Phylogenetic Analysis of Platelet-derived Growth Factor by Radio-receptor Assay

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ABSTRACT Competition between ^{125}I-labeled platelet-derived growth factor (PDGF) and unlabeled PDGF forms the basis of a specific "radio-receptor assay" for quantifying PDGF in clotted blood serum. Human clotted blood serum contains 15 ng/ml of PDGF by radio-receptor assay; this corresponds to a PDGF content of $\sim 7.5 \times 10^{-5}$ pg per circulating platelet, a figure which is corroborated by purification data.

Clotted blood sera from mammals, lower vertebrates and marine invertebrates were screened for homologues of human PDGF by radio-receptor assay. All tested specimens from phylum Chordata contain a mitogenic agent that competes with human PDGF for receptor binding. Sera from tunicates down on the chordate line of evolution and sera from all tested animals on the arthropod line of development were negative. The phylogenetic distribution of PDGF homologue does not correlate with platelet distribution since platelets and their precursor cell—the bone marrow megacaryocyte—are unique to the mammalian hematopoietic system. One anatomical feature appearing coordinately with PDGF on the vertebrate line of development is a pressurized circulatory system. The coincidental appearance of these features may lend support to the hypothesis that PDGF plays a role in maintenance and repair of the vascular lining in vivo.

Platelet-derived growth factor (PDGF) is a heat-stable, cationic polypeptide which has been purified to homogeneity from clinically outdated human blood products (1–3). At concentrations of 10^{-10} M or less, PDGF stimulates the replication of fibroblasts (1), glial cells (2), and other connective tissue cells (4) in vitro. PDGF is contained within the alpha-granules of circulating blood platelets (5–7) and is released into serum when whole blood is allowed to clot (8, 9). Thus clotted blood serum supports the growth of fibroblasts and connective tissue cells in vitro and is conventionally used as a supplement for cell culture media; platelet-poor plasma (prepared from unclotted blood) is deficient in PDGF activity and has been shown to be an inefficient medium supplement for fibroblasts and connective tissue cells (8–11).

Since its discovery, PDGF has been quantified by virtue of its capacity to stimulate the growth of connective tissue cells in vitro (1–3, 8, 9). These "bioassays" are reliable for purified or partially purified preparations. However, whole blood samples, crude platelet lysates, and tissue biopsies contain a variety of agents other than PDGF which stimulate replicative DNA synthesis and cell division in cultured fibroblasts. These include epidermal growth factor (12), prostaglandins (13), insulin (14), and somatomedins (15). Thus, there have been no unambiguous estimates of the PDGF content of common biological fluids such as blood or platelet-poor plasma. A radioimmunoassay for PDGF has been described but the assay has not been applied to blood samples (16).

Recently, it has been shown that PDGF, like other growth factors, exerts its biological effects through interaction with specific, high-affinity receptors associated with its target cells (4, 17–20). We exploited the PDGF:receptor interaction in a sensitive and highly specific "radio-receptor assay" for quantification of PDGF. This assay is based upon the competition of unlabeled PDGF with homogeneous preparations of human ^{125}I-labeled PDGF for binding to the PDGF-specific receptor associated with BALB/c-3T3 cells. Here we describe the radio-receptor assay for PDGF and provide estimates of the PDGF content of human platelets and of human clotted blood serum;

moreover, we provide evidence that a functional homologue of PDGF appeared suddenly with the first chordates and has been highly conserved since that time.

MATERIALS AND METHODS

Cell Culture

Stock cultures of BALB/c-3T3 cells (clone A-31) were maintained as previously described (1). PDGF-binding studies were conducted on confluent, density-arrested monolayers of BALB/c-3T3 cells in 35-mm diameter culture dishes at a density of 4–5 × 10^5 cells/cm^2.

PDGF

Human PDGF was purified to homogeneity by a modification of our previous protocol which employs high performance liquid chromatography (HPLC) at two stages. We employed reverse-phase chromatography on an HPLC C-18 column (Micromeritics Instruments Co., Norcross, GA) and molecular sieving chromatography on an HPLC sizing column (Waters Associates, Milford, MA) in lieu of the isoelectric focussing and SDS gel electrophoresis steps described earlier (1). The revised protocol yields material which appears as a single band of molecular weight 33,000-daltons by SDS gel electrophoresis and is mitogenic at protein concentrations of <1 ng/ml.

Radio-iodination

The human PDGF was labeled with ^{125}I by the Hunter-Greenwood procedure (21). The iodination reaction was terminated and the free ^{125}I was removed by rapidly passing the samples through the Waters HPLC sizing column. Specific activity of the radio-labeled PDGF ranged from 0.2 to 0.3 mol I per mol PDGF in various preparations. The radio-labeling procedure did not alter the mitogenic activity of PDGF as assayed on BALB/c 3T3 cells under standard conditions (1).

Serum and Plasma Samples

Blood was drawn from larger animals by cardiac puncture or venipuncture. Blood was collected from some smaller animals by slicing major vessels with a scalpel. Sea urchins were bled from the coelomic cavity by needle aspiration. Clotted lobster hemolyph was purchased commercially (Gibco Laboratories, Grand Island Biological Chemical Co., Grand Island, NY). Clotted blood serum was prepared by allowing freshly drawn blood to clot at 37°C (warm blooded animals) or at room temperature (cold blooded animals). Unclotted human platelet-poor plasma was prepared as previously described (1). All serum and plasma samples were heat-treated at 56°C for 30 min and dialyzed against Dulbecco's modified eagle's medium (DME) before use in the binding assay.

Receptor Binding Assay

To avoid the complexities introduced by modulation of receptor content at 37°C (22–25), binding studies were conducted at 4°C where true equilibrium conditions can be approached and sustained. At 4°C, cell-associated PDGF approaches saturation within 2 h (data not shown). To initiate the receptor binding assay, 3T3 cell monolayers were washed twice with PBS. The washed monolayer cultures were incubated at 4°C in DME containing 1 mg/ml BSA, ^{125}I-PDGF, and (when indicated) blood samples being assayed for receptor binding activity. Total volume of the binding assay mixtures was 700 μl. The culture plates were gently agitated during the course of the binding action. After 2 h at 4°C, the binding medium was aspirated. Unbound radioactivity was removed by washing the monolayers four times with PBS containing BSA (1 mg/ml). Cell-associated radioactivity was then solubilized in a mixture of 1% Triton X-100 and 10% glycerol and counted. Nonspecific binding was determined by incubating the ^{125}I-PDGF tracer with a 100-fold molar excess of unlabeled PDGF.

RESULTS

Scatchard Analysis of the PDGF:Receptor Interaction

The binding of human ^{125}I-PDGF preparations to confluent monolayers of BALB/c-3T3 cells is saturable (Fig. 1, *inset*). Scatchard plot analysis (26) (Fig. 1) indicates approximately 160,000 PDGF receptors per 3T3 cells with an equilibrium dissociation constant (k_d) of 5 × 10^{-10} M. These binding characteristics of BALB/c-3T3 cells are similar to those reported for Swiss 3T3 cells by Huang et al. (19) and for human fibroblasts by Heldin et al. (17). Data of Bowen-Pope and Ross (4) are in accord with respect to receptor number. However, the later workers have reported dissociation constants some 50-fold lower than those described here and elsewhere (17, 19). The basis of this discrepancy is unclear.

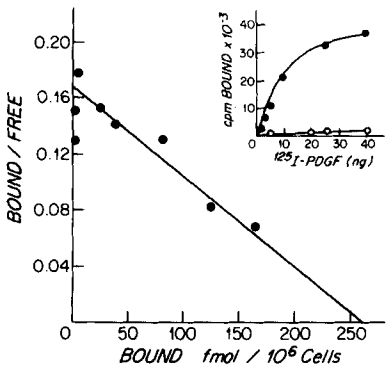


FIGURE 1 Scatchard analysis of the PDGF: receptor interaction. Density arrested monolayer cultures of BALB/c-3T3 cells were incubated at 4°C in DME supplemented with BSA (1 mg/ml) and varying amounts of ^{125}I-PDGF (sp act 8,000 cpm/ng). Binding of ^{125}I-PDGF approached saturation after 2 h, at which time cell-associated radioactivity was determined as described in Materials and Methods. Specific binding (*inset*; ●) is plotted after correction for nonspecific binding (*inset*; ○) determined in the presence of a 100-fold excess of unlabeled PDGF. Although PDGF is a "sticky" protein which binds to laboratory plasticware under some conditions (18), the amount of nonspecific binding in these studies was low as has been noted by others (4, 17, 19, 20). We have found that PDGF does not adhere nonspecifically to plastic culture dishes that have been used for cell culture because serum-supplemented culture medium contains a substance (not PDGF) which coats the dishes and eliminates adventitious binding (Singh and Stiles, unpublished observations).

Specificity of the PDGF:Receptor Interaction

The PDGF receptor exhibits stringent specificity (Fig. 2). A 10-fold molar excess of unlabeled human PDGF quantitatively competes with ^{125}I-PDGF for binding to 3T3 cells. Other growth factors (EGF, pituitary fibroblast growth factor) and other platelet-specific proteins (platelet factor IV, beta-thromboglobulin) do not compete with ^{125}I-PDGF for receptor occupancy even at high molar excess (Fig. 2). Clotted blood serum from humans does compete for receptor occupancy (Fig. 2, *inset*). The dose-response curve of human serum parallels that of the PDGF standard, indicating that the competing material in the serum sample is PDGF.

Quantification of PDGF in Mammalian Blood Preparations

The data presented in Fig. 2 indicate that competition between unlabeled PDGF and ^{125}I-PDGF for occupancy of receptors on BALB/c-3T3 monolayer cultures could allow quantification of PDGF in blood samples. A standard reference can be generated by admixing known quantities of pure PDGF with a fixed concentration of ^{125}I-PDGF before incubation with 3T3 monolayers (Fig. 3). When human serum (100%) is admixed with the ^{125}I-PDGF, competition equivalent to 15 ng/ml of pure PDGF is observed; by contrast, unclotted human platelet-poor plasma contains ~1 ng/ml of PDGF (Fig. 3). The low concentration of PDGF in human plasma is consistent with a variety of data showing that human PDGF is contained in the alpha granules of platelets (5–7) and is released only when blood clots (8, 9). Assuming (*a*) a normal platelet count of 200,000/mm^3 and (*b*) total release of PDGF during clot

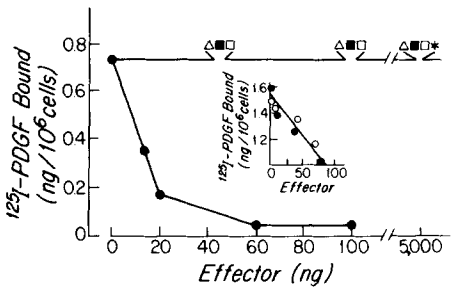
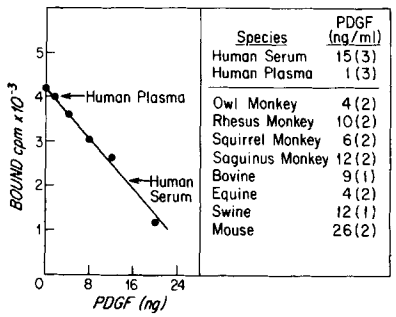


FIGURE 2 Specificity of the PDGF:receptor interaction. 4 ng of ^{125}I-PDGF (specific activity 8,000 cpm/ng) were admixed with the indicated quantities of unlabeled PDGF (●), epidermal growth factor (■), pituitary fibroblast growth factor (□), platelet factor IV (△), or beta-thromboglobulin (*). Binding of the ^{125}I-PDGF to 3T3 cell monolayers at 4°C was then determined as described in Materials and Methods. (*Inset*) Same as above except that 8 ng of the ^{125}I-PDGF tracer were admixed with unlabeled PDGF (●) or human serum (○). The units on the abscissa are ng × 10^{-1} for PDGF and final concentration (percent) for serum.



Species	PDGF (ng/ml)
Human Serum	15(3)
Human Plasma	1(3)
Owl Monkey	4(2)
Rhesus Monkey	10(2)
Squirrel Monkey	6(2)
Saguinus Monkey	12(2)
Bovine	9(1)
Equine	4(2)
Swine	12(1)
Mouse	26(2)

FIGURE 3 Quantification of PDGF in serum samples by radio-receptor assay. Between 300 and 400 μl of each sample were admixed with 8 ng of ^{125}I-PDGF (sp act 6,000 cpm/ng). The samples were adjusted to a final volume of 700 λ with DME—1% BSA. Specific binding of the ^{125}I-PDGF to 3T3 cell monolayers was determined as described in Materials and Methods. The standard curve for the radio-receptor assays was established from binding assays conducted with 8 ng of the ^{125}I-PDGF and the indicated quantity of unlabeled PDGF. The indicated PDGF content of the serum samples was read from the standard curve and normalized to 100% concentration. Human platelet-poor plasma which contains very little PDGF activity was actually assayed at 100% concentration. The numbers in parentheses indicate the number of determinations done for each sample. Experimental variation in these determinations was <5%.

formation, it may be calculated that each circulating platelet contains 7.5 × 10^{-5} pg of PDGF. This figure is in very close agreement with an independent estimate of 5 × 10^{-5} pg/PDGF/platelet which can be calculated from our original PDGF purification data (1). The latter calculation is based upon the normalized recovery of pure PDGF from 500 U of clinically outdated human platelets (where 1 U of platelets is equivalent to ~250 ml of blood).

Clotted blood serum from a variety of other mammals was assayed for homologues of human PDGF. On a volumetric basis, these mammalian sera are all roughly as potent as human serum for competitive inhibition of ^{125}I-PDGF binding. Assuming that the equilibrium dissociation constants for PDGF homologues in animal serum are identical to that of human PDGF, the PDGF homologue content of the samples ranges from 4 to 26 ng/ml (Fig. 3, *inset*).

Phylogenetic Survey for Homologues of Human PDGF

Clotted blood sera from a variety of lower vertebrates and marine invertebrates were screened for homologues of human PDGF by radio-receptor assay and the results are summarized (Fig. 4). Material which competes with ^{125}I-labeled human PDGF for binding to specific receptors can be detected in blood from all members of phylum Chordata. For lower chordates, the potency of the sera and the calculated PDGF homologue concentrations are remarkably similar to those noted for mammalian and human sera. Below phylum Chordata, none of the sera contains a detectable PDGF homologue.

Correlation between Receptor-Binding Activity and Mitogenic Activity

As independent documentation of the PDGF homologue content in blood samples, all sera were assayed for PDGF-like mitogenic activity on microtiter cultures of 3T3 cells. The accord between the radio-receptor assay for PDGF and the bioassay was very good. A detailed comparison of the two assays is shown in Fig. 5 for sera from two lower vertebrates (chicken, skate) and several marine invertebrates (starfish, *Maia*, *Limulus*). Human clotted blood serum and human platelet-poor plasma are also compared. The vertebrate sera inhibit the binding of ^{125}I-PDGF to 3T3 cells (Fig. 5, *upper*) and are potent mitogens in the concentration range of 10–20% (Fig. 5, *lower*). By contrast, the sera from marine invertebrates display neither binding nor mitogenic activity. Human platelet-poor plasma displays very weak binding inhibition activity, as noted previously (Fig. 3), and very weak mitogenic activity.

DISCUSSION

Clotted blood sera from old world monkeys down through jawless fish in phylum Chordata inhibit the binding of human ^{125}I-PDGF to specific receptors on cultured mouse fibroblasts (Fig. 4). The specificity of the PDGF radio-receptor assay (Fig. 2) together with good correlation between the radio-receptor assay and bioassay data (Fig. 5) supports the conclusion that a homologue of human PDGF exists in clotted blood sera from all members of phylum Chordata. In humans, between 5 and 7.5 × 10^{-5} pg of PDGF are contained in the average platelet. The cellular source of PDGF in lower vertebrates is unclear since platelets (and their precursor cell, the bone marrow megacaryocyte) are unique features of the mammalian hematapoietic system (27).

The failure to detect a PDGF homologue outside of phylum Chordata (Figs. 4 and 5) can be interpreted in either of two ways. It is possible that serum from many or all of these animals contains a homologue of PDGF but that below phylum Chordata the proteins are so structurally divergent from human PDGF that they do not bind to the mammalian PDGF receptor. The alternate view is that a PDGF-like peptide appeared suddenly with the first chordates, served a function, and was stringently preserved through subsequent selection. The fossil record indicates that the time interval between the appearance of fishes and the appearance of man (about 350 million years) far exceeds the time interval from invertebrates to the first fishes (no more than 100 million years). The observation that PDGF homologue titers drop abruptly after remaining nearly invariant from mammals down through fish thus argues in favor of the view that PDGF appeared coincidentally with chordates and was subsequently preserved. If this is true (and the conclusion is, of course, speculative) it is of interest to consider developmental advances in the chordates which could have dictated the need for a PDGF-like mitogen.

As noted above, the phylogenetic distribution of PDGF does

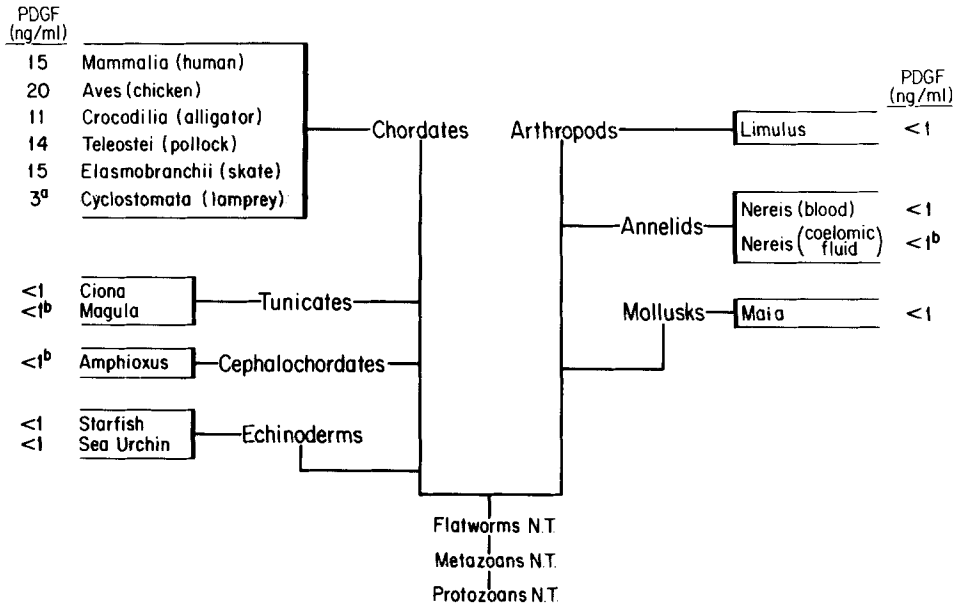


FIGURE 4 Phylogenetic survey of PDGF-like material in clotted blood serum. Clotted blood serum was collected from animal donors and assayed for PDGF content by radio-receptor assay as described in Materials and Methods. All of the invertebrate sera were assayed at final concentrations of 50–70% in an effort to detect trace quantities of PDGF homologue. Results of the radioreceptor assay have been normalized to a serum concentration of 100%. (*a*) Lamprey blood was from *Petromyzon marinus*. The adult form of this animal is a blood-sucking parasite; however, the blood was collected from the filter-feeding juvenile form (ammocoetes) and from newly transformed adults which had never fed upon blood. (*b*) These animals are difficult to bleed because they are small. The blood samples were contaminated with contents of the gut which caused the 3T3 cell monolayers to detach from culture dishes at the high concentration (50–70%) employed in the receptor binding assay. At concentrations of 20% and less there was no toxicity. At these lower concentrations the samples were totally negative for PDGF activity by bioassay. Since the bioassay actually detects some mitogenic activity in human platelet-poor plasma at 20% (see Fig. 5) and since human platelet-poor plasma contains ~1 ng/ml of PDGF by the radio-receptor assay (Fig. 3), we infer that these samples have <1 ng/ml of PDGF homologue.

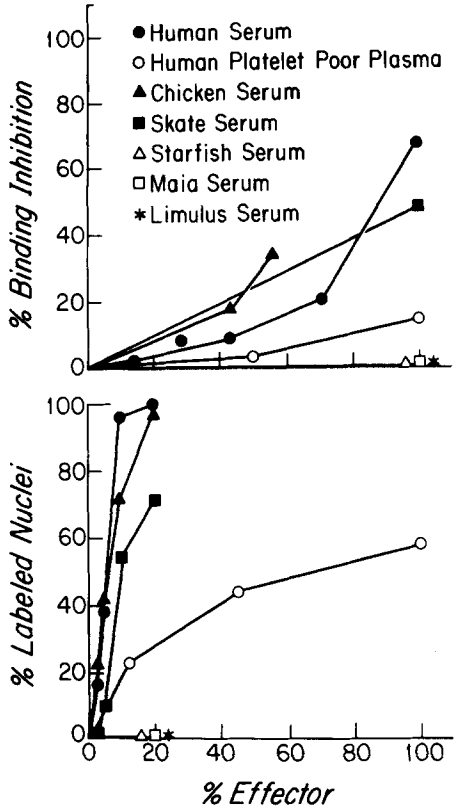


FIGURE 5 Sera which compete with ^{125}I-PDGF for receptor occupancy are mitogenic. *Upper* panel. Representative vertebrate and invertebrate blood samples at the indicated concentrations were admixed with ^{125}I-PDGF (8–10 ng/ml). Binding of ^{125}I-PDGF to 3T3 cell monolayers was then assayed as described in Materials and Methods. Data are plotted as "percent binding inhibition" relative to controls incubated with ^{125}I-PDGF only. *Lower* panel. Quiescent, density-arrested monolayer cultures of 3T3 cells were prepared in culture media supplemented with 5% platelet-poor plasma as previously described (1). Blood samples (symbols as in *upper* panel) were added to these cultures to the final concentration indicated on the abscissa. The cultures were incubated for 24 h at 37°C in the presence of 5 μCi/ml [^{3}H]-thymidine and processed for autoradiography as previously described (1).

not correlate with the distribution of the platelet/megakaryocyte cell system (27). Nor does the PDGF homologue correlate with clotting ability because clotting is a ubiquitous function of vertebrate and invertebrate blood (27). One possibly relevant development coinciding with the appearance of PDGF-like material is the blood pressure produced in animals featuring both a closed circulatory system and a chambered heart. Closed circulatory systems appear at several points in the animal kingdom and are featured by *Nereis*, *Amphioxus*, and all chordates (Fig. 3) (28). Chambered hearts are seen in mollusks (*Maia*) as well as in chordates (Fig. 3) (28). However, only phylum Chordata is uniformly characterized by the combination of a chambered heart and a closed circulatory system (28). The luminal surface of blood vessels in chordates is subject to abrasion from cells and fluid moving under pressure and at high speed. It is possible that a serum mitogen such as PDGF became necessary to regulate repair of such damage. This view of the phylogenetic data is consistent with a hypothesis advanced by Ross and his co-workers (8, 29) on totally independent grounds to the effect that a platelet-derived mitogen such as PDGF functions in vivo to repair damage to the vascular lining.

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