

# Chemotactic Activity of Platelet Alpha Granule Proteins for Fibroblasts

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**ABSTRACT** At sites of blood vessel injury, platelets release numerous substances that may have biological activities influencing cellular responses. In this study we examined separately the chemotactic activity for fibroblasts of three highly purified proteins obtained from platelet alpha granules: platelet factor 4 (PF4), platelet-derived growth factor (PDGF), and  $\beta$ -thromboglobulin (BTG). We observed that each of these proteins was strongly chemotactic for fibroblasts, with maximum chemotactic activity in each instance comparable to that observed with an optimal concentration of the control chemotactic protein, plasma fibronectin. Each protein was active at very low concentrations. The peak chemotactic activities of PF4, PDGF, and BTG occurred at 200 ng/ml, 30 ng/ml, and 6 ng/ml, respectively. Specificity of fibroblast chemotaxis to individual platelet proteins was provided by finding that anti-PF4 immunoglobulin blocked the chemotactic activity of PF4 without affecting the chemotactic activity of PDGF, while anti-PDGF immunoglobulin blocked the activity of PDGF but did not alter the capacity of PF4 to promote fibroblast chemotaxis. These results suggest that in vivo several alpha granule proteins released from platelets may affect wound healing by causing directed fibroblast migration.

Apart from an essential role in hemostasis, platelets appear to be important for inflammation (1). The mechanisms by which platelets mediate inflammation have not been well understood. To help determine whether platelets might influence inflammation by inducing directed cell migration of inflammatory cells to sites of injury, we undertook to examine the chemotactic activities of highly purified platelet secretory proteins for neutrophils and monocytes. Platelet factor 4 (PF4) was shown to be a potent chemotactic agent for human monocytes and for human neutrophils, at concentrations of PF4 present in normal human serum and thus at concentrations present at sites of injury (2). Subsequently, we reported that the platelet-derived growth factor (PDGF) purified to homogeneity is highly active as a chemoattractant for human inflammatory cells (3). Optimal PDGF concentrations for chemotaxis for monocytes and neutrophils were 20 ng/ml and 1 ng/ml, respectively. Coincident with these studies, Grotendorst et al. (4) and Seppa et al. (5) reported that PDGF is chemotactic for fibroblasts and smooth muscle cells, and suggested that PDGF may be in-

involved in wound healing through effects upon fibroblast movement.

The present report extends the observations concerning the activity of platelet proteins upon cell migration by establishing that PF4 and another platelet alpha-granule protein,  $\beta$ -thromboglobulin (BTG), are highly chemotactic for fibroblasts. The results provide additional information in support of the hypothesis that platelets participate in inflammation and wound repair.

## MATERIALS AND METHODS

PF4, PDGF, and BTG were purified from outdated, frozen platelet packs using procedures previously reported (6-8). Both PF4 and BTG migrated as single homogeneous bands in SDS PAGE (6, 8). BTG was established as pure also using N-terminal amino acid analysis. PDGF was resolved into two separate homogeneous species, PDGF I and PDGF II (7), by SDS PAGE; each protein species was tested separately for chemotactic activity. Fibronectin, purified from human plasma (a gift of Dr. John A. McDonald, Washington University and The Jewish Hospital of St. Louis), was used as a positive control.

*Fibroblasts:* Fibroblasts from human skin and bovine nuchal ligament

were cultured in Dulbecco's modified Eagle's medium (DME, The Center for Basic Cancer Research, Washington University School of Medicine, St. Louis, MO), as previously described (9). Confluent fibroblasts (2-6 passages) were detached by exposure to 0.25% (wt/vol) trypsin and 0.1% (wt/vol) ethylenediaminetetraacetic acid (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline for 3 min at 37°C before use in chemotaxis assays. The trypsin was promptly inactivated by dilution into a 5× volume of fresh culture medium containing 10% (vol/vol) fetal bovine serum (KC Biological, Inc., Lenexa, KS). To minimize the potential effects of platelet-derived factors contained in the fetal bovine serum included in the culture medium, the medium was not changed for 48 h before the cells were harvested.

**Chemotaxis Assays:** Assays of fibroblast chemotaxis were performed in triplicate in a multi-blind well apparatus by the method recently reported (9). Each well of the apparatus was separated into two compartments by a pair of micropore membranes, an 8- $\mu$ m micropore membrane (Nucleopore Corp., Pleasanton, CA) overlying a 0.45- $\mu$ m micropore membrane (Millipore Corp., Bedford, MA). To obtain attachment and spreading of the fibroblasts to the membranes, the membranes were pretreated with poly-L-lysine (P-1886, Sigma Chemical Co., St. Louis, MO). The lower compartment of each well was filled with 240  $\mu$ l of solution to be assayed and the upper compartment was filled with 360  $\mu$ l of a cell suspension containing  $1.2 \times 10^5$  cells per ml. After 6 h of incubation at 37°C the chambers were disassembled and the membranes stained with hematoxylin. To quantify cell migration, 5 high-power ( $\times 400$ ) fields (hpf) were counted per membrane pair. In every experiment negative controls were run in parallel. These controls, consisting of wells in which the lower compartment contained medium only, yielded  $\sim 20$  cells per hpf. The cell migration in test chambers was corrected by subtracting the number of cells in the corresponding control assays. Accordingly, the results are reported as net cell migration.

In some experiments, rabbit anti-PF4 IgG or rabbit anti-PDGF IgG was incubated for 60 min at 37°C with either PF4 or PDGF and then the mixture was assayed for chemotactic activity. In these experiments, in addition to the usual negative controls, additional controls of IgG with medium in the lower compartment were also included. The IgG was established as monospecific by immunodiffusion analysis against whole platelet lysates and against the appropriate purified protein.

## RESULTS

Both BTG and PF4 were observed to promote fibroblast migration, with the optimal cell migration at 6 ng/ml and 200 ng/ml, respectively (Fig. 1A and B). The effects of these proteins upon fibroblast migration were established as chemotactic, since cell movement was little above background when the assay chambers were prepared so that the concentration of the test protein was either the same in both compartments, or

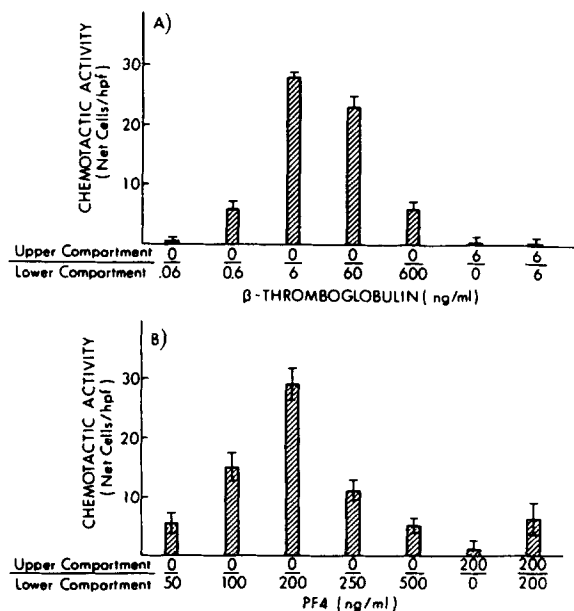


FIGURE 1 Bovine ligament fibroblast migration in response to  $\beta$ -thromboglobulin (A) and PF4 (B). Mean and SE of triplicate observations.

high in the upper compartment. Maximum chemotactic responsiveness of fibroblasts to PF4 and to BTG was comparable to the maximum chemotactic response induced by 1  $\mu$ g/ml plasma fibronectin ( $\sim 35$  net cells per hpf).

The chemotactic activity of homogeneous preparations of PDGF II for fibroblasts was then tested. Table I presents results of examining various concentrations of PDGF in the upper and lower chambers of the in vitro chemotactic assay ("checkerboard analysis"). PDGF is established as chemotactic for fibroblasts in this assay. Maximal chemotactic activity of PDGF occurred at 30 ng/ml, approximately the  $K_d$  of PDGF for fibroblasts (10). The activity of PDGF I was tested also and found to be similar to that of PDGF II.

Fig. 2, depicting the cell migration of human skin fibroblasts in comparison to bovine ligament fibroblasts, indicates that PDGF has effects on both types of fibroblasts; the dose response curves for both cell types are similar although human cells appear to be quantitatively less responsive. We previously observed a similar difference between chemotactic responses of human and bovine cells, using tropoelastin and elastin derived peptides as chemoattractants (9).

Rabbit immunoglobulins raised against PF4 and PDGF were used to help establish the specificity of the chemotactic responses of fibroblasts to the platelet proteins. The chemotactic activity of PF4 for fibroblasts was abolished by anti-PF4 IgG, but was unaffected by anti-PDGF IgG; reciprocally, the chemotactic activity of PDGF was abolished by anti-PDGF IgG but unaffected by anti-PF4 IgG (Fig. 3A and B). In these experiments, in which the same batches of bovine ligament fibroblasts were tested simultaneously with either PF4 or PDGF, as shown, the peak chemotactic activity to PF4 fully equaled the peak chemotactic activity of PDGF, but there is a marked difference between the concentration optimums and the concentration ranges over which PF4 and PDGF are effective. PDGF activity, maximum at 30 ng/ml, is expressed over a 1,000-fold range of concentration (0.3-300 ng) whereas the activity of PF4, maximal at 200 ng/ml, is expressed over a relatively narrow, 10-fold concentration range (50-500 ng).

TABLE I  
Fibroblast Chemotaxis to PDGF

PDGF, lower compartment	PDGF, upper compartment, ng/ml			
	0	3	15	30
ng/ml				
3	17 $\pm$ 0.5*	5 $\pm$ 0.4	0 $\pm$ 0.5	4 $\pm$ 0.6
15	29 $\pm$ 0.6	13 $\pm$ 0.6	6 $\pm$ 0.4	2 $\pm$ 0.4
30	52 $\pm$ 0.9	42 $\pm$ 0.6	8 $\pm$ 0.8	3 $\pm$ 0.3

\* Cells/hpf  $\pm$  SEM,  $n = 15$

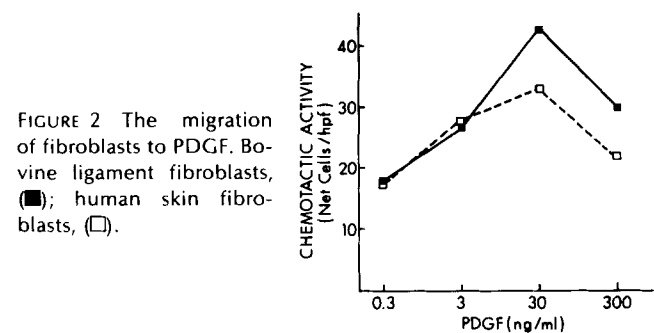
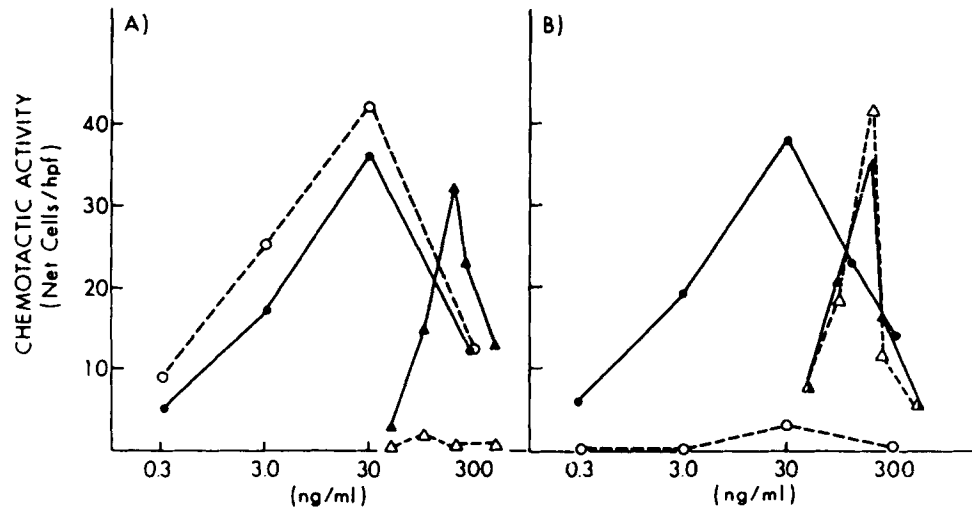


FIGURE 2 The migration of fibroblasts to PDGF. Bovine ligament fibroblasts, (■); human skin fibroblasts, (□).

FIGURE 3 Bovine ligament fibroblast migration to PF4 and PDGF which have been preincubated with (A) anti-PF4 immunoglobulin (PF4 Ab) or (B) anti-PDGF immunoglobulin (PDGF Ab). (A) PDGF, (●); PDGF plus PF4 Ab, (○); PF4, (▲); PF4 plus PF4Ab, (△). (B) PDGF, (●); PDGF plus PDGF Ab, (○); PF4, (▲); PF4 plus PDGF Ab, (△). See Materials and Methods for details.



## DISCUSSION

When blood vessels are injured, circulating platelets adhere to exposed collagen and to the subendothelial basement membrane. Aggregation of adhered platelets follows and the platelets involved release the contents of their alpha and dense granules (11). In addition to fibrinogen, fibronectin, and other proteins also found in plasma, three platelet-specific, alpha-granule proteins, PF4, PDGF, and BTG, are released (12). The present experimental data demonstrate that each of these unique alpha-granule proteins is a strong chemotactic agent for fibroblasts. Thus, platelet-specific secretory proteins have the potential to be highly important mediators of fibroblast migration into injured areas. Other potentially important mediators of fibroblast migration include lymphokines, a complement-related component, collagen, fibronectin, tropoelastin, and elastin-derived peptides (9, 13–16).

PF4 was the first platelet-specific, alpha-granule protein shown to be active in chemotaxis of inflammatory cells, suggesting a vital role for platelets as effector cells in initiating major events of inflammation and repair (3). Additional support favoring a role of PF4 *in vivo* in mediating chemotaxis of inflammatory cells was obtained by the demonstration that PF4 was active in chemotaxis at concentrations found in human serum (17). In contrast, the concentration of PF4 resulting in maximum fibroblast chemotactic activity is less than one-tenth that required for optimal chemotaxis of inflammatory cells—the concentration also found in human serum. This differential in PF4 concentration for chemotactic activity between inflammatory cells and fibroblasts may have relevance to the types of responses initiated by platelets. High-level, acute injury with major platelet release provides concentrations of PF4 favoring inflammation whereas low-level, chronic release may mediate fibroblastic ingress. Also, optimal chemotactic concentrations for monocytes and neutrophils are found at the immediate site of injury, where monocytes and neutrophils are to be found; the substantially lower optimal concentrations of PF4 required for attracting fibroblasts are consistent with the lower concentrations of PF4 found as the distance is increased from the immediate site of injury to the subendothelial site of focus of fibroblasts *in vivo*. The differences in optimal concentrations of PF4 required for cell migration of monocytes and neutrophils on one hand and fibroblasts on the other emphasize the complexity of interactions between cells and chemoattractants.

Each cell type needs to be investigated over a wide range of concentrations before concluding whether or not the cells will respond to a potential chemoattractant.

PF4 antigen rapidly permeates into blood vessel walls following endothelial injury (18). Whether PF4 is subject to proteolytic cleavage in the vessel wall is not clear, however, it is possible that PF4-derived peptides may retain chemotactic activity. Indeed, we recently demonstrated that the carboxyl terminal 13 amino acid residues of PF4 fully express monocyte chemotactic activity of intact PF4, strongly suggesting that this tridecapeptide contains the chemotactically active site of PF4 (19). Release of this peptide by proteolysis in the tissues could provide locally a potent chemotactic agent for inflammatory cells.

The function of BTG heretofore has not been described. The finding that BTG is highly chemotactic for fibroblasts provides one potentially important function for this protein. BTG shares marked amino acid sequence homology with PF4 (20). Despite close similarity in structure, as well as similarities in concentration within the platelet and in the quantity of each protein released from stimulated platelets (21), however, there are a number of differences between BTG and PF4. PF4 does not compete with BTG for receptor binding on endothelial cells (22), an observation suggesting that BTG and PF4 may utilize different receptors in promoting fibroblast chemotaxis. Also, BTG has a longer half-time in the circulation (21), suggesting that BTG may function effectively as a chemoattractant over a longer time. In the experiments reported above, on a mole to mole basis, BTG is a more potent fibroblast chemoattractant than PF4. The maximum effect of BTG occurs at  $1.7 \times 10^{-10}$  M, 30-fold less than the concentration required for optimal effectiveness of PF4.

Recently, PDGF was shown to have fibroblast chemotactic activity (5). In that work, in which the concentrations of PDGF were expressed as units of mitogenic activity, the dose-response curves for the mitogenic and chemotactic activities of PDGF were found to be similar up to the optimal concentration for chemotactic activity. Above that concentration, chemotactic activity declined while mitogenic activity persisted. We now demonstrate that PDGF is at least as potent a fibroblast chemoattractant as fibronectin and that the maximal chemotactic activity occurs at  $10^{-9}$  M, a concentration virtually identical to the dissociation constant ( $0.7 \times 10^{-9}$  M) of PDGF we reported in studies of its binding to fibroblasts (10). The

fibroblast chemotactic activity of PDGF is likely due to an interaction of PDGF with specific cell surface receptors. Such receptors have been suggested in direct binding studies with mouse fibroblasts, human skin fibroblasts, arterial smooth muscle cells, and human glial cells (10, 23, 24). The chemotactic responsiveness of neutrophils and monocytes to PDGF also suggests that these cells have receptors for PDGF (3). PDGF I and PDGF II show comparable chemotactic activity, analogous to the finding that both proteins have similar mitogenic activity (7).

The demonstration of PDGF fibroblast chemotactic activity by Seppa et al. (5) was preceded by their observation that supernatants from thrombin-stimulated platelets were chemotactic for fibroblasts. The possibility that other factors released by stimulated platelets besides PDGF might also have chemotactic activity for fibroblasts was not examined. The present results indicate that at least two other proteins released from platelet alpha granules, PF4 and BTG, also have the capacity to promote fibroblast chemotaxis, and that their effects appear to be at least as great as the effects achieved by either PDGF I or PDGF II. The possibility that platelets contain still other chemotactic factors for fibroblasts needs further study because platelets release fibronectin, a known chemoattractant for fibroblasts (16), and Nakao et al. recently observed that 12-L-5,8,10,14-eicosatetraenoic acid, a platelet lipoxygenase product, produces smooth muscle migration (25). It must be concluded that the role of platelets in inflammation and wound healing is likely to be highly complex, involving a variety of factors with differing potency and cellular specificity.

We acknowledge with gratitude the cooperation of the American Red Cross Blood Banks in Chicago, IL (R. Gilbert), Toledo, OH (F. Courtwright and P. Lau), Tulsa, OK (K. Kasprisin), Waterloo, IA (J. Bender and T. Brown), and Fort Wayne, IN (G. Drew and D. Dunfee), for generously supplying us with outdated human platelet packs.

This work was supported by U.S. Public Health Service Grants HL 16118 (to R. M. Senior and G. L. Griffin); HL 14147, HL 22119, CA 22409, T32-HL07088, and by the American Heart Association, Missouri Affiliate, Inc. (to J. S. Huang and T. F. Deuel); and HL27073 and a Grant-in-Aid from the Michigan Heart Association (to D. A. Walz).

Received for publication 7 September 1982.

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