# Platelet-derived Growth Factor Is Chemotactic for Fibroblasts

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ABSTRACT   Chemotaxis assays in modified Boyden chambers were used to detect fibroblast chemoattractants in materials released from early-stage inflammatory cells, namely, mast cells, platelets, and neutrophils. Strong attractant activity was found in substances released from platelets. This activity was accounted for mainly by the platelet-derived growth factor (PDGF), which is released from the platelets and which was active as a chemoattractant at 0.5–1.0 mitogenic units/ml. The mitogenic activity of purified PDGF, measured by [$^3$H]thymidine incorporation, occurs at a similar concentration range. By varying the gradient of PDGF, we demonstrated that PDGF stimulates chemotaxis rather than random motility. Preincubation of suspensions of fibroblasts in the presence of PDGF decreased the subsequent migration of cells to a gradient of PDGF as well as to a gradient of fibronectin, which is also an attractant for fibroblasts. The chemotactic response of fibroblasts to PDGF was not inhibited by hydroxyurea or azidocytidine but was inhibited by actinomycin D and cycloheximide, suggesting that synthesis of RNA and proteins but not of DNA is required for the chemotactic response to occur. Fibroblast growth factor, epidermal growth factor, nerve growth factor, and insulin were not chemotactic for human skin fibroblasts, suggesting that the chemoattractant activity of PDGF for fibroblasts is not a general property of growth factors and mitogens. These results suggest that PDGF could have two functions in wound healing: to attract fibroblasts to migrate into the clot and then to induce their proliferation.

It is well-known that, in a wound, cells invade the blood clot in a defined sequence. Polymorphonuclear leukocytes arrive first, followed by mononuclear leukocytes, fibroblasts, and finally endothelial cells (1). It is generally thought that the phagocytic cells are attracted to materials present in the wound, such as complement-derived peptides (2–4), products of the clotting system (5), the fibrinolysis pathway (6, 7), or the kallikrein-kinin system (7, 8). Less is known about the factors which cause fibroblasts to migrate into the wound. It has been suggested that proliferation of these cells occurs after loss of contact inhibition, perhaps due to a diffusible "wound hormone," and that the cells subsequently fill the space free of fibroblasts (9).

Recent studies have shown that fibroblasts exhibit directed migration along a concentration gradient, a process known as chemotaxis. Using a Boyden chamber for assaying chemotaxis, migration of fibroblasts across collagen-coated filters has been observed in response to products of activated lymphocytes (10) and macrophages (11) as well as to collagen and to peptides produced by the enzymatic degradation of collagen (12). More recently, it has also been observed that fibroblasts are attracted to fibronectin (13–16) and to the portion of the fibronectin molecule which contains the cell-binding region but not to that containing the collagen-binding region (14–16).

In this study we have investigated the chemotactic response of human skin fibroblasts to substances released by cells which are present in the initial stages of wound repair. These cells included mast cells, neutrophils, and platelets. The materials released from platelets exhibited the highest activity. We demonstrate that the chemoattractant present in this exudate fluid is the platelet-derived growth factor (PDGF). Since this 30,000-dalton, heat-stable basic protein is a potent mitogen in serum for fibroblasts (17–19), we tested other growth factors for their ability to stimulate chemotaxis. No other growth factors were found to be active for these cells, indicating that PDGF is unique among mitogens in this respect. This has led us to postulate that, in the wound, PDGF aids tissue repair both by attracting fibroblasts into the clot and by inducing their subsequent proliferation.

## MATERIALS AND METHODS

### Cell Cultures and Assay of Chemotaxis

Most experiments were carried out using a human fetal skin fibroblast strain, CRL 1475, obtained from the American Type Culture Collection, Rockville, Md. For comparison, cells from five strains of human skin fibroblasts from adults were also used (kind gifts from Drs. B. McCoy and K. Cohen, Division of Plastic Surgery, Medical College of Virginia, Richmond, Va.). All cells were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 5% (vol/vol)

fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were subcultured weekly, and confluent cultures were used in chemotaxis assays since preliminary experiments showed that cells from these were more responsive than cells from less dense cultures.

Peritoneal exudates were induced in rabbits and used as source of polymorphonuclear leukocytes as described earlier (20). Cells were washed twice in DMEM containing 1 mg/ml of bovine serum albumin (DMEM-BSA; Reheis Chemical Co., Phoenix, Ariz.), suspended in the same medium, and incubated with $10^{-9}$ M formylmethionylleucylphenylalanine (FMLP) (Peninsula Laboratories, San Carlos, Calif.) to induce a release reaction (21). Cells were spun down at 800 $g$ and the supernatant was then tested for chemotactic activity. Controls consisted of supernatants from cells incubated without FMLP, and FMLP incubated without cells.

Platelets were obtained from the Blood Bank, National Institutes of Health (NIH) (Bethesda, Md.). The platelets were suspended in DMEM-BSA, washed free of plasma by centrifugation at 1,200 $g$ for 15 min at room temperature, adjusted to a concentration of $2.5 \times 10^{10}$ platelets/ml, and exposed to 1 U/ml of human thrombin (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 5 min. After aggregation, the mixture was centrifuged, and the supernatant was used for chemotaxis assay. Controls consisted of supernatants incubated without thrombin and thrombin incubated without cells.

Rat mast cell media were kindly donated by Dr. M. Kaliner, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, NIH. The mast cells were isolated and purified as previously described (22), and exposed to compound 48/80 at 5 µg/ml for 5 min to activate granule release reaction from cells suspended in DMEM-BSA at $10^6$ cell/ml. The histamine content of the supernatant fluid showed that a release reaction had taken place. The supernatants were used for chemotaxis assays. Controls were supernatants from cells incubated without 48/80, and 48/80 incubated without cells.

The migration of fibroblasts was assayed as described by Postlethwaite et al. (10). The fibroblasts were detached from the tissue culture flasks with 0.1% (wt/vol) trypsin (Worthington Biochemical Corp., Freehold, N. J.) in phosphate-buffered saline at 37°C, using as short a time as possible, usually 3 min or less, to avoid excessive exposure to trypsin. The cells were suspended in DMEM containing 0.5% (wt/vol) bovine pancreatic trypsin inhibitor (Aprotinin; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), washed in DMEM containing 1 mg/ml of DMEM-BSA, and suspended in DMEM-BSA. A portion of the cells were counted with a Coulter counter, and the cell concentration was adjusted to $3 \times 10^5$ cells/ml. Attractant solutions (0.2 ml) were pipetted into bottom wells of Boyden blind-well chambers (type 200-312; Neuroprobe, Bethesda, Md.), and 8-µm-pore-size polycarbonate (Nuclepore, Pleasanton, Calif.) filters coated with gelatin were placed above them. The chambers were assembled and 0.8 ml of the cell suspension was pipetted into the top compartment of each chamber. The chambers were incubated for 4 h at 37°C in a humidified atmosphere of air/5% $CO_2$. At the end of the assay, the filters were removed and the cells were fixed and stained with Diff-Quick (Scientific Products, Columbia, Md.). The filter was rinsed twice with water and placed on a microscope slide, with the lower surface facing the slide. Cells which did not migrate through the filter were wiped off the upper surface with a cotton swab. Migrated cells were counted in consecutive microscope fields along the diameter of the filter, covering a total area of 1 mm². Assays were run in triplicate.

Attachment of cells was determined by counting numbers of cells on the upper surface of filters from quadruplicate chambers.

## Sources of Growth Factors

Purified PDGF and platelet extracts with known mitogenic activities (19) were gifts from C. D. Scher and C. D. Stiles (Sidney Farber Cancer Institute, Boston, Mass.). This growth factor was stable to heating (100°C for 10 min) and was homogeneous by PAGE. PDGF was inactivated by trypsin and by exposure to sulfhydryl reagents. A mitogenic unit (U) of PDGF is defined as that which induces DNA synthesis in 50% of confluent BALB/c 3T3 (clone A31) cells when they are cultured in 5% plasma (19). Partially purified PDGF, epidermal growth factor (EGF), bovine fibroblast growth factor (FGF), and nerve growth factor (NGF) were purchased from Collaborative Research (Waltham, Mass.). Zinc-free porcine insulin was obtained from Elanco Products (Indianapolis, Ind.). On the generation of data in all tables and figures, purified PDGF, not activated platelet supernatants, was used.

## Assay for Growth Factor–induced DNA Synthesis in Fibroblast Cultures

Induction of DNA synthesis in CRL 1475 fibroblasts by growth factors was assayed by measuring the incorporation of [³H]thymidine (23). Cells were plated in multiwell tissue culture plates at 6,000 cells/0.32-cm² well in 0.3 ml of DMEM plus 5% fetal calf serum. After 2 d, cells were washed twice with DMEM without

serum and were incubated in DMEM without serum for an additional 12 h. Growth factors, dissolved in DMEM, were then added to the cells. After 16 h, [methyl-³H]thymidine (25 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) was added to a final concentration of 1 µCi/0.3 ml in each well without removing the growth factor. The incorporation of [³H]thymidine was measured 24 h later by detaching the cells with trypsin solution and harvesting the cells on filters (Whatman 934-AH glass microfiber filters) using a cell harvester (Brandel, Rockville, Md.). The radioactivity on the filters was determined by scintillation counting.

## Inhibition of Synthesis of DNA, RNA, and Protein

Fibroblasts were suspended as described above, and hydroxyurea (Sigma Chemical Co.), azidocytidine (2'-azido-2'-desoxycytidine; Boehringer-Mannheim Biochemicals), or actinomycin D (Sigma Chemical Corp.) was added to give the specified final concentrations. The cell suspensions were incubated at 37°C in a humidified atmosphere of 5% $CO_2$/air for 30 min. Control cells were preincubated without additions. The cells were transferred to chemotaxis chambers and their migration was tested in response to 0.5 U of PDGF/ml in the lower well. To avoid gradients of inhibitors, the lower well also contained the same concentration of inhibitor that was present in the cell suspension. Viability of cells was determined at the termination of the chemotaxis assay using the trypan blue exclusion test in a parallel experiment with the same cell suspensions. The effects of the inhibitors on macromolecular synthesis were determined in parallel multiwell cultures. Inhibition of protein synthesis by cycloheximide was monitored by measuring incorporation of L-[4,5-³H]leucine (76 Ci/mmol; Amersham Corp.); inhibition of RNA synthesis by actinomycin D by measuring [5,6-³H]uridine (40 Ci/mmol; Amersham Corp.) incorporation; and inhibition of DNA synthesis by hydroxyurea and azidocytidine by measuring [methyl-³H]thymidine incorporation.

## RESULTS

In preliminary studies, we assayed materials secreted by mast cells, neutrophils, and platelets for their chemotactic activity toward fibroblasts. There was no detectable chemotactic activity in the supernatant fluids of rat mast cells which had been activated to release their granules by using compound 48/80. Chemotactic activity for fibroblasts was observed in the supernatants of rabbit polymorphonuclear cells that had been activated to secrete materials by means of FMLP. The activity was 210% of the level observed in controls. An even stronger chemotactic response (400% of control) was seen by using supernatant fluid from platelets that had been aggregated with thrombin (24). This activity exceeded the maximal response of fibroblasts to fibronectin and was therefore likely to be distinct from that of fibronectin.

Since PDGF is present in the α-granules of platelets (25), it was possible that a significant fraction of the chemotactic activity in the supernatant from stimulated platelets could be accounted for by this growth factor. We therefore compared the chemotactic activity in the material released from platelets with that of purified PDGF. A strong chemotactic response was obtained with purified PDGF (Fig. 1), although, at concentrations >1 U/ml, chemotaxis was reduced while [³H]thymidine incorporation remained maximal. At concentrations that were mitogenically equivalent, the chemotactic response obtained with the platelet supernatant was equal to that obtained with purified PDGF (data not shown). We were, therefore, able to replace the supernatant from stimulated platelets with PDGF. These studies indicate that PDGF is a potent chemotactic factor for fibroblasts present in the platelet fluids. While the chemotactic response of the cells was reduced at high levels of PDGF, similar requirements for optimal levels of attractants have been observed previously with other cells and other attractants (26). The inhibition is usually attributed to a loss of the concentration gradient guiding the direction in

which the cells migrate and to a deactivation of the cells due to "down regulation" of the receptors for the attractant.

Evidence that PDGF was chemotactic rather than just chemokinetic was obtained by a "checkerboard" analysis (27, 28). Here, different concentrations of PDGF added below and above the filter in the Boyden chamber were varied in a systematic manner to generate various gradients of the material (Fig. 2). Migration of the cells across the filter occurred whenever the concentration of PDGF in the lower compartment was greater than that in the upper chamber, indicating that migration occurs along a concentration gradient of PDGF. It should be noted that the Zigmond-Hirsch (27) procedure for determining chemotactic motility measures the distance traveled by the leading front of cells within a micropore filter for a given period of time. Here, we are measuring numbers of cells that have crossed the width of the filter during a given period of time. Our results, however, are consistent with what would be expected for cells responding to a gradient of attractant (27).

It was possible that the PDGF had bound to the membrane filter separating the two compartments of the Boyden chamber and that the cells were migrating along a gradient of immobilized PDGF, a process known as haptotaxis (29). To test for haptotaxis, filters were incubated with various concentrations of PDGF to allow binding to occur and were then placed in the Boyden chamber. No significant migratory response by fibroblasts was observed when the filters were exposed to PDGF before assay compared to their responses when similar
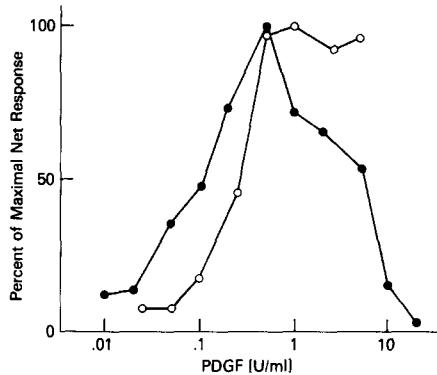


FIGURE 1 Comparison of migratory (●) and mitogenic (O) responses of human skin fibroblasts (CRL 1475) to purified platelet-derived growth factor. The responses, which are expressed as percentage of the maximal response, are given on the ordinate. The maximal migratory response to PDGF was 345 ± 25 cells/mm² and the background in the absence of attractant was 113 ± 8 cells/mm² of filter surface. Each point is the average of triplicate values. Number of experiments performed was three.



FIGURE 2 Migratory responses of fibroblasts to varied gradients of partially purified PDGF. Gradients were created by adding PDGF solutions of given concentrations to wells below filter and to cell suspensions to make given final concentrations. The values in the checkerboard are the numbers of migrated cells ± SEM per mm² area of filter. Each point is the average of triplicate values. Number of experiments performed was four.

concentrations of PDGF were added to the lower chamber (Table I). These experiments indicate that migration of the fibroblasts in response to PDGF is the result of a true chemotactic response and not to haptotaxis.

As noted previously, PDGF is a potent mitogen for fibroblastic cells, and the accumulation of cells on the lower surface of the filter could be due to proliferation. However, the time-course of the chemotactic response (2–4 h) suggests that cell proliferation is not involved (17, 30). In addition, hydroxyurea and azidocytidine had no effect on the migratory response of the cells at concentrations which inhibited DNA synthesis by 93–97% (Table II). In contrast, actinomycin D and cycloheximide inhibited the chemotactic response of the cells in a dose-

TABLE I

*Migration of Fibroblasts on Filters Preincubated in the Presence of PDGF Gradients*

| | Migratory responses | |
|---|---|---|
| PDGF | PDGF present during preincubation only* | PDGF present during chemotaxis assay |
| U/ml | cells/mm² ± SEM | |
| 0 | 84 ± 36 | 110 ± 17 |
| 0.1 | 110 ± 10 | 134 ± 8 |
| 0.5 | 129 ± 19 | 232 ± 30 |

* Filters were preincubated at 37°C in the chemotaxis chambers in the presence or absence of partially purified PDGF in the lower well for 30 min. After incubation, the medium above filter was suctioned off, the filter was removed and washed in three changes of DMEM-BSA, and finally 5 ml of DMEM-BSA was forced through the filter using a syringe and a Millipore filtration unit. Filters were used immediately in chemotaxis assays where no PDGF was added to lower well. Number of experiments performed was four.

TABLE II

*Effect of Inhibiting Synthesis of Protein, RNA, or DNA on Fibroblast Chemotaxis to PDGF*

| Inhibitor | Chemotaxis* | [³H]Leucine incorporation‡ |
|---|---|---|
| | % of control | % of control |
| Cycloheximide | | |
| 0.1 μg/ml | 94 | 16 |
| 5.0 μg/ml | 42 | 3 |
| 20.0 μg/ml | 8 | 1.5 |
| | | [³H]Uridine incorporation‡ |
| Actinomycin D | | |
| 0.1 μg/ml | 90 | 35 |
| 1.0 μg/ml | 50 | 12 |
| 10.0 μg/ml | 6 | 11 |
| | | [³H]Thymidine incorporation‡ |
| Hydroxyurea | | |
| 0.1 mM | 93 | 100 |
| 1.0 mM | 116 | 46 |
| 5.0 mM | 125 | 7 |
| Azidocytidine | | |
| 0.1 mM | 70 | 6 |
| 0.5 mM | 100 | 6 |
| 1.5 mM | 123 | 3 |

* Values give migratory responses expressed as percent of response to 0.5 U/ml of partially purified PDGF observed in the absence of inhibitors.

‡ Values give incorporation of specified radiolabeled substance as percent of incorporation in the absence of inhibitors. Each point is the average of triplicate values, the SEM of which did not exceed 15%. Number of experiments performed was three.

dependent manner. These data indicate that the chemotactic response of the fibroblasts require RNA and protein synthesis but not DNA synthesis.

Next, several other growth factors were tested for chemotactic activity (Fig. 3) and for their ability to stimulate thymidine incorporation into cultured skin fibroblasts (data not shown). At 0.5 μg/ml, fibroblast growth factor, epidermal growth factor, insulin, and PDGF all stimulated DNA synthesis, while nerve growth factor had no effect even at 5 μg/ml. Of these materials, only PDGF showed strong chemoattractant activity (Fig. 3). These results indicate that chemotactic activity is not a general property of mitogens.

Previous studies on leukocytes have shown that preincubation with a chemoattractant can prevent the subsequent chemotactic response to itself and to other attractants (26, 31, 32). To determine whether PDGF has this deactivating effect, cells prepared by the usual methods were divided into two groups
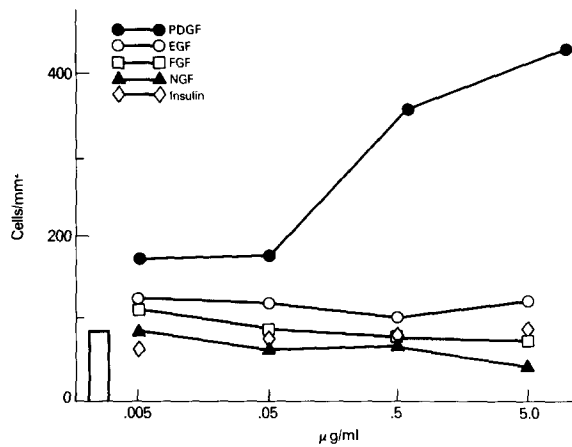


FIGURE 3 Comparison of growth factors as fibroblast chemoattractants. Partially purified preparations of platelet-derived growth factor ( PDGF), epidermal growth factor ( EGF), fibroblast growth factor ( FGF), nerve growth factor ( NGF), and a pure preparation of insulin were dissolved in DMEM containing 1 mg/ml of bovine serum albumin to give the specified concentrations and were tested for chemotactic activity in Boyden blind-well chambers. At these concentrations, the growth factors, except NGF, stimulate incorporation of [³H]thymidine in fibroblasts (not shown). The activities of the growth factors (Collaborative Research Corp.) are as follows: 1 U of PDGF = 3 μg/ml, 1 U of EGF = 100 ng/ml, and 1 U of FGF = 33 ng/ml. The open bar shows the migration in the absence of added growth factor. Each point is the average of triplicate values. Number of experiments performed was four.

TABLE III
Effect of Preincubation of Fibroblasts with PDGF on Their Subsequent Chemotaxis to PDGF

| Conditions of preincubation* | Migratory responses, cells/ mm² | | |
| | Attractants | | |
| | PDGF (2 U/ml) | Fibronectin (20 μg/ml) | None |
| --- | --- | --- | --- |
| PDGF, 2 U/ml in DMEM-BSA | 72 ± 18 | 67 ± 11 | 20 ± 5 |
| DMEM-BSA | 318 ± 15 | 252 ± 12 | 81 ± 12 |

* After trypsinization, cells were preincubated for 30 min at 37°C in DMEM supplemented with 0.1% BSA. The cells after washing were resuspended in the same medium and assayed for chemotaxis as in Methods. Number of experiments performed was three. Partially purified PDGF was used in both the preincubation and attractant solutions.
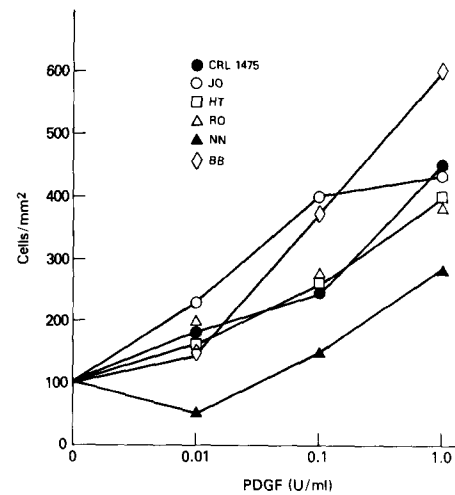


FIGURE 4 Migratory responses of six strains of human skin fibroblasts to partially purified PDGF. CRL 1475 is the fetal line used in all other experiments. The other strains are from adults. Each point is the average of triplicate values. Number of experiments performed was three.

which were preincubated for 30 min either with 2 U/ml of PDGF or with control medium. The cells were then washed and assayed for their chemotactic responsiveness to either PDGF or fibronectin. As seen in Table III, the response of the pretreated cells to both attractants was greatly reduced compared with the controls. This indicated that the fibroblasts, like leukocytes, are deactivated by their own attractants, suggesting that there may be similarities in the regulatory mechanisms of migration in both cell types.

To exclude the possibility that only fetal cells respond chemotactically to PDGF, we tested fibroblasts from adult human skin and found that they also respond to PDGF in a dose-dependent manner (Fig. 4).

## DISCUSSION

Our studies show that platelets release a potent chemoattractant for fibroblasts. Similar chemotactic activity is observed with purified PDGF. Since PDGF is the major mitogenic factor in the platelet-derived fluids, we compared the mitogenic and chemotactic activities in the platelet fluids with those of the purified material. These studies showed that the chemotactic activity of platelet fluids can be attributed to PDGF. PDGF over a wide concentration range stimulated the incorporation of [³H]thymidine into DNA, whereas chemotaxis was inhibited at higher concentrations of PDGF. PDGF is also a chemoattractant for smooth muscle cells (33) but not for endothelial cells or polymorphonuclear leukocytes (Seppä et al., manuscript in preparation). In this regard, there is a correlation between the chemotactic responsiveness of cells and their ability to respond mitogenically. Both responses presumably depend on the presence of receptors for PDGF.

Results of experiments in which fibroblasts were treated with inhibitors of DNA, RNA, and protein synthesis demonstrate that the response of the cells to PDGF depends upon the continuous synthesis of protein. In this regard, fibroblasts differ from leukocytes in which chemotaxis occurs even when protein synthesis (34) or RNA synthesis (E. Schiffman, unpublished results) is blocked. In contrast, DNA synthesis is not required for chemotaxis. Results with hydroxyurea and azidocytidine, furthermore, indicate that the proliferation of cells is not responsible for increased migration in the presence of PDGF.

In addition, the time of assay (4 h) is shorter than the time required to detect a mitogenic response (30 h).

The loss of responsiveness of the fibroblasts when pretreated with an attractant is similar to what has been reported previously for neutrophils (26, 31, 32). It is possible that the loss of response, or deactivation, resembles the "down regulation" observed with various peptide hormones and involves loss of surface receptors either by a direct effect or as a secondary consequence of cytoskeletal changes.

Various peptide growth factors, including EGF, FGF, insulin, and NGF, were not active in the chemotaxis assay although all but NGF are mitogenic for fibroblasts. The experiments reported here do not support the possibility that mitogenic and chemotactic activities of PDGF are directly related although both responses could involve identical receptors. The concentrations of PDGF required for chemotaxis and mitogenesis are similar. However, the cells respond in chemotaxis assays to a gradient of PDGF, where the indicated nominal concentration is present only in the lower compartment of the chamber. The cells, therefore, respond chemotactically to a concentration of PDGF lower than that required for mitotic activity.

The responses of fibroblasts to PDGF may be significant in wound repair. When platelets aggregate early in clot formation they release PDGF, which may recruit fibroblasts into the wound area. The mitogenic activity of PDGF may then cause a rapid proliferation of fibroblasts. Subsequently, these cells would produce collagen and other materials necessary to repair the wound. In this context, PDGF may be considered a wound hormone. When such a response occurs inappropriately, it could lead to fibrosis.

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