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Research Article

Fibrin formation and turnover are intimately associated with inflammation and wound healing. To explore whether fibrin(ogen)-derived peptides exert direct effects upon cells involved in inflammation and tissue repair we examined the capacity of human fibrinopeptide B (hFpB), a thrombin-derived proteolytic cleavage product of the fibrinogen B beta-chain, to stimulate neutrophils (PMN), monocytes, and fibroblasts. hFpB caused directed cell migration of PMN and fibroblasts that was optimal at approximately 10(-8) M. This chemotactic activity was blocked by preincubating hFpB with antiserum to hFpB. hFpB was not chemotactic for monocytes. The chemotactic potency of hFpB for PMN was equivalent to that of anaphylatoxin from the fifth component of human complement (C5a), leukotriene B4 (LTB4), and formyl-methionyl-leucyl-phenylalanine (fMLP), and for fibroblasts its chemotactic activity was comparable to that of platelet-derived growth factor. hFpB did not interact with PMN receptors for C5a, LTB4, or fMLP as (a) desensitization with 10(-7) M hFpB abolished chemotaxis to hFpB but had no effect upon chemotaxis to C5a, LTB4, or fMLP and (b) induction of chemotactic responses to fMLP and LTB4 in neutrophilic leukemic cells (HL-60 cells) by incubation with dimethylsulfoxide did not extend to hFpB. Like fMLP, hFpB caused a rapid, dose-dependent increase in PMN cytoskeletal associated actin, but unlike fMLP, hFpB did not cause PMN aggregation, release of lysosomal enzymes (lysozyme and beta-glucuronidase), or the production […]

Effects of Fibrinogen Derivatives Upon the Inflammatory Response

Studies with Human Fibrinopeptide B

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Abstract

Fibrin formation and turnover are intimately associated with inflammation and wound healing. To explore whether fibrin(ogen)-derived peptides exert direct effects upon cells involved in inflammation and tissue repair we examined the capacity of human fibrinopeptide B (hFpB), a thrombin-derived proteolytic cleavage product of the fibrinogen B β-chain, to stimulate neutrophils (PMN), monocytes, and fibroblasts. hFpB caused directed cell migration of PMN and fibroblasts that was optimal at $\sim 10^{-8}$ M. This chemotactic activity was blocked by preincubating hFpB with antiserum to hFpB. hFpB was not chemotactic for monocytes. The chemotactic potency of hFpB for PMN was equivalent to that of anaphylatoxin from the fifth component of human complement (C5a), leukotriene B_4 (LTB_4), and formyl-methionyl-leucyl-phenylalanine (fMLP), and for fibroblasts its chemotactic activity was comparable to that of platelet-derived growth factor. hFpB did not interact with PMN receptors for C5a, LTB_4, or fMLP as (*a*) desensitization with 10^{-7} M hFpB abolished chemotaxis to hFpB but had no effect upon chemotaxis to C5a, LTB_4, or fMLP and (*b*) induction of chemotactic responses to fMLP and LTB_4 in neutrophilic leukemic cells (HL-60 cells) by incubation with dimethylsulfoxide did not extend to hFpB. Like fMLP, hFpB caused a rapid, dose-dependent increase in PMN cytoskeletal associated actin, but unlike fMLP, hFpB did not cause PMN aggregation, release of lysosomal enzymes (lysozyme and β-glucuronidase), or the production of superoxide anion. These results suggest that hFpB may have a role in recruiting PMN and fibroblasts at sites of fibrin deposition and turnover. The capacity of hFpB to cause PMN chemotaxis without causing concurrent release of lysosomal enzymes or the production of superoxide anion is further evidence for the complexity of PMN responses to chemotactic agents.

Introduction

Fibrin has long been recognized as a histologic feature of both acute and chronic inflammatory processes. While the role of fibrin deposition in inflammatory lesions is not yet well understood, its presence does not appear to be incidental. Fibrin formation is necessary for the full development of delayed hypersensitivity reactions (1, 2). Moreover, fibrin(ogen)-derived proteolytic fragments have been found to produce many effects relevant to inflammation that include changes in vascular tone (3) and permeability (4), retraction (5) and disorganization of endothelial cells (6), suppression of lymphocyte proliferation (7), and chemotaxis of inflammatory cells (4, 8–10).

Human fibrinopeptide B (hFpB),[1] a peptide of 14 amino acids released from the amino-terminus of the B β-chains of fibrinogen by thrombin and also contained within the primary plasmin cleavage product of fibrinogen, B β 1-42, has been reported to have chemotactic activity for neutrophils (PMN) (10, 11). The present study extends these data, showing that the chemotactic activity of hFpB does not involve the receptors involved in responses to anaphylatoxin from the fifth component of human complement (C5a) (12), leukotriene B_4 (LTB_4) (13), or formyl-methionyl-leucyl-phenylalanine (fMLP) (14). Like fMLP, hFpB was found to increase the amount of actin associated with the cytoskeleton, but unlike fMLP it did not cause PMN aggregation, release of lysosomal enzymes or the production of superoxide anion. Fibroblasts were also found to demonstrate chemotactic responses to hFpB, suggesting that the effects of this fibrinopeptide extend to cell types other than inflammatory cells.

Methods

Reagents. hFpB was purchased from Bachem Inc., Fine Chemicals (Torrance, CA), and hFpB without its carboxy-terminus arginine (hFpB des Arg) was prepared by solid-phase peptide synthesis (15). C5a, platelet-derived growth factor (PDGF), and LTB_4 were gifts from Dr. Robert O. Webster, St. Louis University School of Medicine, St. Louis, MO; and Dr. Thomas F. Deuel and Dr. William F. Stenson, Jewish Hospital at Washington University, St. Louis, MO, respectively. Dimethyl sulfoxide (DMSO), fMLP, cytochrome c, superoxide dismutase, cytochalasin B, *Micrococcus luteus,* and *p*-nitrophenyl-β-D-glucuronide were obtained from Sigma Chemical Co., St. Louis, MO. Antibody to hFpB was prepared by immunizing rabbits with synthetic hFpB conjugated to bovine serum albumin, as previously described (15).

Cells. PMN and peripheral blood mononuclear cells were separated on Ficoll-Hypaque gradients from peripheral blood obtained from healthy volunteers (16). Human promyelocytic cells, HL-60 (17), a gift from Dr. Arnold Kahn, Washington University School of Dental Medicine, St. Louis, MO, were grown in RPMI 1640 supplied by The Basic Cancer Center, Washington University, St. Louis, MO, supplemented with 10% fetal calf serum. Fetal bovine ligament fibroblasts were obtained from explants of ligamentum nuchae, as previously described (18), and were used between the second and sixth passages.

Chemotaxis. Chemotaxis was determined in modified Boyden chambers, as previously described (19, 20). Briefly, 1.2×10^6 PMN/ml, 2.5×10^6 mononuclear cells/ml, or 1.2×10^5 fibroblasts/ml were placed

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1. *Abbreviations used in this paper:* C5a, anaphylatoxin from the fifth component of human complement; DMSO, dimethyl sulfoxide; fMLP, formyl-methionyl-leucyl-phenylalanine; Gi, guanine nucleotide inhibitory protein; hFpB, human fibrinopeptide B; HPG, high power grid; LTB_4, leukotriene B_4; PDGF, platelet-derived growth factor; PMN, neutrophil.

in the upper compartment and separated from chemoattractants in the lower compartment by a 2-μm pore size (PMN), 5 μm (mononuclear cells), or 8-μm (fibroblasts) filter (Nucleopore Corp., Pleasanton, CA) overlying 0.45-μm filters (Millipore Corp., Bedford, MA). After 1 h (PMN), 2 h (mononuclear cells), or 6 h (fibroblasts) the chambers were disassembled and the membranes stained with hematoxylin. Chemotaxis was quantified by counting at high dry magnification (× 400) the number of cells per high power grid (HPG), or per high power field in the case of fibroblasts, that migrated to the interface between the membranes. Five fields were counted per experiment, and the results expressed as the mean and standard error, corrected for the number of cells migrating in controls consisting of chambers in which only medium was in the lower compartment. Experiments were performed in triplicate.

In experiments to assess the effects of anti-hFpB IgG, hFpB was incubated with anti-hFpB IgG for 1 h and then used in the chemotaxis assay in the standard manner. Control measurements were made using the same concentration of the IgG incubated with fMLP.

To assess whether hFpB utilizes receptors involved in chemotaxis to C5a, LTB_4 or fMLP, two approaches were used: (*a*) desensitization in which PMN were incubated with hFpB, 10^{-7} M, for 30 min at room temperature, washed gently once, and then tested for chemotactic responsiveness to hFpB, fMLP, LTB_4, or C5a; and (*b*) HL-60 cells were tested for chemotaxis to fMLP, LTB_4, and hFpB before and after exposure to DMSO, 1.12% vol/vol, for 96 h, conditions known to induce chemotactic responsiveness to fMLP (14).

Other assays. Cytoskeletal-associated actin was measured by the method of White et al. (21). Briefly, 1×10^7 PMN were prewarmed to 37°C before the addition of test agents. Ice-cold Triton X-100 was added at time points from 15 s to 10 min after addition of the test agents. The cell lysates were kept on ice for an additional 10 min, then centrifuged (8,000 × *g*, 4 min, 4°C) to pellet the cytoskeleton. The pellet was solubilized in 50 μl of denaturing solution and electrophoresis was performed with a 6–12% polyacrylamide gradient. After staining with Coomassie Blue, the gel was scanned for the intensity of the actin band (42 kD) (Ultrascan, model 2202; LKB Instruments, Inc., Gaithersburg, MD). PMN aggregation was performed as described by Craddock et al. (22). Accordingly, PMN, 2×10^6/ml, were stirred at 1,200 rpm at 37°C in a cuvette in an aggregometer (model 300; Chrono-Log Corp., Havertown, PA) and the change in light transmission was monitored continuously after addition of either hFpB or known PMN aggregants.

PMN lysosomal enzyme release was determined by measuring β-glucuronidase and lysozyme activities in Hank's balanced salt solution after exposure of PMN to hFpB or other agents in the presence or absence of cytochalasin B (5 μg/ml for 5 min at 37°C) (23). Release was expressed as a percentage of the total (100%) cellular enzyme activity liberated from the cells by 1% (vol/vol) Triton X-100. The generation of superoxide anion was determined by reduction of cytochrome c in the presence and absence of superoxide dismutase (24).

Results

Chemotactic activity of hFpB. As shown in Fig. 1, hFpB stimulated migration of PMN, with a peak effect occurring at 10^{-8} M. In contrast, hFpB exerted no effect upon the migration of monocytes under conditions in which monocyte responses occurred, ~100 cells per HPG at 10^{-9} M fMLP (data not shown). The stimulation of PMN migration by hFpB was chemotactic as seen by checkerboard analysis (Table I). The chemotactic activity of hFpB was blocked by anti-hFpB IgG (Fig. 2). The effect of the anti-hFpB IgG was specific for hFpB and did not involve a toxic effect upon the cells as the same concentration of antibody had no effect upon PMN chemotaxis to fMLP. The magnitude of the chemotactic response of PMN to hFpB was comparable to that achieved with C5a, LTB_4, and fMLP (Fig. 3). hFpB displayed the same without its carboxy-terminus arginine chemotactic activity as hFpB, indicating that the carboxy-terminus arginine is not crucial to the activity of the peptide (data not shown).

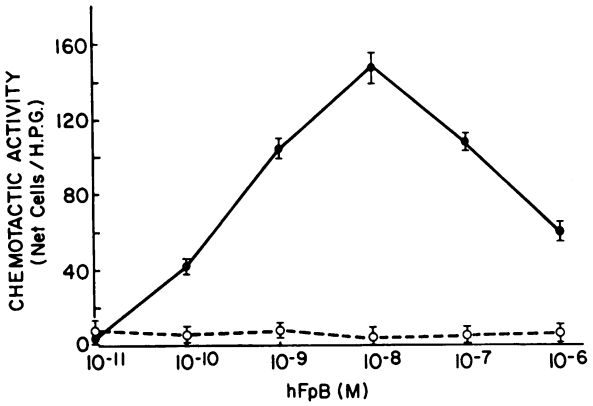

Figure 1. Migration of PMN (●) and monocytes (○) to hFpB. hFpB was added to the lower compartments of the assay chambers at the concentrations shown and the net cell migration from the upper compartments was measured as described in Methods. The error bars indicate the SEM for triplicates.

Pre-exposure of PMN to hFpB at 10^{-7} M abolished the subsequent responsiveness of the cells to hFpB, but other cells similarly exposed to hFpB retained full responsiveness to C5a, LTB_4, and fMLP (Fig. 4). Before incubation with DMSO, HL-60 cells demonstrated no chemotactic responsiveness to fMLP, LTB_4, or hFpB. After exposure to DMSO, HL-60 cells demonstrated chemotactic responsiveness to LTB_4 and fMLP, but still did not respond to hFpB (Fig. 5). Taken together, these observations indicate that PMN chemotaxis to hFpB does not involve the PMN receptors for fMLP, C5a, or LTB_4.

hFpB also exerted chemotactic activity for fibroblasts. As shown in Table II there was fibroblast migration in the direction of a concentration gradient of hFpB. The potency of hFpB was comparable to that observed with PDGF under identical test conditions (25).

Actin polymerization, aggregation, lysosomal enzyme release, and release of superoxide anion. Following exposure to hFpB, PMN demonstrated an increase in cytoskeletal-associated actin occurring within 30 s (Fig. 6 *A*). The response was not as prominent as that observed with fMLP, but it showed a clear dose response with the maximal effect at 10^{-8} M (Fig. 6 *B*).

PMN did not aggregate in response to hFpB over the concentration range 10^{-6}–10^{-10} M, although under identical con-

Table I. The Chemotactic Activity of hFpB for PMN

	Upper compartment			
Lower compartment hFPB	hFPB 0	10^{-11} M	10^{-10} M	10^{-9} M
10^{-10} M	36±5.6*	9±3.0	8±1.7	11±4.3
10^{-9} M	73±6.2	20±4.6	9±4.1	12±3.3
10^{-8} M	160±7.4	104±8.1	46±7.4	9±3.8

* Cells per HPG.

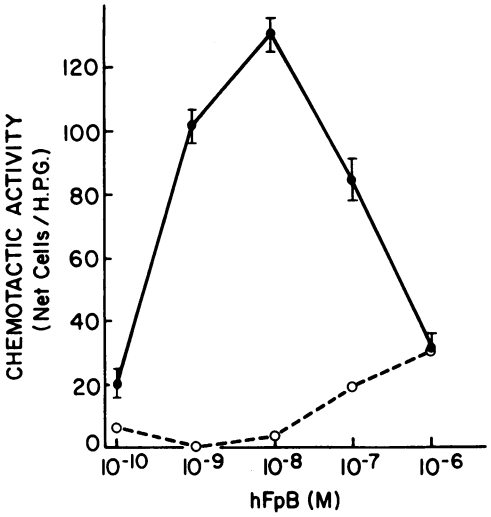

Figure 2. The effect of anti-hFpB IgG on the chemotactic activity of hFpB for PMN. hFpB (●); hFpB + anti-hFpB IgG (○). The error bars are as in Fig. 1.

ditions typical aggregation responses were observed with both C5a and fMLP.

There was no detectable production of superoxide anion after exposure of PMN to hFpB, although such production was readily observed when the cells were stimulated with fMLP (Table III). PMN which had been exposed to cytochalasin B did not release either lysozyme or β-glucuronidase in response to hFpB, even at concentrations 10-fold greater than the optimal concentration of hFpB for chemotaxis, while fMLP caused substantial release of both enzymatic activities.

Discussion

Clot formation begins with activation of the intrinsic or extrinsic coagulation pathways leading to generation of the prothrombin activation complex (prothrombinase) (26). Thrombin produced from these reactions catalyzes the conversion of fibrinogen to fibrin and stimulates platelet activation and the platelet release reaction. Coincident with fibrin formation and platelet activation, a variety of chemotactic and growth promotion substances are released, including complement fragments (27, 28), proteases (29), and platelet proteins such as platelet factor 4 and PDGF (19, 25, 30). Once formed, the fibrin clot undergoes remodeling by proteolysis, notably through the actions of plasmin (31) and PMN elastase (32). Thus, substances released during clot formation, platelet activation, and clot resolution have the capacity to recruit inflammatory cells and other cell types including fibroblasts, and also to exert mitogenic activity. This indicates that fibrin clot formation should be viewed as a dynamic process that involves not only hemostatic functions but also as one that generates a number of substances that are known to play important roles in inflammation and wound healing (Fig. 7).

Possibly even more important than the release of biologically active substances from the site of fibrin formation is the fact that fibrin clots have the potential to hold active compounds in the fluid phase of the fibrin gel and shield them from inhibitors and metabolic turnover. Several groups of investigators have

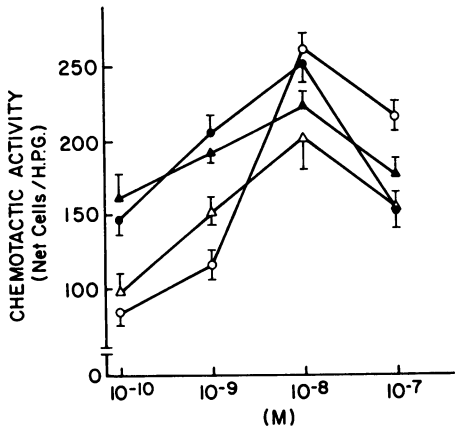

Figure 3. Comparison of the PMN chemotactic activity dose-response curve of hFpB (●) with the dose response curves of C5a (○), LTB_4 (▲), and fMLP (△). The error bars are as in Fig. 1.

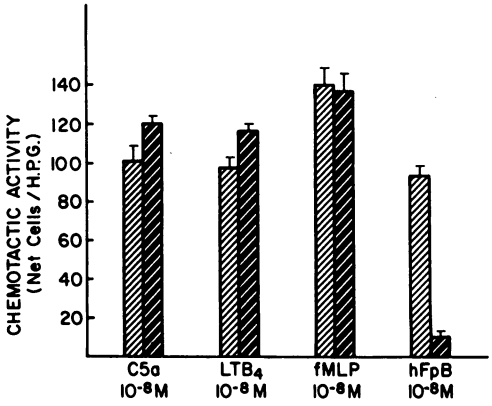

Figure 4. PMN chemotaxis to C5a, LTB_4, fMLP, and hFpB after preincubating the cells with hFpB, 10^{-7} M (■), or with media only (■), for 30 min at room temperature. The error bars are as in Fig. 1.

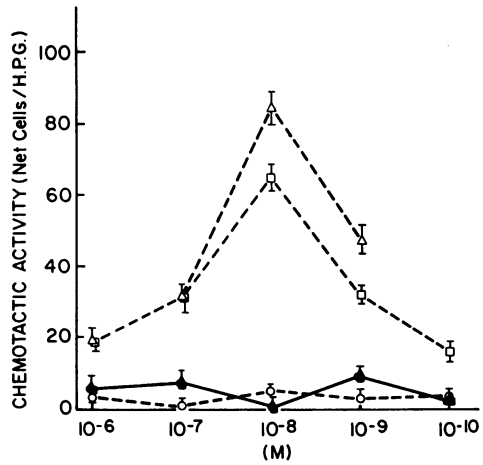

Figure 5. The effects of DMSO upon the chemotactic responsiveness of HL-60 cells to fMLP, LTB_4, and hFpB. See Methods for details of the culture conditions. Responses of the cells before incubation with DMSO are shown in the solid symbols. Responses of the cells after culture with DMSO: fMLP (△); LTB_4 (□); and hFpB (○). The error bars are as in Fig. 1.

Table II. The Chemotactic Activity of hFpB for Fibroblasts

Lower compartment hFPB	Upper compartment hFPB 0	10^{-11} M	10^{-10} M	10^{-9} M
10^{-11} M	21*±1.4	0±1.2	8±2.9	0±1.3
10^{-10} M	36±2.7	9±2.0	4±2.0	0±1.9
10^{-9} M	59±2.7	42±2.4	15±2.1	1±1.1

* Cells per high power field.

demonstrated that substantial quantities of active thrombin can become sequestered within the fibrin gel (33–35). This is noteworthy because active thrombin is a potent stimulator and growth factor for fibroblasts and other mesenchymal cell types (36, 37), and both active and degraded thrombin forms have chemotactic and growth factor activities for cells of monocyte/macrophage lineage (38, 39). Thus, it is possible that a microenvironment of many mediators can be established within the fluid phase of fibrin clots from which many biological activities such as chemotaxis and growth stimulation can be expressed.

This report demonstrates that hFpB, a peptide released from the B β-chains of fibrinogen and contained within the principal plasmin cleavage product of fibrin, may exert potent effects upon

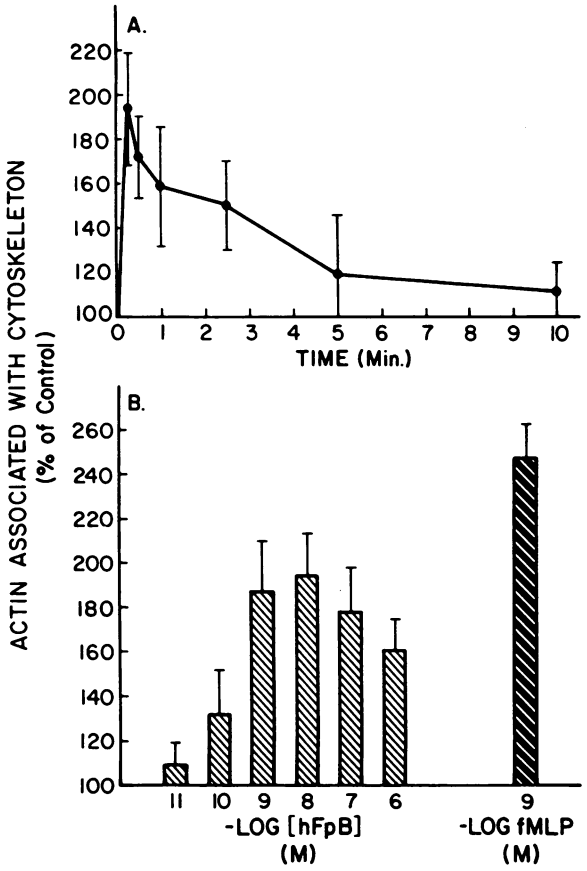

Figure 6. Effect of hFpB upon the quantity of PMN actin associated with the PMN cytoskeleton. (*A*) Time course following addition of hFpB. (*B*) Dose-response effect of hFpB. The maximal response achieved with fMLP is shown for reference. Each point is the mean of five experiments±SEM.

Table III. Superoxide Anion Production and Lysozyme and β-Glucuronidase Release in Response to hFpB and fMLP

	Superoxide anion	Lysozyme§	β-Glucuronidase§
	*nmol/10^7 cells**	*% released*	*% released*
hFpB (*M*)			
10^{-5}	27.1±5.9		
10^{-6}	26.7±5.4		
10^{-7}	24.1±4.9	0.5	4.1
10^{-8}	21.4±3.7		
None‡	21.2±7.6		
fMLP (*M*)			
10^{-6}	59.2±14.8		
10^{-7}	56.9±14.4	71.5	35.5

* = ±1 SD; *n* = 8 separate determinations in three different experiments.
‡ Cells only.
§ Cells were pre-exposed to cytochalasin B, 5 μg/ml, for 5 min at 37°C; in the absence of cytochalasin B release was only trace; *n* = mean of four separate determinations.

migration of PMN and fibroblasts. These results confirm findings of Kay et al. (11). This study has extended that earlier work by showing that hFpB also possesses potent chemotactic activity relative to C5a, LTB_4, and fMLP, and that this high degree of hFpB chemotactic activity occurs in the same concentration range, $\sim 10^{-8}$ M, as that found with these other well defined PMN chemoattractants (12–14).

The present results further demonstrate that the chemotactic activity of hFpB extends to fibroblasts. In this respect, hFpB has activity similar to several platelet alpha granule proteins (30), extracellular matrix components including fibronectin (40), collagen (41) and elastin peptides (20), factors from lymphocytes (42), and from C5 (43). This finding suggests that hFpB, and possibly other fibrin degradation products, contribute to mesenchymal cell recruitment at sites of inflammation and injury.

Two compelling pieces of information indicate that PMN chemotaxis to hFpB does not involve receptors involved in PMN responses to C5a (12), LTB_4 (13), or fMLP (14). First, desensitization of PMN with hFpB abolished the response of the cells to hFpB without affecting their chemotactic responses to these

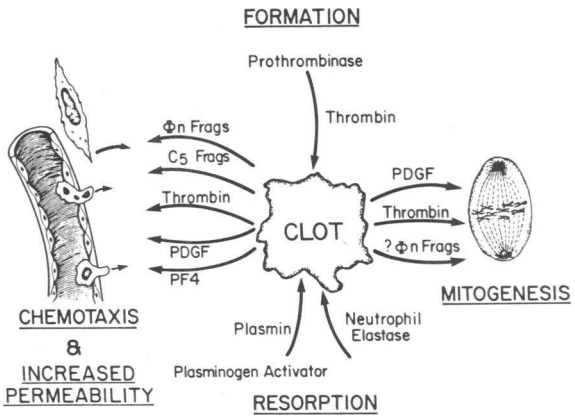

Figure 7. Schematic concept of how fibrin clot formation and dissolution influence inflammation and wound healing. φn represents fibrinogen. Frags refers to fragments.

other chemotactic factors. The fact that desensitization to chemotactic factors is factor-specific has been shown by others (44, 45). Second, HL-60 cells, upon exposure to DMSO, were found to develop chemotactic responsiveness to two chemotactic factors, fMLP and LTB_4, but not to hFpB. Apart from providing further evidence that chemotaxis to hFpB is not mediated by receptors for either fMLP or LTB_4, these data suggest that PMN do not acquire responsiveness to all potential chemoattractants simultaneously during development.

Kay et al. reported that hFpB is chemotactic for monocytes (10). This was not confirmed in the present experiments. There is no obvious explanation for the discrepancy, but it may be noted that chemotactic activity for one type of leukocyte is not necessarily shared by other types of leukocytes. For example, C5a without its carboxy-terminus arginine is as active as C5a for monocytes but much weaker than C5a for PMN (46). Also, thrombin, which is chemotactic for monocytes, is inactive against PMN (47).

A most interesting finding about the effect of hFpB upon PMN is its capacity to cause actin polymerization and to elicit a potent chemotactic response without causing aggregation, the release of lysosomal enzymes, or the production of superoxide anion. In this respect, hFpB contrasts with C5a, fMLP, or LTB_4 and resembles instead a factor generated from plasma by superoxide (48) and the synthetic tripeptide, gly-his-gly (49), which also causes PMN chemotaxis without causing PMN secretory responses.

That PMN chemotactic factors do not always cause lysosomal enzyme release has been supported by other evidence. Snyderman and colleagues have shown that exposure of PMN to certain aliphatic alcohols enhances chemotactic responses while depressing specific granule secretion (50). An interesting example of dissociation of enzyme secretion and chemotaxis was found with equine PMN (51). These cells were observed to bind fMLP and secrete lysosomal enzymes in response to fMLP but failed to show chemotaxis to fMLP.

Not only are chemotaxis and lysosomal enzyme secretion separable in response to some agents, but enzyme release and superoxide production have also been dissociated in studies of rat PMN (52). Moreover, even with the same PMN response, superoxide anion production, multiple pathways appear to exist (53). One must conclude that (*a*) the patterns of PMN responses triggered by C5a or fMLP should not be regarded as applicable to all PMN chemotactic factors and (*b*) factors may cause the same responses in PMN but do not necessarily do so by the same mechanisms.

Recently, there have been important insights into the signal transduction of PMN chemotactic factors. fMLP has been the prototype chemotactic agent in these studies. A role has been proposed for a guanine nucleotide Gi-binding protein in mediating fMLP signal transduction as pertussis toxin, an exotoxin purified from *Bordetella pertussis* which ribosylates the Gi protein and inhibits fMLP-mediated PMN chemotaxis (54–57). The toxin also inhibits superoxide production and lysosomal enzyme release, suggesting a common signal pathway between all of these PMN responses to fMLP. The failure of hFpB to cause either the production of superoxide anion or the release of lysosomal enzymes, although acting as a potent chemoattractant, suggests that hFpB's mode of action may involve a transduction pathway that is different from that described for chemotaxis caused by fMLP. Elucidation of the mechanisms by which hFpB exerts its chemotactic activity may yield new information about PMN function.

Irrespective of the precise way in which hFpB operates as a PMN chemoattractant, the present data indicate that this fibrinopeptide, which is released in abundance during insoluble fibrin formation, has potent activity to recruit both PMN and fibroblasts. Thus, these data support other studies showing that peptides released during fibrinogen turnover should be viewed as having potential influences extending beyond hemostasis.

Acknowledgments

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