Dissociation of the Chemotactic and Mitogenic Activities of Platelet-derived Growth Factor by Human Neutrophil Elastase

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ABSTRACT Because platelet-derived growth factor (PDGF) may be released at sites where neutrophil proteinases may also be released, we examined the effects of neutrophil elastase and cathepsin G upon the chemotactic and mitogenic activities of PDGF. Elastase abolished the chemotactic activity of PDGF for fibroblasts but had no effect on its chemotactic activity for monocytes, or on its mitogenic activity for 3T3 cells or its capacity to bind to 3T3 cells. Cathepsin G had no effect upon the chemotactic or mitogenic activities of PDGF. In contrast, trypsin eliminated the chemotactic activity of PDGF for monocytes and fibroblasts and the mitogenic activity of PDGF. After reduction and alkylation, PDGF retained full chemotactic activity for fibroblasts and monocytes but exhibited no mitogenic activity and only limited binding to 3T3 cells. These results indicate separate domains on PDGF for fibroblast chemotactic and mitogenic activity and for monocyte and fibroblast chemotactic activity and raise the possibility that the biological activities of PDGF may be modified selectively in vivo. The findings further suggest that the majority of PDGF receptors on fibroblasts mediate mitogenic activity and that only a minority of the PDGF receptors on fibroblasts are responsible for chemotactic activity.

Human platelet-derived growth factor (PDGF)[1] is a glycoprotein of ~30,000 mol wt that contains two polypeptide chains joined through disulfide linkages (1, 9, 16). Stored in the alpha-granules of platelets and released when platelets are activated by blood clotting and contact with sites of injury (6), PDGF is a potent chemotactic factor for neutrophils, monocytes, fibroblasts, and smooth muscle cells (11, 13, 14, 30, 33) and a strong mitogen for fibroblasts, smooth muscle cells, and glial cells (27, 35). Thus, PDGF may play an important role in the migration of inflammatory cells and connective tissue cells to sites of inflammation and injury and in the repair and restructuring of injured tissues.

Similar to many other chemotactic factors, PDGF causes neutrophils to release superoxide anion and lysosomal enzymes and to undergo adhesion and aggregation (36, 38). Accordingly, besides recruiting neutrophils, PDGF may also cause neutrophil activation generally. Since neutrophils contain two proteinases with different specificity, human neutrophil elastase (HLE) and cathepsin G, that are released at sites of inflammation (28) and during coagulation (26, 37) and thus may interact with PDGF and other platelet products (23), we thought it would be of interest to examine the effects of these proteinases on the chemotactic and mitogenic activities of PDGF. We observed that HLE abolishes the chemotactic activity of PDGF for fibroblasts without changing either the chemotactic activity of PDGF for monocytes or its mitogenic activity. In contrast, cathepsin G has no effects on either the chemotactic or mitogenic activities of PDGF. These findings were extended using PDGF that was modified by reduction and alkylation. This chemical modification changed the biological activity of PDGF so that the mitogenic activity was

[1] *Abbreviations used in this paper:* FCL, fetal calf ligament fibroblasts; HLE, human neutrophil elastase; PDGF, platelet-derived growth factor.

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abolished without changing chemotactic activity for either fibroblasts or monocytes.

These results indicate complex relationships between the structure and function of PDGF. It appears that the active site of PDGF for chemotaxis is different for different cell types and that different sites on PDGF are involved in its chemotactic activity and mitogenic activity for fibroblasts.

MATERIALS AND METHODS

Materials: Swiss mouse 3T3 fibroblasts (CLL92) were obtained from the American Type Culture Collection (Rockville, MD). Fetal calf ligament fibroblasts (FLC) were obtained from explants of fetal calf ligamentum nuchae, using the procedure of Mecham et al. (25). Fetal calf serum was from KC Biological (Lenexa, KS); other tissue culture reagents were provided by the Basic Cancer Center, Washington University Medical Center, St. Louis, MO. Monocytes and neutrophils were prepared from peripheral venous blood of healthy volunteers (31). Cell culture cluster plates (24 wells, 16-mm well diameter) were obtained from Costar, Data Packaging (Cambridge, MA).

PDGF I and II were purified from human platelet-rich plasma as described previously (9). PDGF II, which has similar chemotactic and mitogenic activities to PDGF I (19, 30), was used in all experiments except in the experiments of reductive alkylation where a mixture of PDGF I and PDGF II was used. PDGF II was labeled with ^{125}I as previously described (18). Approximately, 0.7 mol of iodide was incorporated per mole of PDGF, yielding a specific radioactivity of ~16 μCi/μg ^{125}I-PDGF.

$Na^{125}I$ (17 Ci/mg) and [^{3}H]methyl thymidine (79.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA). HLE and cathepsin G, purified from neutrophils in human sputum, were kindly provided by Dr. Edward J. Campbell (The Jewish Hospital of St. Louis at Washington University Medical Center). The catalytic activities of these enzymes were determined using fibronectin and synthetic peptides as substrates (4, 5, 29). Porcine pancreatic trypsin, dithiothreitol, and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO).

Proteolysis of PDGF: 5 μg of PDGF was dispensed into 1.5-ml polypropylene microfuge tubes, to which was added 50 ng of either HLE, cathepsin G, or trypsin to a final reaction volume of 100 μl in PBS, pH 8.0. Enzyme or PDGF alone served as controls. For reaction mixtures containing HLE or cathepsin G, the reactions were held at 37°C for up to 24 h; aliquots were removed at 0, 1, 2, 6, and 24 h and then rapidly frozen. For the reaction mixtures containing trypsin, incubation was held for a maximum of 2 h at which time the enzyme was inhibited by the addition of 250 μg of aprotinin (Trasylol, Bayer AG, Wuppertal, Federal Republic of Germany). All reaction mixtures were stored at −70°C until use.

Reduction and Alkylation of PDGF: 5 μg of PDGF in 4 μl of 0.5 M ammonium bicarbonate was mixed with 28 μl of 20 mM dithiothreitol also in 0.5 M ammonium bicarbonate. The reaction vessel was gased with nitrogen and kept in the dark at room temperature for 2 h. The reduced PDGF was alkylated for 30 min by addition of 8 μl of 160 mM idodacetamide in 0.5 M ammonium bicarbonate. Reduced and alkylated PDGF was stored at −70°C until use.

Chemotaxis Assay: Chemotaxis was assayed in modified Boyden chambers using either monocytes or FCL, according to methods previously reported (31, 32). Briefly, target cells in the upper compartment (human venous blood mononuclear cells, 2.5 × 10^6 cells/ml or FCL, 1.2 × 10^5 cells/ml) were separated from PDGF in the lower compartment by two micropore membranes, either a 5-μm micropore for mononuclear cells or an 8-μm micropore for FCL overlaying a 0.45-μm micropore membrane. At the completion of the incubation period, 2 h for mononuclear cells and 6 h for FCL, the chambers were disassembled, the membranes stained, and the cells migrating through the upper membrane counted at high dry magnification (× 400). The results were expressed as net cells migrating per grid for monocytes or cells per field for fibroblasts by correcting for migration in the absence of PDGF in the lower compartment. All experiments were done in triplicate and included positive controls of fresh PDGF, 30 ng/ml.

Mitogenic Activity Assay: The mitogenic activity of PDGF was determined by PDGF-dependent incorporation of [^{3}H]thymidine into 3T3 or FCL cell DNA, as described elsewhere (19).

Binding Assay: Binding of ^{125}I-PDGF to 3T3 cells or FCL was performed as described previously (18). Briefly, 50 ng of ^{125}I-PDGF together with either native PDGF, reduced and alkylated PDGF, or PDGF treated with HLE for 24 h at 37°C was incubated in 1 ml of the binding medium with 3T3 cells or FCL that had been grown to confluence in 24-well Costar dishes. Since previous studies had shown equilibrium at 1 h at room temperature (18), we performed assays at room temperature and terminated them after 1 h by washing the cells three times with PBS. The cells were solubilized with 0.4 ml of 0.4 M NaOH; 0.2 ml was taken for measurement of radioactivity. Specific binding was defined as the total radioactivity minus the radioactivity in the presence of 100-fold excess of unlabeled PDGF (5 μg/ml).

Protein Concentration: Protein concentration was determined by Bio-Rad protein microassay (Bio-Rad Laboratories, Richmond, CA) or by the method of Lowry et al. (24).

SDS PAGE: 15% SDS PAGE was carried out according to the procedure of Laemmli (22). Gels were stained with Coomassie Brilliant Blue and destained with 10% 2-propanol/10% acetic acid. In the absence of a reducing agent, the molecular weight of PDGF II was estimated as 28,000. After reduction, two major polypeptide chains (A and B) were observed with apparent molecular weights of 16,000 and 14,000, respectively.

RESULTS

Effects of Proteolytic Modification on the Chemotactic and Mitogenic Activities of PDGF

HLE gradually destroyed the fibroblast chemotactic activity of PDGF over a 24-h incubation (Fig. 1), but it had no influence on the chemotactic activity for monocytes (Fig. 2). The effect of HLE in these assays was not due to change in the fibroblast PDGF receptor since control mixtures of PDGF and HLE without preincubation displayed the full chemotactic activity of PDGF. Cathepsin G showed no effect upon the fibroblast or monocyte chemotactic activities of PDGF even after 24 h of incubation whereas trypsin completely abolished the chemotactic activity for both fibroblasts and monocytes within 2 h (data not shown).

The mitogenic activity of PDGF for Swiss 3T3 cells and FCL after a 24-hour exposure to HLE was identical to the mitogenic activity of unexposed PDGF (Figs. 3 and 4). Notably, FCL showed only one-tenth as much [^{3}H]thymidine incorporation in response to PDGF as did 3T3 cells although the binding characteristics of PDGF for both cells types were essentially the same (Fig. 5). Cathepsin G had no effect upon the mitogenic activity of PDGF, but trypsin completely abol-

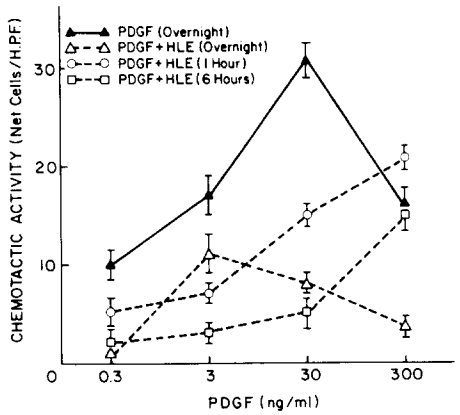

FIGURE 1 The effect of HLE on the chemotactic activity of PDGF for fetal calf ligament fibroblasts. PDGF was incubated at 37°C for the periods as indicated and then placed in the lower compartment of modified Boyden chambers with the test cells placed in the upper compartment. Chemotaxis is expressed as the net number of cells migrating (total cells corrected for background migration) per high power field (*H.P.F.*) with standard errors of the mean. Incubation for 6 h and overnight with HLE produced a significant reduction in chemotactic activity. HLE alone is not chemotactic for fibroblasts and mixtures of PDGF and HLE without preincubation exhibited the same chemotactic activity as PDGF alone (data not shown).

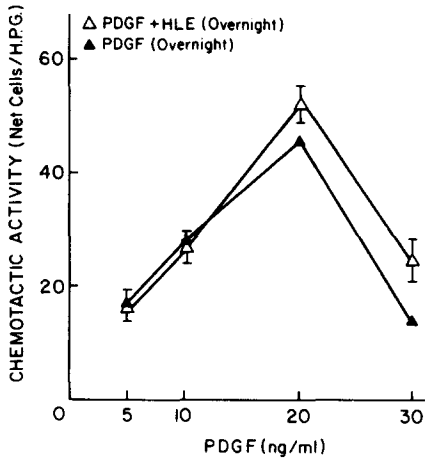

FIGURE 2 The effect of HLE on the chemotactic activity of PDGF for human monocytes. The experimental procedure was as described for Fig. 1 except that the incubation period of the assay was 2 h and the results are expressed as cells per high power grid (*H.P.G.*). HLE alone is not chemotactic for monocytes (data not shown).

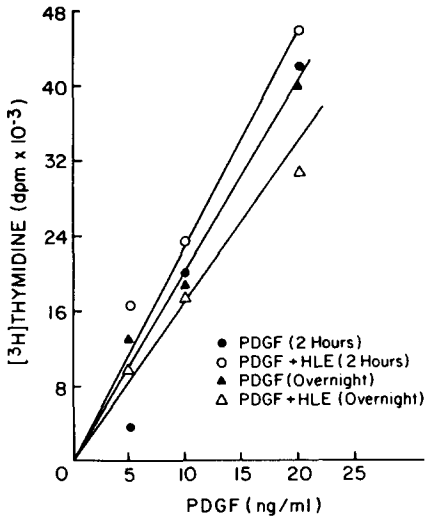

FIGURE 3 The effect of HLE upon the mitogenic activity of PDGF for 3T3 cells. 3T3 cells were incubated for 20 h in Dulbecco's modified Eagle's medium containing [^{3}H]thymidine, 0.2 μCi/μg per ml, and PDGF that either had not been exposed to HLE or had been exposed to HLE for 6 h or overnight. The results represent incorporation of the radiolabel into trichloroacetic acid precipitable material. HLE alone has no mitogenic activity (data not shown).

ished the mitogenic activity of PDGF for both 3T3 cells and FCL. On SDS PAGE, HLE did not influence the mobility of PDGF nor did it significantly change the migration of the polypeptide chains (the A and B chains) after reduction (data not shown), which suggests that the cleavage produced by HLE is located at or near the N- and C-terminal ends of either the A or B chains.

Effects of Reduction and Alkylation on the Chemotactic and Mitogenic Activities of PDGF

While the present investigations were in progress, Williams and his co-workers (38) reported that reductive alkylation of PDGF with dithiothreitol and iodoacetamide resulted in loss of mitogenic activity for fibroblasts without affecting the chemotactic activity for monocytes. Accordingly, they suggested that the active sites for the chemotactic activity and mitogenic activity of PDGF are at different loci on the molecule. However, since different cells types were compared for mitogenic and chemotactic activities, the study did not prove that the active sites on PDGF for mitogenic and chemotactic activity for fibroblasts are different.

Reductive alkylation had no effect on the chemotactic activity of PDGF for either FCL (Fig. 6, left) or monocytes (Fig. 6, right) but abolished the mitogenic activity of PDGF (Table I). Thus, these results support the findings reported by Williams et al. (38) and indicate that their results were not due to comparing different cell types. The present observations with HLE suggest further that the chemotactically active sites on PDGF are different for fibroblasts and monocytes in that HLE abolished chemotactic activity for fibroblasts without changing the chemotactic activity for monocytes.

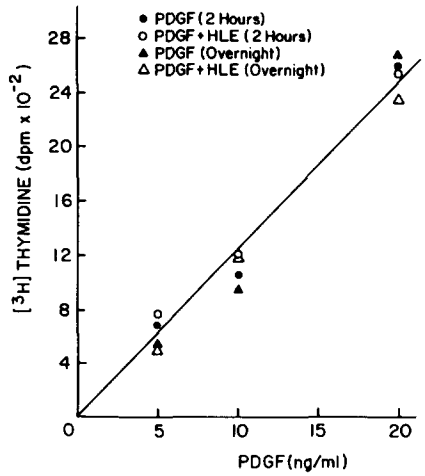

FIGURE 4 The effect of HLE on the mitogenic activity of PDGF for fetal calf ligament fibroblasts. The experimental procedure is as described in Fig. 3.

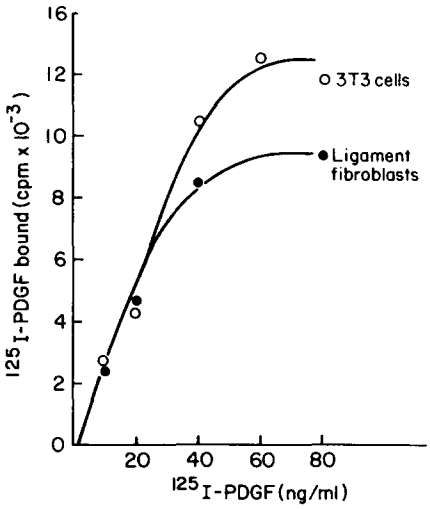

FIGURE 5 Binding of ^{125}I-PDGF to 3T3 cells and fetal calf ligament fibroblasts. Cells were incubated with radiolabeled PDGF for 1 h at 22°C, washed, and lysed with 0.4 M NaOH for 15 min. The radioactivity of the lysates was measured and corrected for nonspecific binding. See Materials and Methods for further details.

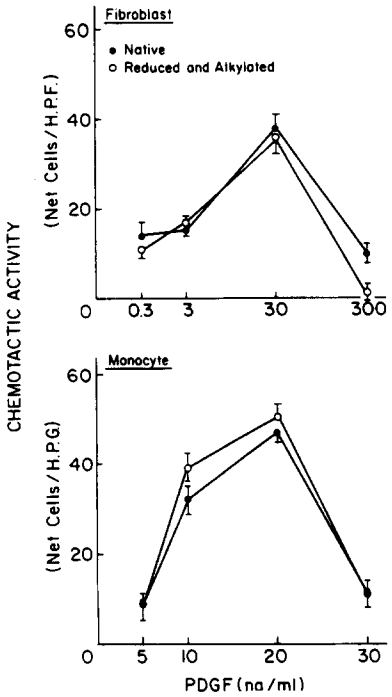

FIGURE 6 The effect of reduction (dithiothreitol) and alkylation (iodoacetamide) upon the chemotactic activity of PDGF. (*Top*) Fetal calf ligament fibroblasts. (*Bottom*) Monocytes. PDGF was chemically modified as described in Materials and Methods and the results are presented as described in Fig. 1.

TABLE I

*Effect of Reduction and Alkylation on the Mitogenic Activity of PDGF**

Assay medium	Mitogenic activity‡
	dpm/well
Control	4,432 ± 1,710
PDGF	17,306 ± 1,388
PDGF + iodoacetamide	16,268 ± 342
PDGF + DTT + iodoacetamide	5,038 ± 368

* 5 μg of PDGF was reduced and alkylated with 0.6 μmol of dithiothreitol (*DTT*) and 1.2 μmol of iodoacetamide as described in Materials and Methods.

‡ PDGF preparations were assayed at a concentration of 5 ng/ml; standard error of triplicate measurements.

Binding of Modified PDGF to Fibroblasts

Although HLE abolished the fibroblast chemotactic activity of PDGF, HLE did not change the mitogenic activity of PDGF. Accordingly, it seemed probable that HLE does not change the binding of PDGF to fibroblasts. Indeed, unlabeled PDGF competed with ^{125}I-PDGF for 3T3 cells binding as effectively for HLE exposure as before (Fig. 7).

Since reductive alkylation completely abolished the mitogenic activity of PDGF, reduced and alkylated PDGF should be an ideal ligand to test whether most of the PDGF receptors on fibroblasts are responsible for mitogenic activity. As shown in Fig. 7, reductive alkylation markedly diminished PDGF binding to fibroblasts as reflected by less effective competition of reduced and alkylated PDGF against ^{125}I-PDGF for fibroblast binding. The partial competition seen with reduced and alkylated PDGF is not due to residual unmodified PDGF because after reductive alkylation no intact PDGF was detected on analysis with 15% SDS PAGE. It is likely that the limited inhibition of ^{125}I-PDGF binding by reduced and alkylated PDGF is through the same mechanism of inhibition seen with a number of basic proteins or polypeptides (18).

DISCUSSION

The presence of both chemotactic and mitogenic activities in PDGF raises the interesting and important question of whether these activities derive from the same site on the PDGF molecule. It is plausible that these two properties of PDGF might involve different sites since it is known that substances may be mitogenic without being chemotactic and vice versa. For example, epidermal growth factor, a mitogen for fibroblasts, does not have fibroblast chemotactic activity (33), whereas platelet factor 4 and β-thromoglobulin are chemotactic but not mitogenic for fibroblasts and PDGF is chemotactic but not mitogenic for inflammatory cells (unpublished results).

The present findings with HLE exposure and with reductive alkylation, together with work of others with reduced and alkylated PDGF (38, 39), indicate clearly that the chemotactic activity and mitogenic activity of PDGF are separable. Table II summarizes the sensitivities of the chemotactic activity and mitogenic activities of PDGF to proteolytic enzymes and reductive alkylation. As shown, the fibroblast chemotactic activity of PDGF is sensitive to HLE and trypsin but not to cathepsin G, whereas only trypsin abolished the chemotactic activity of PDGF for monocytes. The mitogenic activity of PDGF is resistant to HLE and cathepsin G, but not to trypsin.

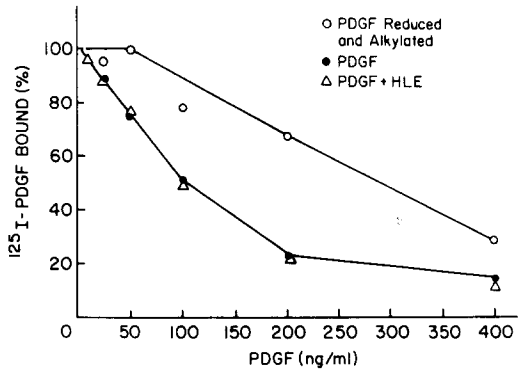

FIGURE 7 The binding of ^{125}I-PDGF to 3T3 cells in the presence of PDGF that had been exposed to HLE for 24 h or had been reduced and alkylated. Confluent 3T3 cells were incubated with various concentrations of modified PDGF and 50 ng/ml of ^{125}I-PDGF. After 1 h at room temperature the cells were washed and assayed for cell-associated ^{125}I-PDGF. The specific binding of ^{125}I-PDGF to 3T3 cells (12,000 cpm/well) was taken as 100%.

TABLE II

Effects of Proteolytic Enzymes and Reduction and Alkylation on the Chemotactic and Mitogenic Activities of PDGF

	Chemotactic activity*		Mitogenic activity*
	Monocyte	Fibroblast	
HLE	–	+	–
Cathepsin G	–	–	–
Trypsin	+	+	+
Reduction and alkylation	–	–	+

* – denotes no effect on the activity; + indicates the activity is markedly reduced or abolished (<5% of control).

In contrast, reductive alkylation has no influence on the chemotactic activity of PDGF for either fibroblasts or monocytes but eliminates its mitogenic activity. Thus, the active sites for mitogenic activity and chemotactic activity in the PDGF molecule are different and the receptors for PDGF chemotactic activity in fibroblasts and monocytes also appear to be different.

Studies of PDGF receptors on fibroblasts including NIH 3T3 cells (17), Swiss mouse 3T3 cells (18), normal rat kidney cells (17), and human fibroblasts (15) have demonstrated a single class of receptors with a K_d of 1 nM and 2–4 × 10^5 receptors per cell. The present study, however, has provided evidence for two functionally distinct receptors in fibroblasts, one for chemotactic activity and the other for mitogenic activity. There are several possible explanations for the apparent discrepancy between the single class of PDGF receptors on fibroblasts and the separate PDGF functions: First, the mitogenic and chemotactic responses of fibroblasts to PDGF may require different percentages of occupancy of PDGF receptors. Thus, mitogenic responses of fibroblasts to PDGF might involve a high percentage of occupancy of PDGF receptors whereas expression of chemotaxis may involve only a low percentage. That HLE abolishes fibroblast chemotactic activity but has no effect on either mitogenic activity of PDGF or PDGF binding to fibroblasts appears to exclude this possibility. Second, there may be another class of PDGF receptors involved in mediating the fibroblast chemotactic activity, but these receptors are few and therefore difficult to detect. This concept is supported by recent studies of inflammatory cells that demonstrate only about 2,000 to 6,000 PDGF receptors per cell (21). However, even by increasing the sensitivity of binding analysis by using a ligand with 10-fold higher specific radioactivity (160 μCi/μg), we could not detect a second, high-affinity receptor. A third possibility, which seems most attractive at this point, is that there are two groups of PDGF receptors, one for chemotactic activity and one for mitogenic activity, which have similar K_d's and thus can not be distinguished by direct binding analysis.

This hypothesis would suggest that the majority of PDGF receptors on fibroblasts mediate mitogenic activity and that only a minority of the PDGF receptors are responsible for chemotactic activity, since HLE-treated PDGF competes with native PDGF for binding to fibroblasts and is mitogenic even though it lacks chemotactic activity for fibroblasts.

The sensitivity of PDGF fibroblast chemotactic activity to HLE but not to cathepsin G is of interest, suggesting that the active site for chemotactic activity is located in a small region(s) of the PDGF molecule. HLE preferentially cleaves at Val-X bonds and to a lesser extent at Ala-X bonds (2) whereas cathepsin G cleaves peptide bonds adjacent to the carboxyl group of phenylalanine, leucine, tyrosine, isoleucine, and methionine residues (3, 40). The inactivation of PDGF chemotactic activity by HLE but not by cathepsin G is similar to the capacity of HLE but not cathepsin G to cleave IgG (34).

In their study showing that after reductive alkylation PDGF has no mitogenic activity for fibroblasts but retains chemotactic activity for monocytes, Williams et al. (38) observed that reduced PDGF was less effective than native PDGF in causing lysosomal enzyme release from cytochalasin-treated inflammatory cells. This finding indicated that reduced PDGF only partially activates inflammatory cells even though it is fully active as a chemoattractant. Whether proteolysis of PDGF by HLE impairs the effectiveness of PDGF to cause lysosomal degranulation from inflammatory cells, even though it does not alter the chemotactic activity of PDGF for monocytes, remains to be determined.

PDGF contains two homologous but not identical polypeptide chains, A and B, which are linked by disulfide linkages. The amino acid sequence of the A chain is 60% homologous to that of the B chain (7, 20). This novel, two polypeptide structure raises the question of whether the active sites for mitogenic activity and chemotactic activity are located on different chains. Since $p28^{v\text{-}sis}$, the transforming protein of the simian sarcoma virus, which is 92% homologous to the A chain of PDGF, has identical specific mitogenic activity (units per nanomole) with PDGF (8), it is likely that the A chain carries the active site for the mitogenic activity of PDGF. At this time there is no evidence to suggest which chain(s) carries the chemotactic activity of PDGF. The B chain may contain the active site for chemotactic activity or the A chain may have the active sites for both the mitogenic activity and the chemotactic activity.

The C-terminal part of the A chain is rich in basic amino acid residues which are preferred cleavage sites for trypsin compared with the N-terminal part of that chain (7). In addition, protamine has been shown to be a strong inhibitor for ^{125}I-PDGF binding to monocytes, neutrophils, and fibroblasts and a potent inhibitor of chemotactic activity of PDGF for these cells (11, 18, 21). These results are compatible with the potential role of basic amino acid residues at the C-terminal part of either the A chain or the B chain in the binding of PDGF to receptors for mitogenic activity and chemotactic activity.

While the present study emphasizes modifications of PDGF that may produce alterations in its activity as a chemoattractant, Grotendorst (12) has shown that target cells for PDGF chemotactic activity can also be modified in their responsiveness to PDGF. Thus, 3T3 cells showed differences in chemotaxis to PDGF depending upon their rate of proliferation and whether they had been transformed with phorbol myristate acetate. These changes appeared to be specific for PDGF since they were associated with decreased PDGF binding and did not extent to chemotaxis to fibronectin.

The capacity of HLE to dissociate the chemotactic and mitogenic activities of PDGF may be important in vivo since neutrophils may be attracted by PDGF to sites of inflammation and HLE is a secretory product of neutrophils. In any event, the present study suggests that the biological effects of PDGF can be modulated selectively by factors that are released at the same sites as PDGF.

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