Fibrinogen-Derived Peptide Bβ1-42 Is a Multidomained Neutrophil Chemoattractant

By William F. Skogen, Robert M. Senior, Gail L. Griffin, and George D. Wilner

The formation and degradation of fibrin play a central role in hemostasis, but other activities have been associated with fibrinogen-derived peptides, which suggests that products of fibrinogen turnover may be involved in inflammation and wound healing. The present study was undertaken to determine whether the plasmic fibrinogen-derived peptide Bβ1-42 has effects on inflammatory cells and fibroblasts (FB). Bβ1-42 was found to be a potent chemotaxin for neutrophils (PMN) and FB, maximally stimulating PMN migration at 10^{-7} mol/L peptide. Unlike the chemotactic factors f-Met-Leu-Phe and C5a, Bβ1-42 did not release the lysisosomal hydrolases and superoxide anion from PMN, nor did it stimulate directed movement of monocytes (MN). These features of Bβ1-42 resemble the properties of human fibrinopeptide B (hFpB), the 14-residue, thrombin-cleaveable fragment that constitutes the amino terminus of Bβ1-42, and suggested that the chemoattractant effects of Bβ1-42 are mediated through its hFpB domain. Against this conclusion, however, were observations that (a) desensitization of PMN with 10^{-7} mol/L hFpB ablated chemotaxis to Bβ1-42; (b) antiserum to hFpB, which recognizes the Bβ1-14 sequence both free and bound to larger fragments of the Bβ chain, blocked hFpB chemotactic activity but did not affect Bβ1-42-mediated chemotaxis; (c) desensitization of PMN with equimolar amounts of hFpB and β15-42 (10^{-7} mol/L), the isolated carboxyterminal sequence of Bβ1-42 remaining after the removal of hFpB, completely inhibited Bβ1-42-mediated chemotaxis; and (d) β15-42 itself was chemotactic for PMN. These data indicate that PMN recognize several independent domains within the amino terminal region of the human fibrinogen Bβ chain and that these biologic effects extend to mesenchymal cells.

MATERIALS AND METHODS

Materials

Reagents. The following were obtained from the sources listed: acrylamide, sodium dodecyl sulfate, and other gel electrophoretic reagents from Bio-Rad, Richmond, CA; human fibrinogen (Kabi, grade L) from Helena Laboratories, Jamaica, NY; hFpB from Bachem, Torrance, CA; Hanks’ balanced salt solution (HBSS) from GIBCO, Grand Island, NY; and cytochalasin B, cytochrome C, f-Met-Leu-Phe-OH (fMLP), n-p-nitrophenyl-b-D-glucuronide, Triton X-100, and superoxide dismutase from Sigma Chemical Co, St Louis. PDGF was a gift from Thomas F. Deuel, Jewish Hospital at Washington University Medical Center, St Louis. PDGF was a gift from Thomas F. Deuel, Jewish Hospital at Washington University Medical Center, St Louis.

Cells. PMN and monocytes (MN) were separated by the Ficoll-Hypaque gradient method of Boyum from peripheral blood samples obtained from healthy volunteers. FB (bovine ligamentum nuchae fibroblasts) were derived from explants of the fetal tissue as described by Mecham et al. The FB were maintained in culture by using Dulbecco’s minimal essential medium and used between the first and fourth passages.

Peptides Bβ1-42 and β15-42. Peptide Bβ1-42 was isolated from plasmic digests of human fibrinogen by preparative reverse-phase, high-performance liquid chromatography (HPLC) as previously described. This antiserum reacts both to free and bound hFpB. Against this conclusion, however, were observations that (a) desensitization of PMN with 10^{-7} mol/L hFpB ablated chemotaxis to Bβ1-42; (b) antiserum to hFpB, which recognizes the Bβ1-14 sequence both free and bound to larger fragments of the Bβ chain, blocked hFpB chemotactic activity but did not affect Bβ1-42-mediated chemotaxis; (c) desensitization of PMN with equimolar amounts of hFpB and β15-42 (10^{-7} mol/L), the isolated carboxyterminal sequence of Bβ1-42 remaining after the removal of hFpB, completely inhibited Bβ1-42-mediated chemotaxis; and (d) β15-42 itself was chemotactic for PMN. These data indicate that PMN recognize several independent domains within the amino terminal region of the human fibrinogen Bβ chain and that these biologic effects extend to mesenchymal cells.

Methods

Chemotaxis. Chemotactic assays were carried out by using two-filter methodology in modified Boyden chambers as previously described.

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chemoattractants in the lower compartment by 2-µm pore size (PMN), 5-µm (MN), or 8-µm (FB) filters (Nucleopore Corp., Pleasanton, CA) overlaying 0.45-µm filters (Millipore, Bedford, MA). After one (PMN), two (MN), or six hours (FB) at 37°C in 5% CO2/95% air, the chambers were disassembled and the membrane pairs stained with hematoxylin. The number of cells per high-power grid (HPG), or per high-power field (HPF) in the case of FB, that migrated to the interface between the membranes was determined by counting at high dry magnification (×400). Five fields were counted for each experiment. The results, expressed as the mean and SEM, were corrected for the number of cells migrating in control experiments in which only medium was in the lower compartment. Experiments were performed in triplicate at least twice.

To demonstrate the effect of the specific antiserum R22 on Bß1-42-mediated PMN chemotaxis, Bß1-42 was preincubated with either the specific IgG fraction or IgG from nonimmune rabbit serum for one hour before testing the mixture for residual chemotactic activity. Control experiments were conducted by using the same concentrations of specific and nonimmune IgG incubated with fMLP.

To assess whether Bß1-42 used receptors involved in chemotaxis to other peptides, desensitization experiments were carried out. Accordingly, PMN were incubated with various peptides at the concentrations stated for 30 minutes at room temperature, washed three times, and then tested for chemotactic responsiveness.

Other assays. Cytoskeletal-associated actin was measured by the method of White et al. Briefly, 1 x 106 PMN were prewarmed at 37°C before the addition of test agents. Ice-cold Triton X-100 was added at time points from 15 seconds to ten minutes after addition of the test agents. The cell lysates were kept on ice for an additional ten minutes and then centrifuged (8,000 g, four minutes, 4°C) to pellet the cytoskeleton. The pellet was solubilized in 50 µL of denaturing solution, and electrophoresis was performed with a 6% to 12% polyacrylamide gradient. After staining with Coomassie blue, the gel was scanned for the intensity of the actin band (42 kd) (LKB Ultrascan, model 2202), Pharmacia-LKB, Piscataway, NJ.

PMN lysosomal enzyme release was determined by measuring β-glucuronidase and lysozyme activities in HBSS after exposure of PMN to hFpB or other agents in the presence or absence of cytochalasin B (5 µg/mL for five minutes at 37°C). Release was expressed as the 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.

RESULTS

Chemotactic Activity of Bß1-42

Bß1-42 was a potent stimulant of PMN movement over a concentration range of 10⁻⁷ through 10⁻¹¹ mol/L (Fig 1). Maximal stimulation of PMN migration occurred at 10⁻⁹ mol/L peptide. On a molar basis, the potency of Bß1-42 as a neutrophil chemotaxin is equivalent to the chemotactic peptide fMLP. Similarly, FB appeared responsive to this peptide, although the level required for maximal stimulation of movement in these cells is higher than that required for PMN; however, the FB responses were comparable to those elicited with PGDF. In contrast, MN did not show significant chemotactic responsiveness of Bß1-42 (Fig 1) under identical conditions where these cells were responsive to fMLP (data not shown). PMN migration inducible by Bß1-42 is dependent on a concentration gradient, as indicated by “checkerboard” analysis (Table 1), and therefore represents a chemotactic rather than a chemokinetic response.

Effects of Bß1-42 on PMN Cytoskeleton.

After exposure of PMN to Bß1-42, a time- and concentration-dependent increase in cytoskeletal-associated actin was observed. The increase is maximal within 30 seconds and returns to baseline levels within five to ten minutes after stimulation of the cells with peptide (Fig 2A). A dose-response analysis demonstrated that a maximal increase in cytoskeletal actin association occurs at 10⁻⁷ mol/L (Fig 2B). The effect seen is comparable to that observable when using fMLP when the latter is tested at its optimal concentration (data not shown).

There was no detectable production of superoxide anion after exposure of PMN to Bß1-42, although such production was readily observed when the cells are stimulated with fMLP. PMN that had been exposed to cytochalasin B did not release either lysozyme or β-glucuronidase in response to Bß1-42, even at concentrations tenfold greater than the optimal concentration of Bß1-42 for chemotaxis, whereas fMLP caused substantial release of both enzymatic activities (Table 2).

Further Characterization of the PMN Fibrinogen Amino Terminal Bß Chain Chemotactic Domain

Because the amino terminal, 14-residue sequence of Bß1-42 is hFpB, a potent PMN chemoattractant, it was impor-
Bβ1-42 CHEMOTACTIC ACTIVITY

Figure 2. The effect of Bβ1-42 (10^{-3} mol/L) on the cytoskeletal association of PMN actin. Top, time course; bottom, dose response.

Figure 3. The effect of preaddition of the anti-hFpB IgG fraction on the chemotactic activities of Bβ1-42 or hFpB. The error bars are as in Fig 1.

(Fig 5). Thus, we conclude that the 1-14 and 15-42 sequences each contribute independently to the chemotactic activity of fragment Bβ1-42.

DISCUSSION

Studies of plasmic derivatives of fibrin and fibrinogen have centered on their importance as markers for proteolytic events associated with diverse thrombotic disorders; however, these derivatives have intrinsic biologic or pharmacological activities that include potent stimulation of leukocyte migration, suppression of in vitro mitogenic effects in lymphoid cells, enhanced vasoactive effects, and endothelial cell injury. These effects are, for the most part, mediated by derivatives of the amino terminal 50 residues of the fibrinogen Bβ chain.

Fragment Bβ1-42 represents one of the earliest cleavage products resulting from the action of plasmin on fibrinogen. When using sensitive and specific immune reagents, this peptide may be measured independently of its parent peptide.

Table 2. Lysozyme and β-Glucuronidase Release in Response to Bβ1-42

<table>
<thead>
<tr>
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<th>Lysozyme (%)*</th>
<th>β-Glucuronidase (%)</th>
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<tbody>
<tr>
<td>Bβ1-42</td>
<td>1.7</td>
<td>5.9</td>
</tr>
<tr>
<td>fMLP</td>
<td>68.1</td>
<td>35.2</td>
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*Cells were preexposed to cytochalasin B, 5 μg/mL, for five minutes at 37°C; hFpB and fMLP were used at 10^{-3} and 2 x 10^{-7} mol/L for lysozyme and β-glucuronidase, respectively. In the absence of cytochalasin B, release was only a trace; n = mean of three separate determinations.
protein or larger proteolytic fragments containing this sequence. The median plasma level in normal individuals is 1.2 nmol/L, and elevated levels are noted in patients with various thrombotic disorders. Concentrations of this peptide are, of course, likely to be much higher at sites of inflammation and wound healing. Once released, Bβ1-42 potentially may undergo secondary cleavage by thrombin to peptides corresponding to hFpB (Bβ1-14) and β15-42, but as recently reported by Weitz et al., secondary cleavage occurs infrequently. Thus, Bβ1-42 circulates as a relatively stable proteolysis product of either fibrinogen or fibrin I, whereas the main source of β15-42 is likely to be from plasmin action on fibrin II.

In the present study, we demonstrate that Bβ1-42 is a potent chemotaxin for PMN and FB, analogous to the responses produced with hFpB. Also, as with hFpB, Bβ1-42 exerts its chemotactic effect on PMN without stimulating the release of lysosomal enzymes or superoxide anion. In addition, neither Bβ1-42 nor hFpB elicit MN chemotaxis. Thus, both of these Bβ aminoterminal fibrinogen derivatives differ from the well-characterized chemotactic agents C5a and fMLP, which cause release of lysosomal hydrolases and superoxide anion and stimulate MN as well PMN.

From the similarity of effects mediated by Bβ1-42 and hFpB, one might assume that (a) the chemotactic activity of Bβ1-42 is simply a function of its Bβ1-14 (ie, hFpB) domain and (b) the chemotactic activities of both peptides are mediated through the same domain and involve the same receptors on the neutrophil cell surface. PMN responsiveness to Bβ1-42 persists, however, when the chemotactic assay to this peptide is carried out in the presence of immune globulin specific for the Bβ1-14 (hFpB) sequence, although this antibody is effective in blocking PMN responses to hFpB. Moreover, preincubation of PMN with hFpB desensitizes the cells to hFpB but not to Bβ1-42-mediated chemotaxis. Similarly, peptide β15-42 is chemotactic for PMN (Fig 5) but alone is incapable of completely desensitizing PMN to Bβ1-42; however, a mixture of hFpB and β15-42 will completely desensitize PMN to Bβ1-42 (Fig 4), thus providing proof for two separate independent chemotactic domains within this plasmic fragment. The implications of these findings are that proteolysis of fibrinogen Bβ chain by thrombin and plasmin, either singly or in concert, releases potent chemotactic fragments capable of mobilizing PMNs to the sites where these enzymes are active.

In conclusion, many factors liberated by cells involved in hemostasis and wound repair have now been found to have the capacity to affect wound repair by stimulating cell division and migration of inflammatory and mesenchymal cells. The present observation that the amino terminal plasmic derivatives of the fibrinogen Bβ chain elicit chemotactic movement of human PMN through multiple domains lends additional support to the concept that fibrinogen turnover can be a source of diverse biologic effects other than the ones related to hemostasis. Potentially, this gains increasing importance in view of our recognition that inflammatory cells express enzymes that can generate fibrinogen turnover products and these products, in turn, may modulate wound repair.

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