Human Platelet-derived Growth Factor

PURIFICATION AND RESOLUTION INTO TWO ACTIVE PROTEIN FRACTIONS*

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Human platelets secrete a factor that stimulates cultured human cells to initiate DNA synthesis and to divide. This human platelet-derived growth factor (PDGF) has been purified ≈100,000-fold into two equally active homogeneous fractions, PDGF I (Mr = 31,000) and PDGF II (Mr = 28,000). The amino acid compositions of each are similar, highly basic, and show 18% half-cystine residues. Both PDGF I and II are glycoproteins, but differ in their carbohydrate compositions. The data suggest that PDGF II may be a proteolytic cleavage product of PDGF I but do not rule out that the proteins may be separate but very similar gene products. Purified PDGF is active in stimulating DNA synthesis at 0.2 ng/ml.

Fibroblasts and smooth muscle cells cultured in vitro require serum for normal growth. Without serum, cell growth stops in the G0/G1 phase of the cell cycle. Human serum, when added back to growth-arrested fibroblasts, restores stimulating DNA synthesis and cell proliferation. The major activity when added back to growth-arrested fibroblasts, restores stimulating DNA synthesis and cell growth in human serum stops in the Go/G1 phase of the cell cycle. Human serum, healing for tissue repair, and in the development of atherosclerosis.

Each protein contains a large number of disulfide bonds, providing substantial stability to the unreduced protein.

Previous reports on the purification of PDGF have appeared, but limited quantities of purified material have made assessment of the degree of purity difficult in the preparations obtained (21, 22). Large scale purifications have not been reported and the chemical composition of the protein is not known. Furthermore, as noted by Heldin (22), a broad distribution of PDGF has been noted previously in isoelectric focusing (14), gel filtration (14), and SDS-polyacrylamide gel electrophoresis (22). This apparent heterogeneity may be a result of inadequate separation of PDGF I and II in previous studies because of the small amounts of materials available. PDGF is known to be cationic (pI 9.8-10.2), to have M, of 25 to 32,000, and to lose mitogenic activity by exposure to trypsin or by reduction (21-24).

EXPERIMENTAL PROCEDURES

Methods—Protein concentration was determined by absorbance at 280 nm, by the Bio-Rad protein microassay (25), or by the method of Lowry et al. (26). Amino acid analyses were performed according to the methods of Spackman et al. (27). Half-cystine residues were determined as cysteic acid in separate analyses after performic acid oxidation and hydrolysis (28). Neutral and amino sugars were determined by gas-liquid chromatography following derivatization as described previously (29). Polyacrylamide gel electrophoresis in SDS was carried out as described by Laemmli (30). Liquid-phase preparative isoelectric focusing was run in an LKB Ampholine column 8101 (110 ml).

Mitogenic activity was measured by the PDGF-dependent incorporation of [methyl-3H]thymidine into trichloroacetic acid-precipitable material by mouse 3T3 fibroblasts (5). One unit of PDGF activity is that activity with a net incorporation of 10,000 dpm in excess of the control culture. All quantitative determinations were carried out in the linear range of the assay (to a net of 5 units or 50,000 dpm).

Materials—Swiss mouse 3T3 fibroblasts (C3H 92) were obtained from the American Type Culture Collection. [Methyl-3H]Thymidine (78.4 Ci/mmol) was purchased from New England Nuclear. Chromatography materials were obtained from Pharmacia. Protein markers, Bio-Gel P-100, and gel electrophoretic materials were purchased from Bio-Rad. Phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, at pH 6.1-6.5, centrifuged (13,000 × g for 30 min), and the supernatant mixed with 500
was added in phoretic pattern in SDS-polyacrylamide gels of individual fractions.

The column used was Bio-Gel P-100 column (2.0 x 90 cm) equilibrated with 0.01 Na-phosphate, pH 7.4, containing 0.1 NaCl. PDGF activity is localized to two overlapping protein peaks. Fractions containing only one protein, at each molecular weight of ~31,000 and PDGF with a molecular weight of ~27-33,000. Between 1 and 2 mg of each protein was added to the appropriate lane. Standards used for estimations of molecular weights (data not shown) included lysozyme (14,300), soybean trypsin inhibitors (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase G (94,000).

The preparation of platelet-derived growth factor which was obtained from Blue-Sepharose was directly subjected to the preparative isoelectric focusing, pH 9-11.

FIG. 1. Elution profile of PDGF from a Bio-Gel P-100 column. The column was packed into a column (5 x 90 cm), washed with 0.5 M NaCl in 0.01 M Na-phosphate, pH 7.4, containing 5 mM EDTA, until the A280 was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05. PDGF was eluted stepwise with 1.5 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, until the absorbance at 280 nm was below 0.05 and was below 0.05.

Properties of the Platelet-derived Growth Factor

| Fraction from | Total volume | Total protein | Total activity | Specific activity | Yield | Purification |
|---------------|--------------|---------------|---------------|------------------|-------|--------------|
| 1) Platelet-rich plasma | 16,000 | 837.280 | 50 x 10^6 | 6 | 100 | 1 |
| 2) Sulfadex G-50 | 2,480 | 952 | 29 x 10^5 | 3,946 | 58 | 508 |
| 3) Supernatant after heat treatment | 224 | 448 | 19 x 10^5 | 4,241 | 38 | 707 |
| 4) CM-Sephadex | 240 | 15 | 9.6 x 10^5 | 6.4 x 10^5 | 19 | 10,667 |
| 5) Blue-Sepharose | 108 | 5 | 6.6 x 10^5 | 1.3 x 10^5 | 13 | 21,667 |
| 6) Bio-Gel P-100 | | | | | | |
| PDGF I | 8 | 0.26 | 1.6 x 10^4 | 6.2 x 10^5 | 9.6 | 1 x 10^5 |
| PDGF II | 10 | 0.5 | 3.2 x 10^4 | 6.4 x 10^5 | 10 | 1 x 10^5 |

PDGF with a molecular weight of ~28,000 (SDS-gel electrophoresis) were designated PDGF I and PDGF II, respectively. PDGF I and II were extracted from the SDS gels and shown to have biological activity correlating precisely with the protein-staining bands, although recoveries of activity are quite low, averaging 10%. All other areas of the gels have been tested and found to be

FIG. 2. Nonreduced SDS-gel electrophoretic pattern of purified PDGF I (left) and PDGF II (right). Between 1 and 2 mg of each protein was added to the appropriate lane. Standards used for estimations of molecular weights (data not shown) included lysozyme (14,300), soybean trypsin inhibitors (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (68,000), and phosphorylase G (94,000).
Properties of the Platelet-derived Growth Factor

Amino acid composition of human platelet-derived growth factors I and II

| Amino acid     | Platelet-derived growth factor* |
|----------------|---------------------------------|
|                | I                         | II                        |
| Lysine         | 21                        | 19                       |
| Histidine      | 6                         | 8                        |
| Arginine       | 23                        | 24                       |
| Aspartic acid  | 24                        | 24                       |
| Threonine      | 19                        | 19                       |
| Serine         | 20                        | 18                       |
| Glutamic acid  | 36                        | 36                       |
| Proline        | 20                        | 20                       |
| Glycine        | 17                        | 17                       |
| Alanine        | 19                        | 18                       |
| Half-cystine   | 18                        | 18                       |
| Valine         | 27                        | 29                       |
| Methionine     | 1                          | 1                        |
| Isoleucine     | 13                        | 13                       |
| Leucine        | 16                        | 16                       |
| Tyrosine       | 3                         | 3                        |
| Phenylalanine  | 8                         | 8                        |
| Tryptophan     | N.D.                      | N.D.                     |
| Total          | 291                       | 291                      |

*The data shown are the nearest integers of the averages from four separate analyses. To allow comparison, an M_r of 31,000 was used with both PDGF I and PDGF II in the calculations of the amino acid residues.

**Half-cystine residues were determined as cysteic acid from a separate analysis after performic acid oxidation and hydrolysis.

N.D., not determined.

Carbohydrate composition of human platelet-derived growth factors I and II

| Carbohydrate          | Platelet-derived growth factor* |
|-----------------------|---------------------------------|
|                      | I                         | II                        |
| Mannose               | 4.92 (5)                   | 3.57 (4)                 |
| Galactose             | 3.55 (4)                   | 2.06 (2)                 |
| N-Acetylgalactosamine | 2.12 (2)                   | 1.38 (1)                 |
| N-Acetylgalactosamine | 1.01 (1)                   |                           |
| Fucose                | 1.07 (1)                   |                           |

*The numbers in parentheses were obtained from the average of analyses of three preparations.

deviate from growth-promoting activity. PDGF I and II have nearly identical specific activities in the standard growth factor assay. Reduction of PDGF I and II by 2-mercaptoethanol completely abolishes growth-stimulating activity.

The amino acid compositions of PDGF I and of PDGF II are nearly identical (Table II) showing a striking predominance of basic amino acids, consistent with the isoelectric point of the intact protein (21). Each protein contains three tyrosine residues and 18 half-cystine residues. Preliminary evidence suggests all half-cystine residues are present in the disulfide form.

Both PDGF I and PDGF II stain positively with periodic acid-Schiff reagent. Direct carbohydrate analysis was performed, using gas-liquid chromatography of the hydrolyzed proteins (Table III). PDGF I and II have significant amounts of covalently bound carbohydrate; ~7% of PDGF I is estimated to be neutral and amino sugars. Significant differences are found in the carbohydrate analysis of PDGF I and PDGF II.

Fig. 3 shows the results of isoelectric focusing of PDGF I and II in 4 M urea. The isoelectric point of the purified growth factor is pH 10.2 ± 0.1. No separation of PDGF I and PDGF II was achieved. PDGF I and II appear either to have an identical isoelectric point or to migrate together, held by forces resistant to 4 M urea. This latter possibility is supported by preliminary results of gel filtration under non-denaturing conditions, which suggest that non-denatured PDGF migrates with an approximate molecular weight of 60,000.

DISCUSSION

Human PDGF has been purified approximately 100,000-fold, with an apparent recovery of ~10%. We now report that the purified protein may be separated into two separate protein peaks, designated PDGF I (M_r = 31,000) and PDGF II (M_r = 28,000). PDGF I and PDGF II appear to have approximately equal mitogenic potency. Both proteins are strongly basic, as reflected in the isoelectric point at 10.2 and in the strong predominance of basic amino acids in the amino acid composition analysis. The proteins contain only three tyrosyl residues and 18 half-cystine residues. Preliminary results suggest the half-cystine residues are all in disulfide linkage. This large number of disulfide bonds is unusual in growth factors and most likely explains the extreme stability of PDGF to heat denaturation.

The platelet-derived growth factor is shown also to be a glycoprotein, as demonstrated by positive periodic acid-Schiff staining of PDGF I and II in SDS gels and by direct analysis of hydrolyzed samples of isolated PDGF I and II. Covalently bound carbohydrate has not been reported previously, perhaps because of the limited quantities of PDGF available for analysis.

The amino acid analyses were calculated using an estimated molecular weight of 31,000 for both PDGF I and PDGF II to emphasize the striking similarities between the two proteins (Table II). These data suggest either that PDGF II is the proteolytic cleavage product of PDGF I, or that PDGF I and PDGF II are separate gene products, or that the two proteins were subject to different post-translational processing.

Our results suggest that the two proteins isolated are the PDGF activity first reported by Ross et al. (9) and subsequently by Antoniades et al. (21) and by Heldin et al. (22). The present results now identify two separate PDGF activi-
ties, establish that PDGF I and PDGF II are glycoproteins of different carbohydrate compositions, and provide an amino acid composition of the two proteins. Previously published purifications (21, 22) do not agree on all the properties of PDGF, especially on the apparent molecular weight of PDGF. Broad bands of PDGF were found in gels. Analysis of gel patterns in these works suggest that with the detection methods used and with the limited quantities of PDGF available for study, the broad distribution of PDGF may reflect the two forms of PDGF we have purified and separated.

Note Added in Proof—During the initial submission of this manuscript, Heldin et al. (Heldin, C.-H., Westermark, B., and Wasteson, A. (1981) Biochem. J. 193, 907-913) presented the amino acid composition of PDGF as purified previously (22). Their preparation was not resolved into separate protein components. Their amino acid composition was similar to ours except our results show 18 half-cystine residues compared to 11 found in their analysis.

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