Platelet-Derived Growth Factor In Vivo: Levels, Activity, and Rate of Clearance

By Daniel F. Bowen-Pope, Thomas W. Malpass, David M. Foster, and Russell Ross

Platelet-derived growth factor (PDGF) is a potent mitogen for many cultured connective tissue cells. It is present in concentrated form within the platelet alpha-granules and is believed to be released during platelet degranulation at sites of vascular injury. We have used a sensitive radioreceptor assay to measure PDGF levels in whole blood serum from normal humans [17.5 ± 3.1 (SD) ng/mL] and baboons (2.7 ± 1.2 ng/mL). PDGF was not detected in plasma from either species. In addition, plasma was found to substantially reduce the ability of added purified PDGF to bind to the cell surface PDGF receptor on cultured cells, suggesting that plasma may contain a PDGF-binding protein that would serve to inactivate PDGF released into plasma. Calculations of PDGF concentrations in serum have been corrected for the effects of the binding protein.\(^{125}\) PDGF injected intravenously into normal baboons was cleared rapidly from the plasma (t\(_{\frac{1}{2}}\) = two minutes). The rapid clearance of \(^{125}\)PDGF did not result from iodination damage, as purified unlabeled PDGF was cleared with comparable kinetics. The rapid clearance of purified and iodinated PDGF did not result from changes in PDGF structure during purification or from removal of PDGF-associated proteins during purification, as PDGF present in freeze–thaw lysates of fresh platelets was cleared equally rapidly. We conclude that release of PDGF at sites of vascular injury would greatly increase the local concentration of PDGF and that PDGF not localized to the site of injury would be rapidly cleared from the circulation.

Until recently, the growth of diploid animal cells in monolayer culture has required the presence of whole blood serum in the nutrient medium.\(^{1-3}\) It was found that the concentration of serum in the nutrient medium was a major determinant of the growth rate and final population density of these cultures.\(^{4}\) Recently, chemically defined media able to support the multiplication of some strains of diploid animal cells have been developed,\(^{5}\) and it has become clear that serum contributes both nutrients and growth-stimulating molecules (mitogens). The development of defined medium able to support the growth of a given cell type can be used to elucidate the basic categories of substances needed to support cell replication by determining the minimum number of additions needed to support growth. Nevertheless, such studies do not necessarily reveal which substances might actually function as key growth regulatory substances in vivo. The major source of mitogenic factors in whole blood serum has been shown by cell fractionation studies to be the blood platelet.\(^{5-7}\) A major such mitogen for connective tissue cell types, platelet-derived growth factor (PDGF), has been highly purified in several laboratories\(^{8-11}\) and shown to be a glycoprotein of 27,000 to 31,000 daltons composed of two peptide chains linked by disulfide bonds.\(^{8,11}\) Purified PDGF has been radioiodinated with retention of mitogenic activity in a cell culture assay\(^{12}\) and shown to bind with high affinity to specific cell surface receptors on many types of cultured connective tissue cell.\(^{12-14}\)

The plateau of PDGF has been proposed to play an important role in the processes of normal wound repair and of atherosclerotic lesion formation.\(^{15}\) The basic postulates behind this proposal are that PDGF is normally present sequestered within the alpha-granule of the platelet,\(^{16-18}\) and that it circulates in blood in this state until the platelet is induced to degranulate, eg, as a result of contact with subendothelial surfaces\(^{19,21}\) exposed as a consequence of injury to the vascular endothelial cells. Once released, PDGF is proposed to bind to, and stimulate, nearby connective tissue cells. To ensure localization of the response to the site of release, PDGF that escapes into the circulation should be rapidly cleared. This scheme differs from that envisioned for most hormones, in which the hormone is conveyed from source to target via the plasma. For these hormones, finite plasma concentrations would be expected. The half-times for the survival of many radiolabeled peptide hormones have been determined and range from one to five minutes for epidermal growth factor,\(^{22,23}\) alpha-melanocyte stimulating factor\(^{24}\) growth hormone\(^{25,26}\) to ten minutes or longer for insulin\(^{27,28}\) melanocyte stimulating hormone-inhibiting factor,\(^{29}\) and chorionic gonadotrophin.\(^{30}\) The concentration of PDGF circulating in plasma is not known. The literature regarding the measurement of the other platelet-specific and granule...
proteins platelet factor-4 (PF4), and beta-thromboglobulin (βTG) indicates that in normal individuals, plasma levels of PF4 and βTG represent less than 0.3% of the whole blood serum levels. Even this relatively small amount may include some artifact due to unavoidable in vitro platelet release during plasma preparation. In this article, we report measurements of the steady-state levels of PDGF and of other platelet alpha-granule proteins in plasma and whole blood serum from normal baboons and humans and the effect of plasma components on PDGF interaction with cells in culture. We then describe studies on the rate of clearance of injected native and radiolabeled PDGF from plasma and discuss the relevance of these measurements to the possible role of PDGF in vivo.

MATERIALS AND METHODS

Materials

Highly purified bovine serum albumin (BSA; Miles-Yeda Laboratories, Elkhart, Ind), highly purified platelet-derived growth factor (prepared as described by Raines and Ross),12 and mitochondrial malate dehydrogenase (gift of Dr Ralph Bradshaw) were radioiodinated to a specific activity of 20,000 to 40,000 cpm/pg using the iodoine monochloride method as described by Bowen-Pope and Ross.12 PDGF-deficient calf serum (CMS-I) was prepared as described by Vogel et al.13

Determination of Plasma Levels of Unlabeled Platelet Proteins

Plasma was prepared as previously described.12 Measurement of PF4 was performed by competitive radioimmuno assay.12 Measurement of βTG was performed using a commercial assay (Amersham, Arlington Heights, Ill.). The antisera used in the radioimmunoassays were raised against human platelet proteins. Material released from baboon platelets activated by thrombin gave parallel standard curves, indicating that baboon PF4 and βTG were recognized by the anti-human PF4 and βTG antibodies. Concentrations of platelet proteins from both species were determined relative to purified human standards. PDGF levels were determined using the radioreceptor assay described by Bowen-Pope and Ross.12 Subconfluent cultures of human foreskin fibroblasts were prepared in 2-cm2 Costar 24-well culture dishes. The cultures were rinsed once with ice-cold phosphate-buffered saline, then incubated at 4°C on an oscillating table with 1.0 mL/well of binding medium containing the test substance, or standard concentrations of pure PDGF, or both. Binding medium consisted of HEPES-buffered Ham’s medium F12, pH 7.4, containing 2% PDGF-deficient calf serum. Two percent BSA can be used with identical results.14 After three hours, the test solutions were aspirated and the wells rinsed once with phosphate-buffered saline. Incubation at 4°C was then continued for one hour using 1.0 mL/well binding medium containing 0.5 ng/mL 125I-PDGF. Binding was terminated by rinsing three times with phosphate-buffered saline containing 0.1% BSA and cell-associated 125I-PDGF extracted with 1% Triton X-100 in 0.1% BSA. PDGF in whole blood serum is stable for at least 2 weeks at 4°C. Assays performed on different days agree within 10%.

Platelets were counted with an electronic particle counter using whole blood collected in EDTA (2 to 4 mg/mL).

Clearance of Radiolabeled Proteins

The clearance of 125I-PDGF was studied after intravenous injection into adult male baboons (Papio cynocephalus). Caged animals received an intramuscular injection of Ketamine HCl (0.1 mg/kg) for initial anesthesia, and 0.05 mg/kg every 13 to 30 minutes for the duration of the experiment (150 minutes total). Fifty nanograms iodinated proteins (1.5 to 3.0 × 10⁶ cpm) was diluted to a final volume of 5 mL in 20% baboon plasma in 0.9% NaCl. The solution was exhaustively dialyzed against 0.9% NaCl to remove trichloroacetic acid (TCA)-soluble radioactivity. The solution containing radiolabeled protein was injected into the brachiocephalic vein. At timed intervals after injection, samples of blood were drawn into EDTA (0.05% final concentration) from peripheral venous sites on a rotating basis, excluding the injection site.

In order to determine cell-associated radioactivity, blood was drawn into a mixture of inhibitors of platelet activation as described for preparation of plasma for analysis of PDGF content. The blood was kept on ice for not more than 30 minutes, then centrifuged at 2,500 g for 15 minutes. The plasma was aspirated, and the resulting cell pellet was washed with cold Ringer’s citrate dextrose. This resuspending was then repeated and a measured sample of pelleted cells taken for determination of cell-associated radioactivity by gamma-spectrophotometry.

For analysis of plasma radioactivity, total radioactivity in a 1-mL sample was determined by gamma-spectrophotometry. One milliliter of ice-cold 20% TCA was added, TCA-insoluble material sedimented at 2,500 g for 5 minutes, and the radioactive content of 1 mL of supernatant was determined. The amount of TCA-precipitable radioactivity in the original plasma was determined by the difference between total and TCA-soluble radioactivity.

Preparation of Fresh Platelet Lysate

Two hundred milliliters of blood was drawn from a normal human donor into inhibitors of platelet alpha-granule release, as described for preparation of plasma for analysis of PDGF content. The blood was immediately centrifuged for 10 minutes at 200 g to sediment erythrocytes. The supernatant was recentrifuged for 10 minutes at 700 g, and the pelleted platelets resuspended in 20 mL of supernatant plasma. The platelets were lysed by three cycles of rapid freezing and thawing (accomplished within 15 minutes) and cellular debris removed by centrifugation for 10 minutes at 700 g. The resulting platelet lysate was used within two hours.

Data Analysis

The plasma radioactivity curves obtained following the injection of 125I-PDGF were analyzed by a multicompartamental model developed using the SAAM computer program [SAAM Manual; Berman M and Wess MF, eds; DH EW Publication No. (NIH) 78-178] and a VAX 11-780 digital computer (Maynard, Mass).

RESULTS

Steady-State Levels of PDGF in Normal Plasma and the Presence of PDGF-Binding Components in Plasma

To determine the concentration of PDGF in plasma and in whole blood serum (WBS) we initially used a radioreceptor assay protocol12 in which test samples and a tracer concentration of 125I-PDGF were incubated together at 4°C on cultures of human fibroblasts. Human fibroblasts have previously been shown...
to express specific high-affinity PDGF receptors.\textsuperscript{12–14} Competition by added PDGF for \(^{125}\)I-PDGF binding to test cultures under these conditions is a sensitive assay for PDGF between about 0.1 and 0.5 ng/mL (Fig 1A), and test samples containing higher levels of PDGF are always serially diluted to give at least two concentrations falling in this range. Figure 1A shows that in this radioreceptor assay, whole blood serum can be calculated to contain about 20 ng/mL PDGF.

Plasma samples were prepared with care to minimize release of platelet alpha-granule components (see Materials and Methods). As will be discussed below, little, if any, platelet activation occurred during preparation of the plasma, at least as determined by levels of other alpha-granule proteins. Plasma reduced \(^{125}\)I-PDGF binding by an amount consistent with the presence of 1.3 ng/mL PDGF. However, this is not actually due to the presence of PDGF in plasma. This is demonstrated by Fig 1B, in which the radioreceptor assay was performed using a different protocol.\textsuperscript{34} Cultures were preincubated with test substance at 4 °C for three hours, then nonbound material was rinsed away and the cultures incubated at 4 °C in medium containing \(^{125}\)I-PDGF but without test substance. PDGF is bound with such high affinity at 4 °C that PDGF receptors occupied and blocked during the preincubation with test substance remain blocked during subsequent incubation with \(^{125}\)I-PDGF.\textsuperscript{34} This is demonstrated by the similar standard curves obtained for PDGF using the two protocols (compare Fig 1, parts A and B). In fact, the sensitivity of the latter protocol is superior, probably due to the fact that it allows PDGF receptors to be blocked by unlabeled PDGF before the \(^{125}\)I-PDGF can bind (see Rodbard et al\textsuperscript{35} for a discussion of the mathematical basis of this difference). The two assay protocols give comparable values for PDGF concentrations in human whole blood serum (compare Fig 1, parts A and B). By contrast, no PDGF can be demonstrated in normal human or baboon plasma using this latter protocol. The presence of PDGF apparently demonstrated by the simultaneous incubation of cultures with test plasma and \(^{125}\)I-PDGF is probably due to the presence in plasma of components that are able to bind a significant fraction of the added \(^{125}\)I-PDGF.\textsuperscript{34} These PDGF-binding components reduce binding of \(^{125}\)I-PDGF to test cultures by competing with the cell-surface receptor for \(^{125}\)I-PDGF, rather than by competing with unlabeled PDGF for the receptor, and thereby give incorrectly high values for PDGF content. Using the protocol of Fig 1B, the test sample and \(^{125}\)I-PDGF are never present together so that this type of interference is not seen. All assay results reported in this article (except in Fig 1A) were therefore obtained from assays in which test substances were present only during preincubation. In order to compensate for PDGF binding to plasma and to obtain valid estimates of plasma PDGF concentrations, all calculations were made from internal PDGF standards, ie, from the effects of known concentrations of PDGF in the presence of the assayed concentration of plasma. This allows us to determine the fraction of PDGF in the sample that would be masked by binding to plasma. Table 1 shows that the levels of PDGF in normal human and baboon plasma are below the detection limit of our assay, ie, less than 0.2 ng/mL PDGF in the presence of 60% plasma, or less than the concentration of PDGF in 1% human whole blood serum.

![Fig 1](image_url)

**Fig 1.** Radioreceptor assays for PDGF. Confluent (4.7 × 10⁴ cells/2.4 cm² culture well) cultures of human foreskin fibroblasts were incubated for three hours at 4 °C with gentle shaking with 1.0 mL/culture of the indicated concentration of test substance: (C) human plasma; (Δ) human whole blood serum; (●)PDGF; (●) PDGF in 80% human plasma. (A) 0.12 ng/mL \(^{125}\)I-PDGF was present with each test substance. The incubation was terminated, and bound \(^{125}\)I-PDGF determined as described in Materials and Methods. (B) The three-hour incubation with test substance was terminated with one rinse with "binding rinse" and followed by a one-hour incubation at 4 °C with gentle shaking in medium without test substance containing 0.25 ng/mL \(^{125}\)I-PDGF. The incubation was terminated and bound \(^{125}\)I-PDGF determined as described in Materials and Methods. All data points represent the mean of determinations from three cultures. The standard deviations from the mean were usually ≏ 10% and always ≏ 20%.

### Levels of PDGF in Whole Blood Serum

The concentration of PDGF in whole blood serum from 12 healthy human volunteers, measured using the radioreceptor protocol of Fig 1B, was calculated to be 17.5 ± 3.1 (SD) ng/mL (Table 1). At the concentrations of WBS used (0.5% to 2.0%), reversible interactions with plasma proteins do not significantly affect detection of PDGF by radioreceptor assay. This value for the PDGF content of human whole blood serum
Table 1. Levels of Alpha-Granule Proteins in Normal Human and Baboon Whole Blood Serum and Plasma

<table>
<thead>
<tr>
<th></th>
<th>Whole Blood Serum</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDGF</td>
<td>PF4</td>
</tr>
<tr>
<td></td>
<td>ng/mL Serum</td>
<td>ng/10^8 Platelets</td>
</tr>
<tr>
<td>Platelet Count (x 10^4/mL)</td>
<td>Platelet Count (x 10^4/mL)</td>
<td>Platelet Count (x 10^4/mL)</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>254 ± 63</td>
<td>17.5 ± 3.1</td>
</tr>
<tr>
<td>Range</td>
<td>186–293</td>
<td>14.4–24.8</td>
</tr>
<tr>
<td>Baboon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>363 ± 159</td>
<td>2.7 ± 1.2</td>
</tr>
<tr>
<td>Range</td>
<td>167–709</td>
<td>1.3–5.1</td>
</tr>
</tbody>
</table>

Samples were obtained from healthy adult human volunteers or from healthy adult baboons as described in Materials and Methods. Levels of PDGF were determined by radioreceptor assay and levels of PF4 and bTG by radioimmunoassay, as described in Materials and Methods. PDGF was not detected (ND) in the plasma samples when assayed at 60%. The concentration of platelets was determined using a separate sample of whole blood collected in EDTA (2 to 4 mg/mL) and analyzed using an electronic particle counter. To calculate the quantity of PDGF per platelet, the number of platelets per milliliter of serum obtained was calculated from the blood values by correcting for the hematocrit (mean for human volunteers, 43%; mean for baboons, 35%).
agrees very closely with the values reported by Singh et al. The number of platelets per milliliter whole blood varied over a larger range (254 × 10⁶ ± 63), as did the calculated amount of PDGF per platelet (0.042 ± 0.009 ng/10⁶ platelets). Whole blood serum from 11 healthy adult baboons contained only 15% as much PDGF (2.7 ± 1.2 ng PDGF/mL) as did human whole blood serum. The platelet count in the 11 baboons varied over a wide range (167 to 709 × 10⁶ platelets/mL whole blood, mean ± SD = 363 ± 159) and accounted for some of the variation seen in PDGF levels. The mean baboon platelet content of PDGF was only 10% that of humans (0.005 ± 0.001 ng/10⁶ platelets).

**Steady-State Levels of Other Alpha-Granule Proteins in Plasma and Whole Blood Serum**

The plasma and whole blood serum samples were also analyzed by radioimmunoassay for their content of two other proteins present in the platelet alpha-granule—βTG and PF4. Table 1 compares the levels of these proteins in plasma and whole blood serum samples drawn from normal baboon or human subjects. In all cases the concentrations of these alpha-granule proteins are very low relative to their concentrations in whole blood serum. This also confirms that very little release of alpha-granule proteins could have occurred during preparation of the plasma.

**Kinetics of Clearance of 125I-PDGF From Plasma**

In order to determine whether PDGF released from platelets would remain in the plasma compartment for an extended period of time, we injected 125I-PDGF intravenously into normal baboons and determined plasma levels of TCA-insoluble radioactivity at intervals after injection. In each individual studied, plasma radioactivity decreased rapidly and in a complex fashion (Fig 2). Preliminary analysis showed that the curve could not be described by a two-compartment model without producing systematic differences between the data and the model predictions. Hence, we fitted the data from each animal to a three-compartment model (Fig 3) with the three exponentials having t½ values of 1.6, 11, and 140 minutes. When each curve was fitted to this model, two facts were apparent: (1) The calculated space of distribution of injected 125I-PDGF was about 4.5% of body weight, ie, approximately equal to the plasma volume. This implies that the injected 125I-PDGF rapidly mixes with the subjects entire plasma volume and is neither excluded from a significant portion of the vascular system nor able to exit from the plasma compartment without some restraint. (2) The rate constants were essentially identical for each animal studied. Hence, all curves were fitted simultaneously to the same model, with the space of distribution fixed at 4.5% of body weight. The resulting rate constants are shown in Table 2.

**Association of 125I-PDGF With Blood Cells**

In order to determine whether 125I-PDGF was being removed from the plasma by binding to the cellular elements of blood, we determined radioactivity associated with washed cell pellets. To prevent the release of 125I-PDGF, which might have been taken up into the platelet alpha-granules, the blood was drawn and processed in the presence of inhibitors of platelet activation (see Materials and Methods). We did not detect any significant association of radioactivity with the cellular fraction of blood at any time point after
Surface Mitogen Receptor on Clearance of 125I-PDGF

In order to determine whether the rapid clearance of 125I-PDGF was an artifact of a greatly accelerated clearance of radioiodinated soluble proteins in our system, we used the same radiolabeling technique to study the clearance of radioiodinated highly purified bovine serum albumin. We found that t/2 for removal of 125I-BSA from the plasma is approximately 500 minutes—about 350-fold longer than the t/2 for 125I-PDGF clearance (data not shown).

Kinetics of Clearance of Other Radioiodinated Proteins From Plasma

In order to determine whether the rapid clearance of 125I-PDGF was an artifact of a greatly accelerated clearance of radioiodinated soluble proteins in our system, we used the same radiolabeling technique to radioiodinate highly purified bovine serum albumin. We found that t/2 for removal of 125I-BSA from the plasma is approximately 500 minutes—about 350-fold longer than the t/2 for 125I-PDGF clearance (data not shown).

Effect of Inhibitors of 125I-PDGF Binding to Its Cell Surface Mitogen Receptor on Clearance of 125I-PDGF From Plasma

Using PDGF-responsive cultured connective tissue cells, it is possible to block the high-affinity binding of 125I-PDGF by adding relatively low concentrations of unlabeled PDGF or protamine sulfate either prior to, or simultaneous with, addition of the 125I-PDGF. In an attempt to block interaction of injected 125I-PDGF with this class of very high-affinity binding sites, we injected either 25 mg of protamine sulfate (giving a calculated initial plasma concentration of 103 to 129 μg/mL) or 5 μg of purified PDGF (final plasma concentration 10 ng/mL) one minute before injecting 50 ng of 125I-PDGF. Figure 4 and Table 2 show that protamine sulfate significantly affected the clearance of 125I-PDGF from plasma. The effect of unlabeled PDGF was small and not reproducible.

Kinetics of Clearance of Purified PDGF Studied by Radioreceptor Assay

Radioiodination of PDGF by the procedure used for these studies does not reduce the ability of PDGF to bind with very high affinity to PDGF receptors on PDGF-responsive cells in culture. Nevertheless, radioiodination could alter the fate of 125I-PDGF in vivo by promoting, or interfering with, other types of interactions with other cell types or surfaces. In addition, some of the TCA-insoluble radioactivity measured in the plasma could represent radiolabeled contaminants present in the preparation of 125I-PDGF or biologically inactive fragments of 125I-PDGF resulting from partial degradation in vivo. In order to more directly measure the survival of biologically active PDGF in plasma, we injected large amounts (2.5 μg) of unlabeled PDGF from plasma.

Table 2. Model Rate Constants

<table>
<thead>
<tr>
<th>Preinjection</th>
<th>Baboon (No.)</th>
<th>Plasma (vol. mL)</th>
<th>L(0,1)(SD) (min^-1)</th>
<th>L(2,1)</th>
<th>L(1,2)</th>
<th>L(3,2)</th>
<th>L(2,3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>006</td>
<td>495</td>
<td>0.043 (0.024)</td>
<td>0.55 (0.15)</td>
<td>0.34 (0.18)</td>
<td>0.098 (0.037)</td>
<td>0.303 (0.016)</td>
</tr>
<tr>
<td>048-1</td>
<td>450</td>
<td>0.051 (0.004)</td>
<td>0.50 (0.09)</td>
<td>0.32 (0.09)</td>
<td>0.054 (0.011)</td>
<td>0.020 (0.004)</td>
<td></td>
</tr>
<tr>
<td>048-2</td>
<td>437</td>
<td>0.025 (0.006)</td>
<td>0.27 (0.05)</td>
<td>0.22 (0.08)</td>
<td>0.050 (0.015)</td>
<td>0.017 (0.007)</td>
<td></td>
</tr>
<tr>
<td>048-3</td>
<td>455</td>
<td>0.038 (0.003)</td>
<td>0.53 (0.07)</td>
<td>0.51 (0.07)</td>
<td>0.080 (0.015)</td>
<td>0.030 (0.005)</td>
<td></td>
</tr>
<tr>
<td>321</td>
<td>432</td>
<td>0.049 (0.003)</td>
<td>0.33 (0.03)</td>
<td>0.13 (0.02)</td>
<td>0.028 (0.011)</td>
<td>0.028 (0.010)</td>
<td></td>
</tr>
<tr>
<td>172-1</td>
<td>495</td>
<td>0.038 (0.014)</td>
<td>0.34 (0.04)</td>
<td>0.16 (0.04)</td>
<td>0.038 (0.007)</td>
<td>0.014 (0.006)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>0.041 (0.009)</td>
<td>0.42 (0.12)</td>
<td>0.25 (0.09)</td>
<td>0.058 (0.026)</td>
<td>0.023 (0.007)</td>
<td></td>
</tr>
<tr>
<td>10 μg PDGF</td>
<td>172-2</td>
<td>495</td>
<td>0.026 (0.005)</td>
<td>0.32 (0.03)</td>
<td>0.18 (0.04)</td>
<td>0.073 (0.012)</td>
<td>0.020 (0.003)</td>
</tr>
<tr>
<td>25 mg Protamine</td>
<td>114</td>
<td>450</td>
<td>0.023 (0.001)</td>
<td>0.30 (0.02)</td>
<td>0.23 (0.03)</td>
<td>0.063 (0.010)</td>
<td>0.034 (0.004)</td>
</tr>
<tr>
<td>sulfate</td>
<td>128</td>
<td>562</td>
<td>0.017 (0.001)</td>
<td>0.45 (0.06)</td>
<td>0.78 (0.15)</td>
<td>0.18 (0.037)</td>
<td>0.047 (0.006)</td>
</tr>
</tbody>
</table>

Column 1 indicates whether a test substance was injected one minute prior to injecting 125I-PDGF. The three-digit number in column 2 identifies the individual baboon used. When a single animal was used for more than one study, the study number is noted to the right. For each individual, the plasma volume was calculated as 4.5% of total body weight. The rate constants describe rates of exchange of TCA-insoluble radioactivity between the pools diagrammed in Fig 3 and were calculated as described in the Results section.

Fig 4. Effects of unlabeled PDGF and protamine sulfate on clearance of 125I-PDGF. Baboon No. 172 was injected with 10 μg of unlabeled PDGF one minute before injecting 125I-PDGF (—p—). At the indicated times after injection of 125I-PDGF, blood samples were withdrawn for analysis of plasma TCA-insoluble radioactivity, as described in Materials and Methods. One month later baboon No. 172 was reinjected with 125I-PDGF alone (—d—) and plasma levels followed. No radioactivity was detected in the plasma before injection. Baboon No. 114 was injected with 25 mg of protamine sulfate one minute before injecting 125I-PDGF (—Δ—). The lines are the model predictions.
of purified unlabeled PDGF into baboons and drew samples of blood at intervals thereafter, essentially as described for studies with $^{125}$I-PDGF, except that the blood samples were drawn into the mixture of substances designed to minimize release of PDGF from the animal’s own platelets. Determination of PF4 levels by radioimmunoassay (RIA) were made for all plasma samples obtained and gave values consistent with release of less than 0.2% of platelet PF4, both before and after bolus injection of PDGF. This indicates that release of PDGF from the subject’s own platelets did not occur either during preparation of the plasma samples or as a consequence of the injection procedure itself. Plasma PDGF levels before injection were below the detection limit of the radioreceptor assay employed. Figure 5 shows that half of the injected PDGF has been removed from plasma, or has become otherwise inactive in this assay, by about two minutes and that levels have decreased below the detection limit by ten minutes. Levels of injected purified PDGF have been followed in three additional individuals with an average half-survival time of 1.9 ± 0.5 minutes. In addition to the large concentration of unlabeled PDGF, the two individuals studied in Fig 3 also received a tracer concentration of $^{125}$I-PDGF, so that the fate of radiolabeled material and of radioreceptor-assayable PDGF could be compared under exactly the same circumstances. Figure 5 shows that although plasma TCA-insoluble radioactivity and radioreceptor-assayable material are initially removed at essentially the same rate, a significant amount of TCA-insoluble radiolabeled material remains after all detectable radioreceptor-assayable PDGF has been removed.

**Kinetics of Clearance of PDGF and PF4 After Injection of Fresh Platelet Lysate**

The PDGF used to prepare $^{125}$I-PDGF, and for the studies of survival of radioreceptor-assayable PDGF, was purified from outdated human platelet-rich plasma using the multistep procedure reported by Raines and Ross. The purified PDGF obtained by this procedure is essentially identical in size (determined by sodium dodecylsulfate polyacrylamide gel electrophoresis) to PDGF purified from fresh human platelets using the procedure reported by Heldin et al and Antoniades. Nevertheless, the clearance of many proteins has been shown to depend on very small changes in the structure of the protein—changes that would not always be detected as a change in size or function, eg, removal of a terminal sialic acid residue from glycoproteins. It therefore remained possible that the purified PDGF and $^{125}$I-PDGF were subtly modified during preparation. Ideally, we would like to determine the survival of PDGF released directly from the platelets into the plasma of the experimental animal. Unfortunately, this was not possible for several reasons. In particular, the PDGF content of baboon platelets is too low (Table 1) to permit us to follow PDGF release and survival unless a large fraction of the subject’s platelets was to degranulate instantaneously, a situation that would produce its own set of artifacts. As an alternative, we prepared human platelets by differential centrifugation of human blood freshly drawn into inhibitors of platelet degranulation. To minimize the time needed to release the alpha-granule proteins from the platelets, we employed three cycles of freezing and thawing in rapid succession, a procedure shown to release essentially all platelet PF4 and $\beta$TG (Malpass, unpublished observations). After removing platelet debris by centrifugation, this platelet extract was injected into anesthetized baboons and plasma samples drawn and analyzed as described for injection of purified PDGF. To determine whether injections of this bolus of platelet extract were causing platelet aggregation in response, for example, to the adenosine diphosphate (ADP) content of the extract, we determined platelet counts for each sample of blood, both before and after injections, and found no decrease at any time point (data not shown). Figure 6 shows that the radioreceptor-assayable PDGF injected as platelet lysate disappeared rapidly from the plas-
assay to determine the levels of PDGF in cell-free plasma and in whole-blood serum. Whole blood serum from 12 normal humans contained an average of 17.5 ng/mL PDGF (Table 1). This is very close to the value (15 ng/mL) obtained by Singh et al13 and relatively close to the value (50 ng/mL) obtained by Huang et al14 for human whole blood serum. Because PDGF is active in stimulating the growth of cultured human fibroblasts at much lower concentrations (ED50 = 0.3 ng/mL),12 blood contains a very large potential source of growth-promoting activity. The observation that baboon whole blood serum contains 6.5-fold less PDGF per milliliter is probably not explained as a result of the use of human PDGF standards and human test cells in the radioreceptor assay. The interaction between PDGF and its cell surface receptor seems to be highly conserved in evolution.125I-PDGF binds with equal affinity to cells from mouse, cow, monkey, and man,12 and serum from all chordates contains material that can compete for 125I-PDGF binding to mouse cells.37 This question will not be completely resolved, however, until purified baboon PDGF standards are available. In any case, the levels of PDGF detected in baboon whole blood serum are still high relative to the known potency of PDGF on cultured cells, and no PDGF is detected in baboon plasma.

The presence in plasma of components that can reversibly bind PDGF reduces our ability to detect low concentrations of PDGF in high concentrations of plasma. As discussed in the Results section, we used internal standards to evaluate the magnitude of this effect and to calculate that we would be able to reliably detect 0.2 ng/mL in plasma. If plasma contained PDGF bound irreversibly to an inactivating plasma component, we would not detect it nor be able to evaluate the possible magnitude of this pool. However, such PDGF would, by the same token, be unlikely to have any physiologic significance as a mitogen. Because human whole blood serum contained 17.5 ng/mL PDGF, less than 1.2% of the PDGF content of blood is present in the plasma compartment before platelet activation. Parallel measurements of two other platelet alpha-granule proteins, PF4 and βTG, in human and baboon plasma gave values less than 0.15% of those in whole blood serum from the same species. The quantities of PF4 and βTG present in platelets greatly exceed that of PDGF (Table 1), so that although the sensitivity of the radioreceptor assay for PDGF exceeds the sensitivity of the radioimmunoassays for PF4 and βTG, our ability to detect platelet activation by measuring PDGF levels in plasma is not as great as our ability to detect PF4 and βTG. After a thorough study of PF4 and βTG levels in plasma from

Fig 6. Clearance of PDGF and PF4 after injection of fresh platelet lysate. A freeze-thaw lysate of human platelets was prepared as described in Materials and Methods. The platelet lysate contained 46.2 ng/mL PDGF (by radioreceptor assay) and 6.050 ng/mL PF4 (by radioimmunoassay). Eleven milliliters of platelet lysate was injected into each of two adult baboons (anesthetized as for 125I-PDGF clearance studies). At the times indicated on the abscissa, samples were withdrawn for normal in vivo PDGF clearance studies. At the times indicated on the abscissa, samples were withdrawn for normal in vivo PDGF clearance studies.

DISCUSSION
Levels of PDGF in Plasma and Whole Blood Serum

The existence of platelet-derived mitogens was first suspected on the basis of the greatly reduced mitogenic potency of serum prepared from cell-free plasma compared with the potency of serum prepared from whole blood.34-36 One mitogenic protein, PDGF, has now been purified from platelets8,9 and from platelet-rich plasma.35,36 We have used a very sensitive radioreceptor
normal humans, or from patients with conditions known or suspected to show increased platelet activation or turnover in vivo, Files et al. concluded that such measurements were probably not sensitive enough as indicators of platelet activation to be useful in clinical diagnosis of chronic conditions. Because PDGF concentrations are even less sensitive indicators of platelet activation in vivo, it is unlikely that this assay will be useful clinically. Files et al. reported finding the highest plasma levels of PF4 and βTG in patients undergoing cardiopulmonary bypass operations. We could detect only a small and statistically insignificant elevation of plasma PDGF levels in two such patients (unpublished observations). Nevertheless, as will be discussed below, the significance of PDGF released in the immediate vicinity of a potentially responsive cell is probably great.

**Clearance of PDGF From Plasma**

The absence of detectable PDGF in human or baboon plasma suggests that PDGF is rapidly cleared from the plasma compartment after release from the platelet. We found that radioiodinated purified human PDGF injected intravenously into baboons was cleared very rapidly (t½ < two minutes, Fig 2). This rate is comparable to the rate of clearance of PF4 and βTG (Fig 6) and is considerably greater than the rate of clearance of βTG. Although we cannot entirely rule out some effects resulting from the use of human PDGF in a baboon model system, the extremely brief existence of the PDGF in plasma argues against clearance by an induced immune response, as does the very poor antigenicity of human PDGF in several species (unpublished observations). Other human proteins, including kallikreins and fibrinogen have been shown to be cleared with comparable kinetics in monkeys and humans. Finally, as discussed above, the interaction between PDGF and its receptor on connective tissue cells seems to be very highly conserved during evolution.

Following the disappearance of TCA-insoluble radioactivity after injecting 125I-PDGF was simple and very sensitive, ie, it allowed us to follow tracer concentrations of PDGF with enough accuracy to permit adequate computer modeling (Figs 2 to 5). Nevertheless, this system is susceptible to several types of artifacts, as detailed in the Results section. Given these possible problems in the interpretation of 125I-PDGF clearance data, we decided to confirm our results using an independent technique. Unlabeled purified PDGF, or freshly prepared platelet lysate, were injected and plasma levels were followed by radioreceptor assay. The results of these studies (Figs 5 and 6) agree well with the 125I-PDGF studies, with the exception that the slow component of 125I-PDGF clearance was not seen. The sensitivity of the experiments with unlabeled material was limited by the sensitivity of the radioreceptor assay and by our desire to keep the amount of injected material as low as possible to avoid possible hemostatic episodes and to remain as close as possible to concentrations of PDGF that could be achieved locally in vivo as a result of platelet activation. Nevertheless, the sensitivity would have been great enough to detect a slowly removed unlabeled component if it represented material recognized by the PDGF receptor. Our tentative conclusion is that the slow component of the 125I-PDGF clearance may represent clearance of radiolabeled contaminants or degradation products but that the rapid clearance of the major portion of the radioactivity does reflect removal of PDGF from the plasma compartment.

**Association of PDGF With Different Cell Types**

The 125I-PDGF did not leave the plasma compartment by associating with the cellular elements of blood—we found very little association of injected radioactivity with the washed cell pellet at any time after injection. Because the total surface area of blood cells is extremely large, this finding argues against the possibility that the 125I-PDGF is cleared by nonspecific adsorption to surfaces—a phenomenon that can be demonstrated in vitro using artificial surfaces. This finding also argues against the possibility that platelets acquire their PDGF by accumulating it from plasma sources.

Another platelet alpha-granule protein, PF4, is also cleared extremely rapidly from the plasma (Fig 6). Some removal may result from binding of PF4 to the vascular endothelium. It has been demonstrated that significant 125I-PF4 binds to cultured vascular endothelial cells in culture and that this bound 125I-PF4 can be released with heparin. Similarly, bolus injection of heparin causes plasma levels of PF4 to increase greatly within a few minutes (Fig 5), which is consistent with the interpretation that PF4 is present bound to the vascular endothelium where it can be readily released by injected heparin. βTG has also been reported to bind to cultured vascular endothelial cells, but with a lower affinity. However, in the case of 125I-PDGF, we do not find measurable binding to aortic or umbilical vein endothelial cells in culture, nor does bolus injection of heparin increase plasma levels of PDGF, even if injected soon after clearance of a large concentration of injection PDGF (Fig 5).

Although endothelial cells cultured from large vessels do not bind 125I-PDGF, vascular smooth muscle cells and many types of connective tissue cells do have high-affinity cell surface receptors for PDGF.
Because PDGF binding to its receptor on these cell types seems to result in the activation of many cell processes, including DNA synthesis and cell division, it is of considerable interest to determine whether the clearance of injected $^{125}$I-PDGF results in part from binding of the $^{125}$I-PDGF to its mitogen receptor. If this were to occur, it would suggest that circulating PDGF could gain access to responsive cell types. The data we have obtained to date are not sufficient to evaluate this possibility. Preinjection of enough unla-
belled PDGF to give an initial plasma concentration of 20 ng/mL did not alter clearance of $^{125}$I-PDGF injected one minute later (Fig 4), even though this concentration of PDGF reduces $^{125}$I-PDGF binding to its receptor on cultured fibroblasts by over 80% (Fig 1). This does not rule out clearance via specific PDGF receptors, however, as Fig 5 demonstrates that high plasma concentrations of PDGF are not maintained for long following bolus injection of comparable amounts of PDGF. Clearance of other hormones has also been reported to be independent of hormone concentration within a wide range. The great capac-
ity of the clearance system may thus prevent evaluation of its affinity. Preinjection of enough protamine sulfate to give an initial plasma concentration of 103 to 129 $\mu$g/mL did significantly decrease clearance of $^{125}$I-PDGF injected one minute later (Fig 6). This concentration of protamine reduces $^{125}$I-PDGF binding to its receptor on cultured fibroblasts by over 98%; (our unpublished observations). In this case, we do not know the concentration of unneutralized protamine sulfate remaining during clearance of the $^{125}$I-PDGF, so that the relatively small effect on $^{125}$I-PDGF clearance observed in vivo may be consistent with the relatively large effects on receptor-mediated $^{125}$I-
PDGF binding observed in culture, where concentrations can be controlled. It is also possible, however, that the observed effect of protamine sulfate on $^{125}$I-PDGF clearance does not result from effects on the PDGF mitogen receptor. Protamine is used to neutralize the anticoagulant effects of injected heparin and may interact with cell surface or extracellular anionic matrix compounds, eg, heparin sulfate, and affect their interactions with the very cationic PDGF molecule.

**Interaction of PDGF With Plasma Components**

A factor that may greatly influence the potency and fate of PDGF in plasma is its interaction with other soluble plasma constituent(s). PDGF has been shown to interact reversibly with plasma components in vitro. Data derived from experiments like that of Fig 1B, in which the presence of 60% human plasma greatly reduces the ability of PDGF to bind to the PDGF receptor on cultured human fibroblasts, suggest that the “complexed” form of PDGF is unable to interact with the PDGF mitogen receptor. The formation of inactive complexes with binding proteins in plasma has been reported for other hormones, for example, the insulin-like growth factors. Whether complexed PDGF would be more or less able to interact with possible binding sites other than the PDGF receptor is not known.

**PDGF Acts Locally**

The rapid clearance of injected $^{125}$I-PDGF from plasma and the very low (undetectable) steady-state concentration of PDGF in normal plasma has significant implications for its function in vivo. Many connective tissue cell types throughout the body, including vascular smooth muscle cells, fibroblasts, and glial cells, can respond mitogenically to PDGF in culture. Adherent platelets are seen in vivo in many circumstances in which vascular integrity is compromised, and in some cases degranulation can be demonstrated morphologically. Goldberg et al. have shown that PF4, presumably released from adherent platelets, is present within the media of vessels deendo-
theialized by balloon catheterization. As PF4 and PDGF both seem to be present in the platelet alpha-granules and are both released in response to various stimuli, the strong inference is that vascular connective tissue cells can be exposed to PDGF as well. It seems likely that the localization of response to PDGF in vivo results from a limitation on the exposure of responsive cells to regions in which the platelets are induced to degranulate and release alpha-granule contents, eg, at the site of damage to the vascular endothelium. PDGF that did not bind locally would then be rapidly inactivated by binding to the plasma component and/or cleared, maintaining the very low circulating levels of PDGF to which uninjured tissues were exposed.

**ACKNOWLEDGMENT**

We thank Thomas Kirkman, Delnora Williams, and Esther Yee for technical assistance; Carrol Harris for helping with the computer analysis; and Mary Hillman for typing the manuscript.

**REFERENCES**


44. Balk SD: Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. Proc Natl Acad Sci USA 68:271, 1981


Platelet-derived growth factor in vivo: levels, activity, and rate of clearance

DF Bowen-Pope, TW Malpass, DM Foster and R Ross