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Research Article

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Platelet-derived Growth Factor Stimulates Mouse 3T3 Cell Mitogenesis and Leukocyte Chemotaxis Through Different Structural Determinants

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ABSTRACT Platelet-derived growth factor (PDGF) stimulates both proliferation of fibroblasts and chemotaxis of leukocytes. In this study we compared the mitogenic and chemotactic activities of native PDGF and reduced PDGF. Reduction of PDGF (M_r = 32,000) to its constituent polypeptides (M_r = 14,000 and 17,000) caused a loss of the ability to stimulate proliferation of Balb/c 3T3 cells. However, reduced PDGF retained virtually all of its activity as a chemotactic agent for human neutrophils and monocytes. A half-maximal chemotactic response to both native and reduced PDGF occurred at a concentration of ~0.08 nM for neutrophils and 0.1 nM for monocytes. The maximal chemotactic response to reduced PDGF was at least as great as the maximal response to native PDGF. Both native and reduced PDGF stimulated the release of the lysosomal enzyme, β-glucosaminidase, from neutrophils with a half-maximal response at <0.1 nM. However, the net maximum release of this enzyme by PDGF (and reduced PDGF) was significantly less than that stimulated by a maximal concentration of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine. These results indicate that different structural determinants are required for the proliferative response of 3T3 cells to PDGF and for the chemotactic response of leukocytes to PDGF.

INTRODUCTION

Platelet-derived growth factor (PDGF)[1] is a 32,000-D polypeptide that is a potent mitogenic agent for fibroblasts, vascular smooth muscle cells, and glial cells. After PDGF is released from the alpha granules of activated platelets, it presumably acts locally to stimulate the growth of vascular smooth muscle cells and fibroblasts, thereby playing a role in wound healing and atherogenesis (1). The mitogenic effect of PDGF on fibroblasts is initiated by its interaction with a specific 165,000-D receptor, which has been labeled by affinity cross-linking of ^{125}I-PDGF to the cell membranes of 3T3 fibroblasts (2). Reduction of native PDGF yields at least two polypeptides that are not capable of stimulating DNA synthesis (3, 4).

In addition to its mitogenic activity, PDGF also has the ability to stimulate chemotaxis of human neutrophils and monocytes (5), an effect that may be important in the inflammatory response at sites of thrombosis. Neither the mechanism by which PDGF stimulates leukocyte chemotaxis nor the relationship of this effect to the mitogenic effect of PDGF has been elucidated. Since a distinctive feature of the mitogenic response to PDGF is the loss of biological activity after reduction of PDGF, we have studied the ability of native and reduced PDGF to stimulate chemotaxis of leukocytes. The results, which indicate that reduced PDGF retains its activity as a chemotactic agent, suggest that the chemotactic response and mitogenic response are mediated by different structural determinants.

METHODS

Preparation of PDGF. PDGF was purified from human platelets essentially as described in reference 4. Platelet ex-

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[1] *Abbreviations used in this paper:* C_5fr; chemotactic fragments of the fifth component of complement; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PDGF, platelet-derived growth factor.

tracts were subjected to ion-exchange chromatography on carboxymethyl Sephadex, absorption chromatography using Blue Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), and gel filtration using Biogel P-150. In some preparations, low molecular weight contaminants were present after these steps and were removed by high-pressure liquid chromatography using a phenyl micro Bondapack column (Waters Associates, Millipore Corp., Milford, MA) that was developed with a linear acetonitrile gradient in 1% trifluoroacetic acid. At each step, fractions were selected on the basis of their ability to stimulate [^{3}H]thymidine uptake into Balb/c 3T3 cells. The product was homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis, as assessed by Coomassie Blue dye staining or by iodination (Fig. 1).

DNA synthesis. [^{3}H]Thymidine uptake by Balb/c 3T3 cells was used as a measure of DNA synthesis and was determined as previously described (4). For these assays, cells were plated in 96-well plates at a density of 2×10^5 cells/ml and allowed to deplete the media of PDGF activity for 7–9 d. The confluent cells were then incubated for 16 h with the indicated concentration of PDGF or reduced PDGF. [^{3}H]Thymidine was then added for an additional 6 h and the cells were fixed and counted (4).

Chemotaxis assays. 0.5-ml aliquots of suspensions of $4.0 \pm 0.4 \times 10^6$ neutrophils or monocyte-containing mononuclear leukocytes per milliliter were added to the Teflon upper compartment of microchambers (Neuroprobe, Inc., Bethesda, MD) over 3-μm pore or 8-μm pore cellulose acetate filters that separate the respective types of leukocytes from the 0.2-ml stimulus compartments (6, 7). After a 45-min and 90-min incubation at 37°C for neutrophils and monocytes, respectively, the filters were washed, fixed, and stained. The leukocytes were counted as described (6, 7) and chemotaxis was expressed as net leukocytes per high-power field after subtraction of the corresponding value for filters of control chambers lacking a stimulus. Chemotactic fragments of the fifth component of complement (C_5fr) were purified partially from zymosan-activated human serum (6) and synthetic *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) was purchased from Sigma Chemical Co., St. Louis, MO.

Assessment of neutrophil lysosomal degranulation. Replicate suspensions of 2×10^6 neutrophils or mononuclear leukocytes in 1 ml of Hanks' solution–0.1 g human serum albumin/100 ml containing 0.5 mg of cytochalasin B/100 ml were incubated for 20 min at 37°C without and with PDGF or other stimuli and then centrifuged at 1,000 *g* for 5 min at 4°C. 20 μl each of the 1,000-*g* supernate and of a sonicate of the leukocyte pellet in 1 ml of release buffer were mixed separately with 75 μl of Hanks' solution and added to 0.9 ml of a solution of 0.2 mM 4-methyl-umbelliferyl-*N*-acetyl-β-D-glucosaminide in Hanks' solution with 0.04 M sodium citrate and 0.1 g human serum albumin/100 ml (pH 4.4). After 30 min at 37°C, each tube received 3 ml of 0.2 M sodium glycine buffer (pH 10.65) and fluorescence was determined with emission wavelength 448 nm and excitation wavelength 364 nm as described in reference 8.

Reduction of PDGF. Purified PDGF (10 μg) was exposed to 20 mM dithiothreitol in 0.5 M ammonium bicarbonate at 23°C for 2 h in the dark under nitrogen. The mixture was then treated with 40 mM iodoacetamide for 30 min at 4°C and dialyzed in 1 M acetic acid at 4°C. The dialyzed material was lyophilized and used in the chemotaxis, mitogenic, and binding assays. For every reduced preparation, a control unreduced preparation was treated in an identical fashion, except for the exposure to dithiothreitol and iodoacetamide and was tested in the appropriate assays. In one preparation, the PDGF was reduced by exposure to 5% β-mercaptoethanol rather than dithiothreitol.

RESULTS

When PDGF was exposed to the reducing agent dithiothreitol, the native 32,000-mol wt polypeptide was converted to at least two smaller polypeptides with molecular weights of ~14,000 and 17,000 (Fig. 1) as previously reported (3). In four preparations of ^{125}I-PDGF, 80–95% of the total number of counts present in the native preparation before reduction (32,000 D) were recovered as the reduced polypeptides (14,000–18,000 D). After reduction, 5% of the radioactivity remained at the gel position of native PDGF (n = 4). Although native PDGF was a very potent mitogen for 3T3 cells (half-maximal stimulation of thymidine uptake occurred at 0.2 nM), reduced PDGF had no detectable mitogenic activity at concentrations up to 1 nM (Fig. 2). To insure that the lack of mitogenic activity of the reduced material was not caused by a nonspecific toxic effect on the cells, we tested the combination of native and reduced PDGF, each at a concentration of 1 nM (Fig. 2). The presence of reduced PDGF did not inhibit the ability of native PDGF to stimulate thymidine uptake. Hence, the polypeptide chains formed by reduction of PDGF lost the ability to stimulate DNA synthesis, presumably because they no longer were able to interact with the cellular receptor sites that mediate the mitogenic effect of PDGF.

The leukocyte chemotactic response to native and reduced PDGF demonstrated a specificity that was entirely different from that of the 3T3 cell mitogenic response. Reduced PDGF was a very potent chemotactic agent that elicited half-maximal stimulation of neutrophil migration at a concentration <0.1 nM and was indistinguishable from native PDGF in this respect

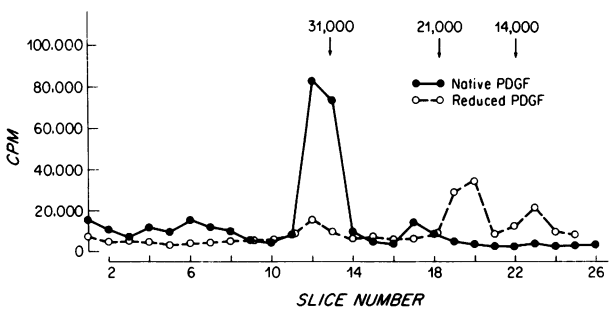

FIGURE 1 Polyacrylamide gel electrophoresis of ^{125}I-PDGF and reduced ^{125}I-PDGF. PDGF was radioiodinated by the iodogen method (13). Native and reduced ^{125}I-PDGF were then analyzed by SDS-polyacrylamide gel electrophoresis using 12% gels, which were sliced and counted.

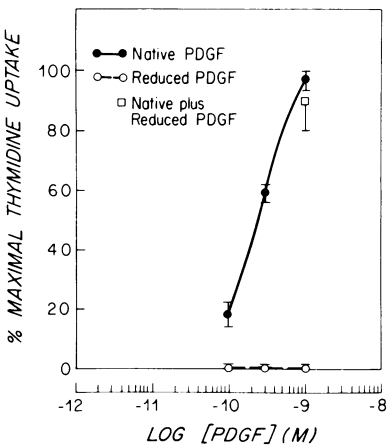

FIGURE 2 Mitogenic effect of native and reduced PDGF on fibroblasts. Balb/c 3T3 cells were incubated with the indicated concentrations of native and reduced PDGF and [^{3}H]thymidine uptake was measured as described in Methods. The maximal response refers to the uptake stimulated by 10% fetal calf serum and was approximately 10-fold higher than unstimulated uptake which was 10,000–20,000 cpm. Each value represents the mean ± SEM of three experiments each done in triplicate. Two different preparations of PDGF and reduced PDGF were used for these experiments.

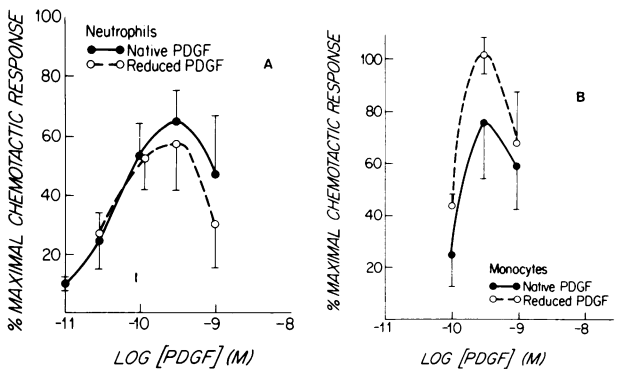

FIGURE 3 Chemotactic effect of native and reduced PDGF on neutrophils and monocytes. Chemotaxis was measured in modified Boyden chambers as described previously (6, 7). The indicated concentrations of native and reduced PDGF were tested for chemotactic activity, which was compared with the maximal response elicited by FMLP (for neutrophils, *A*) or C_5fr (for monocytes, *B*). Each value represents the mean ± SEM of four separate experiments. Four different preparations of native and reduced PDGF were used, two of which were identical to the preparations used in Fig. 2. The background was one to four cells per high-power field for both neutrophils and monocytes. The maximal response C_5fr was 24–32 cells per high-power field for neutrophils and 12–16 cells per high-power field for monocytes. The maximal response to FMLP was 28–36 cells per high-power field for neutrophils.

(Fig. 3 *A*). The maximal response to both PDGF and reduced PDGF was consistently somewhat less than the maximal response to 10^{-7} M FMLP and C_5fr, which are known potent chemotactic agents for neutrophils (9). Reduced PDGF was at least as effective as native PDGF in evoking monocyte chemotaxis (Fig. 3 *B*) and caused a maximal response comparable to the maximal response to C_5fr. To eliminate the possibility that the reduced polypeptide chains were recombining to form native PDGF during the incubations for the chemotaxis assay, the reduced preparations were alkylated with iodoacetamide to prevent disulfide formation. In each case, the same reduced preparations that failed to evoke fibroblast mitogenesis (Fig. 2) elicited optimal leukocyte chemotaxis (Fig. 3). Radioligand-binding studies have demonstrated that native PDGF, but not reduced PDGF, competes for PDGF receptors labeled in 3T3 membranes with radioiodinated PDGF. Native PDGF half-maximally occupies the binding sites at a concentration of 0.1 nM, whereas reduced PDGF does not occupy the sites at concentrations up to 10 nM (Williams, L. T., submitted for publication). Reduced radioiodinated PDGF does not bind to specific high affinity sites on 3T3 cell membranes (Williams, L. T., submitted for publication). We have not been able to perform receptor-binding studies in leukocytes because of the low ratio of specific to nonspecific binding of ^{125}I-PDGF in these cells.

Native PDGF stimulated the release of the lysosomal enzyme, β-glucosaminidase, from human neutrophils (Fig. 4). The peak response elicited by PDGF was only about half of the peak response that could be elicited by 10^{-7} FMLP (Fig. 4). Reduced PDGF stimulated β-glucosaminidase release over the same concentration range required for the release of the enzyme by native PDGF (0.03–1 nM). However, the maximal release attainable using reduced PDGF was less than the maximal release induced by native PDGF.

DISCUSSION

These results demonstrate that the polypeptides generated by reduction of PDGF have full activity as chemotactic agents but are devoid of activity as mitogens. Thus, the structural requirements for the interaction of PDGF with fibroblasts in eliciting a mitogenic response are different from those that are important for the interaction of PDGF with leukocytes in stimulating chemotaxis. Apparently, a single polypeptide chain of PDGF contains the structural information necessary for stimulating a chemotactic response, whereas at least two interconnected polypeptides are required for the mitogenic response. Our preliminary data, using PDGF polypeptide chains separated by SDS-polyacrylamide gel electrophoresis, lead us to suggest that the high-

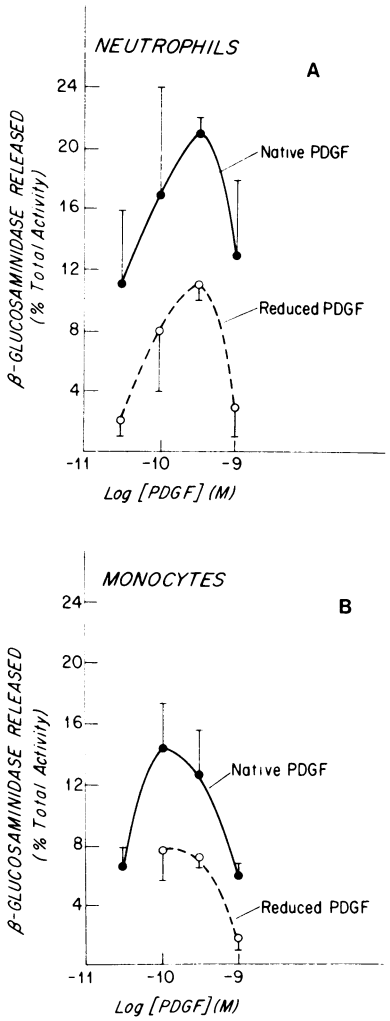

FIGURE 4 Release of β-glucosaminidase from human leukocytes by native and reduced PDGF. Enzyme release was measured as described in Methods. Values shown represent the mean ± SE for the release of β-glucosaminidase from neutrophils (*A*) and monocytes (*B*). The background release of β-glucosaminidase was 15.9% for human neutrophils and 18.4% for monocytes. The maximal response to FMLP (10^{-7} M) was 27–46% for neutrophils and 14–29% for monocytes.

molecular-weight (17,000) chain has most if not all of the chemotactic activity (not shown), although further experiments are necessary to insure that this finding is not attributable to activity induced by exposure of the polypeptides to SDS.

The data in Fig. 4 indicate that PDGF stimulates the release of lysosomal enzymes from neutrophils. Reduced PDGF shared equal potency with native PDGF in this respect. However, both native and reduced PDGF achieved less than half the degree of enzyme release that could be stimulated by a maximal concentration of FMLP (Fig. 4). The concentrations of PDGF that elicited lysosomal enzyme release were similar to the concentrations that stimulated chemotaxis. This is unusual in that for several other chemotactic stimuli the concentrations required for enzyme release are often substantially higher than the concentrations required for a chemotactic response (10). These findings suggest that for PDGF the stimulation of chemotaxis and enzyme release are often mediated by the same sites, whereas multiple sites might be involved in the responses to other chemotactic stimuli, such as FMLP. At high concentrations (≥1 nM) of PDGF there was an attenuation of the release of β-glucosaminidase. This phenomenon, which is observed for a variety of chemotactic stimuli, may be due to receptor down-regulation, stimulation of "deactivating" enzyme, or other inhibitory effects of high concentrations of chemotactic agents. Reduced PDGF stimulated lysosomal enzyme release over the same concentration range as that required by native PDGF to stimulate enzyme release (Fig. 4). However, the maximal response of reduced PDGF in stimulating lysosomal enzyme release was not as great as the maximal response of native PDGF in stimulating enzyme release. This is in contrast with the ability of reduced PDGF to stimulate chemotaxis to the same extent as native PDGF (Fig. 3).

These findings raise the possibility that the sequence of steps between receptor binding and intracellular response is different for chemotaxis and lysosomal enzyme release. Thus, the interaction of reduced PDGF with the leukocyte cell surface might effectively trigger the events that lead to chemotaxis but only partially activate the sequence of steps leading to lysosomal enzyme release. Alternatively, it is possible that different receptor sites or different states of the same sites mediate chemotaxis and lysosomal enzyme release. Reduced PDGF may bind to both states but fully activate only the receptor state that is coupled to the chemotactic response. Additional work will be necessary to resolve these issues and to determine whether the stimulation of lysosomal enzyme release is important in vivo in the inflammatory response at sites of platelet aggregation and thrombus formation.

The mechanism by which PDGF initiates mitogenesis is unknown. Recent studies (11, 12) have demonstrated that one of the early steps after binding of PDGF to its receptor is the stimulation of a protein kinase that specifically phosphorylates tyrosine residues of a 170,000-D membrane protein, which may be the PDGF receptor. Whether a similar reaction is associated with the action of PDGF on leukocytes remains to be determined. It is likely that the chemotactic effect of PDGF on leukocytes is mediated by an interaction of PDGF with specific sites on the leukocyte membrane. Further studies should elucidate the nature of the sites

through which PDGF acts to elicit chemotaxis and should determine the relationship, if any, of these sites to the PDGF receptors that mediate the fibroblast mitogenic response.

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