufficient 0.01 M sodium phosphate/0.08 M NaCl at pH 7.4 was added to bring the final volume to 4 ml per original platelet unit. A unit of platelets by convention is defined as the total platelet content of 1 unit (500 cc) of blood. The platelet suspension was heated at 100°C for 10 min and centrifuged, and the supernatant fluid was collected. The precipitate was extracted four times with 1 M sodium chloride (2 ml per original platelet unit). For each extraction, precipitate was stirred with 1 M sodium chloride overnight. Supernatant fluids were collected by centrifugation at 7500 × *g* for 30 min and dialyzed for 48 hr against 20 vol of 0.08 M NaCl/0.01 M sodium phosphate, pH 7.4.

Ion-Exchange Chromatography. After dialysis, we combined all supernatant fluids and subjected them to ion-exchange chromatography by using CM-Sephadex C-50. The combined PDGF fractions were stirred overnight with 0.5 vol of CM-Sephadex C-50 previously equilibrated with 0.08 M NaCl/0.01 M sodium phosphate, pH 7.4. The mixture was then poured into glass columns (8 cm × 55 cm, 2-liter capacity) and washed with 6 bed vol of 0.08 M NaCl/0.01 M sodium phosphate, pH 7.4. PDGF activity was eluted from the column with 2 bed vol of 1 M NaCl and concentrated by ultrafiltration with Millipore Pelicon membranes.

Bio-Gel P-150 Filtration in 1 M Acetic Acid. CM-Sephadex-purified PDGF was dialyzed against 1 M acetic acid and lyophilized in plastic tubes. Lyophylized material was dissolved in a minimal volume of 1 M acetic acid, and 0.5- to 0.8-ml aliquots were applied to a Bio-Gel P-150 (mesh 100–200) column (1.2 × 100 cm) equilibrated with 1 M acetic acid. After elution with 1 M acetic acid, 2-ml fractions were collected; aliquots of each fraction were diluted 1:100 with 0.15 M sodium chloride containing 1% human serum albumin and were assayed for PDGF activity.

Isoelectric Focusing. The active fractions from the Bio-Gel columns described above were pooled and lyophilized. The salt-free dry powder was reconstituted with H_2O. Preparative isoelectric focusing (pH 9–11) was conducted in a vertical LKB column (110 ml) according to the manufacturer's protocol. Aliquots of each fraction were diluted with 0.15 M sodium

Abbreviations: PDGF, platelet-derived growth factor; $NaDodSO_4$, sodium dodecyl sulfate.

§ Acid/citrate/dextrose buffer was prepared according to National Institutes of Health Formula A: 8 g of citric acid monohydrate/22 g of dextrose (anhydrous)/26 g of sodium citrate (dihydrate), made up to 1 liter with distilled water.

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chloride/1% human serum albumin and assayed for PDGF activity.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Electrophoresis. Electrophoresis on 15% polyacrylamide gels was conducted according to the method of Laemmli (17). For preparative runs, samples of PDGF and molecular weight marker proteins were disaggregated by heating to 100°C for 2 min in 2% $NaDodSO_4$/0.01 M sodium phosphate, pH 7.8, without 2-mercaptoethanol; for some analytical runs, the samples were boiled in 2% $NaDodSO_4$ with 10% 2-mercaptoethanol. After cooling to 25°C, all samples were conjugated with fluorescamine dye (100 μg of fluorescamine per 1–20 μg of sample) (18). Fluorescent bands of protein, visualized under long-wave black light, were excised from the gels at the termination of each run. Protein bands were eluted from $NaDodSO_4$ gel sections in 6 M urea/0.05 M Tris acetate, pH 7.8. Before assaying the samples for PDGF activity, we removed the $NaDodSO_4$ by absorption to Dowex 1-X2 (19). The samples were diluted with 0.15 M sodium chloride/1% human serum albumin and assayed for PDGF activity.

Biological Assay for PDGF. PDGF activity was quantitated by the stimulation of DNA synthesis as determined by autoradiography in confluent BALB/c 3T3 (clone A31) cells (16, 20). Assays were done in the presence of 5% plasma, which is required for the optimal replicative response (16, 20). A unit of PDGF activity is defined as the amount that induces 50% (approximately 10^4) of the cells to synthesize DNA.

RESULTS

Extraction of PDGF from Heated Platelet Suspension. About 60% of the potency of crude platelet lysates prepared by freeze–thaw was lost after 2 min at 100°C; however, additional heat treatment resulted in no additional loss of activity (Table 1). For purification purposes, PDGF was extracted by boiling platelet lysates for 10 min. Of the total PDGF activity remaining after the boiling step, 40% was soluble but 60% was trapped by a precipitate of denatured protein that formed during heating. Recovery of this activity was achieved by three extractions with 1 M sodium chloride. As indicated in Table 1, the heat treatment allows an 8-fold purification of PDGF with a recovery of 40% of the initial activity. In addition, the heated extracts were not toxic to cells, whereas the unheated extracts were.

Ion-Exchange Chromatography. Over 90% of the PDGF activity absorbed to CM-Sephadex C-50 and could be eluted with 1 M NaCl. CM-Sephadex chromatography allowed a 45-fold further purification and resulted in preparations containing about 12,000 units of PDGF per mg of protein (Table 2).

Bio-Gel P-150 Filtration in 1 M Acetic Acid. Preparations of PDGF were purified further by gel filtration on Bio-Gel P-150 in 1 M acetic acid (Fig. 1). PDGF eluted from Bio-Gel P-150 columns in fractions containing substances lighter than chymotrypsinogen A (25,000 daltons) and heavier than insulin (6,000 daltons), with the most active fractions in the molecular weight range of 13,000–16,000. For preparative purposes, all fractions in the molecular weight range of 10,000–25,000 were collected and pooled. The specific activity of the pooled PDGF fractions was 72,000 units per mg of protein (Table 2).

Table 1. Heat treatment of crude platelet lysates

Boiling time, min	Protein, mg/ml	PDGF, units/ml	Specific activity, units/mg
0	30.7	1000	32.6
2	1.2	400	333
10	1.5	400	266

Suspensions of clinically outdated human platelets were washed and lysed by freezing and thawing (0 time). The crude platelet lysates were boiled for the indicated times; the soluble material was collected and the precipitate was washed three times with 1 M NaCl. The 1 M salt solution extracts were combined with the soluble material, and the mixture was assayed for PDGF activity. Volume, 10 ml.

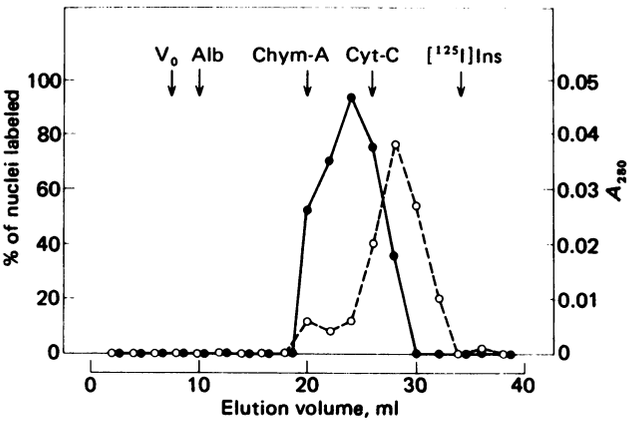

FIG. 1. Molecular gel filtration of PDGF. PDGF was purified through the CM-Sephadex stage, dissolved in 1 M acetic acid, and chromatographed on Bio-Gel P-150 pre-equilibrated with 1 M acetic acid. Fractions were collected and assayed for PDGF activity (●) and absorbance at 280 nm (○). The elution volume of molecular weight markers is indicated by arrows.

Isoelectric Focusing. Initial isoelectric focusing studies (pH 3.5–10) demonstrated that the isoelectric point of PDGF was about 9.8 with no activity in other pH regions (data not shown). Subsequent preparative runs were then made in the 9–11 pH range. As shown in Fig. 2, the PDGF focused between pH 9.6 and 10.2, with the peak of activity at pH 9.8. The pooled active fractions from preparative isoelectric focusing columns yielded PDGF preparations with a specific activity of about 400,000 units per mg of protein (Table 2).

Preparative $NaDodSO_4$/Acrylamide Electrophoresis. Isoelectrically focused preparations of PDGF were submitted to preparative $NaDodSO_4$/acrylamide gel electrophoresis with a fluorescamine dye indicator. Under long-wave black light, $NaDodSO_4$/acrylamide gels of these PDGF preparations displayed a single discrete band of protein at an apparent molecular weight of 38,000. A series of poorly resolved bands and residual ampholine appeared in the lower molecular weight regions (Fig. 3). The band at 38,000 daltons and all the other regions of the gel were excised, eluted, and tested for PDGF activity. Only the single band of protein at the 38,000-dalton region exhibited PDGF activity. By comparing the fluorescence intensity of the PDGF band to the intensity of a graded dilution series of known marker protein (chymotrypsinogen A) on the same gel, we estimated that the specific activity of the PDGF eluted from the gel was about 5 million units/mg of protein.

PDGF that had not been conjugated to fluorescamine was also purified on $NaDodSO_4$/10% acrylamide tube gels (8 cm in length) prepared according to the method of Laemmli (17). The gels were sliced into uniform 1-mm sections, and the individual sections were assayed for PDGF activity as described for fluorescamine-treated samples. The apparent molecular weight of PDGF that was not treated with fluorescamine was consistently somewhat smaller (35,000) than that of fluorescamine-treated material (38,000). Since PDGF is a very basic protein, it probably binds proportionately somewhat more fluorescamine than the molecular weight markers used on our

Table 2. Purification of PDGF from clinically outdated human platelets

Purification step	Volume, ml	Protein, mg	PDGF, units	% recovery	Specific activity, units/mg
Clotted serum	1.5×10^5	1.1×10^7	2.6×10^6	—	2.4×10^{-1}
Platelet lysates	2.0×10^3	7.8×10^4	2.6×10^6	100	3.25×10^1
Heat (100°C)	1.0×10^4	3.8×10^3	1.0×10^6	40	2.66×10^2
CM-Sephadex	1.5×10^1	6.0×10^1	7.2×10^5	28	1.2×10^4
Bio-Gel P-150	4.0×10^1	1.0×10^1	7.2×10^5	28	7.2×10^4
Electrofocusing	9.0×10^1	5.0×10^{-1}	2.0×10^5	7.8	4.0×10^5
$NaDodSO_4$ gel	4.0×10^1	1.8×10^{-2}	3.7×10^4	1.5	5.0×10^6

The PDGF activity from 500 units of clinically outdated human platelets (about 25,000 ml) was purified to homogeneity. For comparative purposes only, the table includes the data that would be indicated for whole human serum containing PDGF activity equal to that of 500 units of platelets.

gels, accounting for the slightly greater apparent molecular weight of the fluorescamine-conjugated PDGF.

Analytical $NaDodSO_4$ Gel Electrophoresis. Homogeneity of the purified PDGF preparations was established by analytical $NaDodSO_4$ gel electrophoresis. PDGF was purified by the sequence of procedures described above; the final step, preparative $NaDodSO_4$ gel electrophoresis, was conducted without the use of fluorescamine dye to obtain material for analytical purposes. The pooled active fractions from preparative $NaDodSO_4$/acrylamide gel electrophoresis were suspended in 2% $NaDodSO_4$ with or without 10% 2-mercaptoethanol and boiled for 2 min. After boiling, aliquots of the PDGF samples were serially diluted and assayed for mitogenic activity on BALB/c 3T3 cells; the remainder of the PDGF samples was conjugated with fluorescamine dye and subjected to $NaDodSO_4$/acrylamide gel electrophoresis.

Table 3 demonstrates that more than 95% of the PDGF activity was lost after reduction with 2-mercaptoethanol. $NaDodSO_4$/acrylamide gel electrophoresis of the samples (Fig. 4) demonstrated that unreduced PDGF contained a single component with an apparent molecular weight of 38,000. Moreover, reduction with 2-mercaptoethanol, which eliminated the mitogenic activity of the PDGF (Table 3), also eliminated the band at 38,000 daltons. Reduced preparations of PDGF displayed a single new band of protein at 13,000 daltons. The reduced PDGF component reacted weakly with the fluorescamine but was readily visualized with Coomassie blue dye (Fig. 4). The protein content of the PDGF samples was estimated by comparing the fluorescence intensity of the unreduced PDGF preparations to the fluorescence intensity of dilutions of molecular weight markers. By interpolation of the data from Table 3, approximately 0.2 ng of unreduced PDGF in 0.2 ml of medium stimulated DNA synthesis in 50% of the cells in culture. Thus, pure PDGF has a specific activity of approximately 5,000,000 units/mg of protein (Table 2).

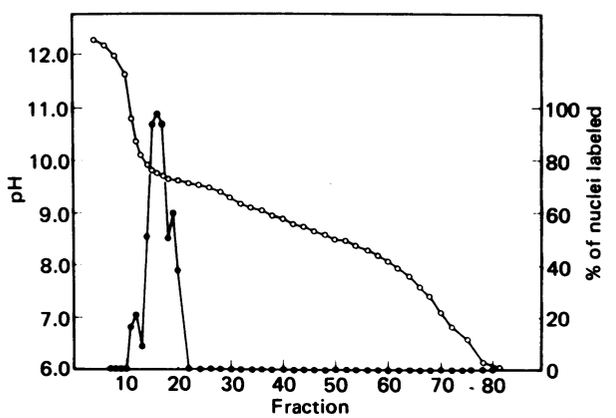

FIG. 2. Preparative isoelectric focusing of PDGF. PDGF (333,000 units) purified through the Bio-Gel P-150 stage (specific activity of 72,000 units/mg) was subjected to preparative isoelectric focusing over the pH range 9–11. Fractions were collected and read for pH (○) and assayed for PDGF activity (●).

DISCUSSION

The procedures described here allow purification of the human PDGF to a specific activity 20 million times greater than that found in human serum. Eighteen micrograms of pure PDGF was obtained from approximately 500 units of clinically outdated human platelets; this quantity of human platelets represents the circulating platelet content of approximately 50 adult humans. Concentrations of the pure PDGF as low as 0.1 nM (1 ng/ml) stimulate replicative DNA synthesis in quiescent cultures of BALB/c 3T3 cells. Approximately 10^6 PDGF molecules per cell stimulate a round of replicative DNA synthesis and cell division.

Potency of these purified preparations of PDGF is similar to that of homogeneous preparations of other growth factors,

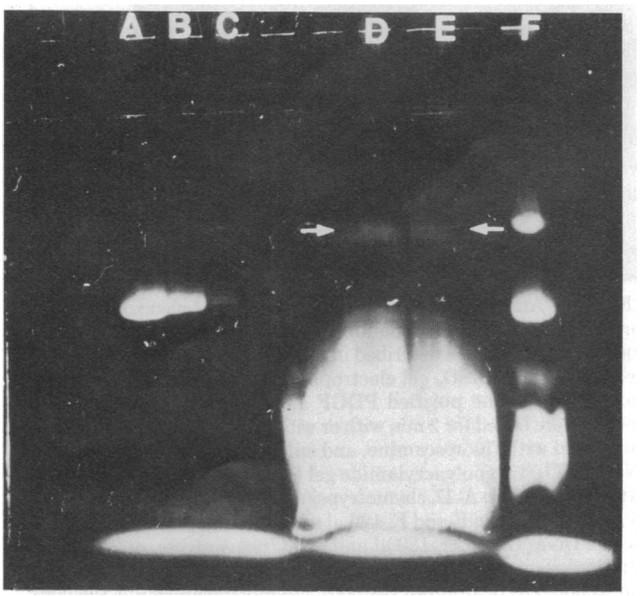

FIG. 3. Preparative $NaDodSO_4$ gel electrophoresis of PDGF. Isoelectrically focused PDGF and molecular weight marker proteins were conjugated to fluorescamine and applied to a $NaDodSO_4$/15% polyacrylamide gel (direction of migration is from top to bottom). Channels A, B, and C contain 15, 5, and 1 μg of chymotrypsinogen A, respectively. About 6000 units of isoelectrically focused PDGF was applied to channel D and channel E. Channel F contained the molecular weight marker proteins (listed in order of distance from origin) ovalbumin (41,000), chymotrypsinogen A (25,000), cytochrome C (13,000), and insulin (6000).

Table 3. Titration of purified PDGF in the native and reduced forms

PDGF, ng	% labeled nuclei: Unreduced PDGF	% labeled nuclei: Mercaptoethanol-treated PDGF
1.0	100	72
0.3	85	7.5
0.1	35	2.0
0.03	15	3.3
0.00	1.8	1.7

PDGF was purified by the sequence of procedures described in *Results* except that the final step, preparative $NaDodSO_4$ gel electrophoresis, was conducted without fluorescamine. The active fractions from the gel were pooled and boiled for 2 min with or without 10% 2-mercaptoethanol, conjugated to fluorescamine, and run on a $NaDodSO_4$/acrylamide gel; protein concentration was estimated by using fluorescenated standards (Fig. 4). For duplicate samples, the $NaDodSO_4$ was removed, and PDGF was assayed on BALB/c 3T3 cells.

such as epidermal growth factor (21), fibroblast growth factor (22), and somatomedins (23, 24). The biologic activity of purified, unreduced PDGF corresponds to the position of a single protein band which can be visualized in the 38,000-dalton region of $NaDodSO_4$/acrylamide gels. The biologic activity of PDGF is destroyed by reduction with 2-mercaptoethanol, and this treatment also eliminates the 38,000-dalton, biologically active PDGF band on $NaDodSO_4$/acrylamide gels. The polypeptide nature of PDGF is indicated from the destruction of its biologic activity by trypsin (data not shown).

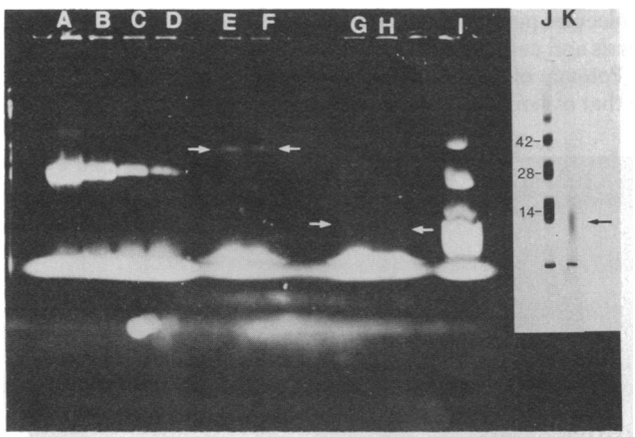

FIG. 4. Analytical $NaDodSO_4$/acrylamide gel electrophoresis of purified PDGF. Left gel, channels A–I: PDGF was purified by the sequence of procedures described in *Results* except that the final step, preparative $NaDodSO_4$ gel electrophoresis, was conducted without fluorescamine. The purified PDGF and molecular weight marker proteins were boiled for 2 min with or without 10% 2-mercaptoethanol, conjugated with fluorescamine, and subjected to electrophoresis on a $NaDodSO_4$/15% polyacrylamide gel (direction of migration, top to bottom). Channels: A–D, chymotrypsinogen A at 15 μg, 5 μg, 1 μg, and 0.5 μg, respectively; E and F, 120 μl and 60 μl, respectively, of unreduced PDGF; G and H, 120 μl and 60 μl, respectively, of 2-mercaptoethanol-reduced PDGF; I, molecular weight standards, which are (top to bottom) ovalbumin (41,000), chymotrypsinogen A (25,000), cytochrome C (13,000), and insulin (6000). Right gels, channels J and K: The same preparation of PDGF depicted in channels E through H in the left gel was reduced by boiling in the presence of 2% $NaDodSO_4$ and 10% 2-mercaptoethanol and then was subjected to electrophoresis on $NaDodSO_4$/12% polyacrylamide tube gels and stained with Coomassie blue. The direction of migration is from top to bottom. Channel J contains synthetic molecular weight markers at 42,000, 28,000, and 14,000 as indicated; channel K contains the reduced PDGF preparation, which migrates with an apparent molecular weight of 13,000.

By the criterion of $NaDodSO_4$/gel electrophoresis under nonreducing conditions, PDGF has a molecular weight of 35,000–38,000. However, by molecular gel filtration in 1 M acetic acid and by $NaDodSO_4$ gel electrophoresis under reducing conditions, PDGF has a molecular weight of 13,000–16,000. The indicated 35,000–38,000 molecular weight obtained from $NaDodSO_4$ gel electrophoresis under nonreducing conditions may reflect formation of multiple aggregates of PDGF; this interpretation would be consistent with the wide spread of PDGF activity noted by molecular gel filtration (Fig. 1). It has also been noted, however, that very basic proteins and proteins that contain intrachain disulfide residues often give spuriously high molecular weights on nonreduced acrylamide gels, due to intrinsic net positive charge and abnormal $NaDodSO_4$ binding (25). We are inclined to have more confidence in the molecular weight value of 13,000–16,000, which is obtained both by gel filtration in 1 M acetic acid and by $NaDodSO_4$ gel electrophoresis under reducing conditions. We have used the molecular weight value of 13,000 to calculate the potency of pure PDGF preparations, because this figure gives the more conservative values for potency. The molecular weight values that we have obtained for pure PDGF preparations are in agreement with reported values for partially purified PDGF that were determined by similar analytical techniques. Heldin *et al.* (26) reported a molecular weight of 26,000–32,000 for partially purified unreduced PDGF on $NaDodSO_4$ gels, and Ross *et al.* (27) found a molecular weight range of 10,000–25,000 for PDGF partially purified by gel filtration.

PDGF fulfills the biological criteria of a polypeptide hormone. It is an informational molecule, carried in the blood, which acts at nanomolar concentrations on specific target cells. Unlike other polypeptide hormones, however, PDGF is not found in plasma (13–16), but is carried in the α-granules of platelets (28, ¶). PDGF is in a cryptic state in the platelets and is not available in that state to stimulate the replication of cells. Platelets specifically adhere to injured areas and release PDGF; connective tissue cells respond to PDGF and initiate the process of replication. Packaging a growth stimulation hormone within the platelet may provide a mechanism for the selective, hormone-modulated growth stimulation of connective tissue cells, which are ubiquitously distributed throughout the body.

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