The Amino-Terminal 29- and 72-Kd Fragments of Fibronectin Mediate Selective Monocyte Recruitment


Proteolytic fragments of fibronectin (Fn) can possess properties not inherent to intact Fn. Previously, only mixtures of low molecular weight Fn fragments, and the 120-Kd fibroblastic cell-binding segment, but not intact Fn, were shown to be selectively chemotactic for human monocytes (MOs). In order to determine if other structural domains of Fn were responsible, we tested six Fn fragments. The amino-terminal 72-Kd fragment at 1.5 μm was about 75% as potent as zymosan-activated serum (ZAS). Its aminoterminal 29-Kd degradation product at 1.0 μm was about one third as potent as ZAS. Checkerboard analysis confirmed chemotaxis. Complexing gelatin to 72-Kd fragments reduced MO chemotaxis by 28% to 30%. Reducing disulfide bonds in 29- and 72-Kd segments had no effect. A synthetic peptide containing the thrombin cleavage site between the 29- and 50-Kd segments of the 72-Kd fragment was chemotactic. The 50-, 190/170-, 35-, and 160/150/120-Kd fragments, and intact Fn were not chemotactic for MOs. The data suggest that the 72-Kd fragment and its 29-Kd subfragment are additional Fn fragments that mediate selective MO chemotaxis. We speculate that proteases present at inflammatory sites can liberate such fragments that selectively recruit MOs.

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FIBRONECTIN (Fn) is present on cell surfaces and in extracellular matrices, extravascular fluids, and plasma. This family of high molecular weight glycoproteins has multiple adhesive functions, such as the ability to bind collagen, heparin (Hep), DNA, bacteria, and most cells. Its multiple biologic functions include cell spreading, adhesion, and motility,1-3 wound healing,4 and mononuclear phagocyte clearance.5,6 Limited proteolysis of Fn in vitro yields fragments that possess one or more of these biologic functions. Many of these properties have been localized to certain domains of intact Fn by studying preparations of such liberated fragments. However, these fragments often have additional biologic properties not present in intact Fn.8-16 For example, the 29- and 35-Kd fragments are potent inhibitors of endothelial cell growth in vitro, whereas native Fn is not.8,9 Recently, Fn was shown to mediate chemoattractant-stimulated polymorphonuclear neutrophil (PMN) adherence,12 and to facilitate hyaluronic acid-mediated PMN migration.13 Neither intact Fn at physiologic concentrations nor mixtures of Fn fragments obtained by proteolytic degradation of Fn were chemotactic for PMN.3,14 However, these same mixtures, but not intact Fn, were chemotactic for human monocytes (MOs), suggesting that proteolytic cleavage of Fn during inflammation produced fragments that selectively recruited MOs. Clark et al reported that the 120-Kd fibroblastic cell-binding fragment was chemotactic for human MOs.15 Previously we reported that the amino-terminal 29-Kd fragment of Fn also mediated MO chemotaxis.16 In this report, we identify the amino-terminal 72-Kd fragment of plasma Fn, its amino-terminal 29-Kd subfragment, and a related synthetic Fn fragment as additional selective chemoattractants for human peripheral blood MOs.

MATERIALS AND METHODS

Chemicals. All common chemicals and reagents were from Fisher Chemical (Pittsburgh, PA). All electrophoretic supplies were from Bio-Rad Laboratories (Richmond, CA). Cathepsin D, Hep, pepstatin, proteinase inhibitors, and conventional chromatography resins were from Sigma Chemical (St. Louis, MO). The tetrapeptide Arg-Gly-Asp-Ser was purchased from Peninsula Laboratories (Belmont, CA). Zymosan-activated human serum (ZAS) was prepared as previously described.17 Murine monoclonal antibodies to Fn and to Fn fragments were a generous gift of Dr. David Hasty (University of Tennessee, Memphis).

Plasma fibronectin and fibronectin fragment preparation. Fragments of Fn were generated from cathepsin D and thrombin digests of human plasma Fn, as described previously.8,9,15 The ligand binding properties and activities of these fragments were demonstrated previously.8,9,16 The following fragments, listed in order of their placement on the Fn subunit from the amino- to carboxy-terminal, were the amino terminal Hep-binding (Hep I) 29-Kd, collagen-binding 50-Kd, central fibroblast-binding and Hep-binding (Hep II) 160/150/120-Kd, and carboxy-terminal high Hep-affinity (Hep I) 35-Kd fragments.18 Larger multidomain segments included the amino-terminal 72-Kd fragment containing the amino-terminal 29-Kd and collagen-binding 50-Kd subfragments, and the 190/170-Kd fragment that lacks both the 29-Kd segment and the carboxy-terminal interchain disulfide region. Methods for generation of Fn fragments, which are reported elsewhere,6,5,18 are briefly summarized here.

In order to generate 72-Kd, 160/150/120-Kd, and 35-Kd fragments, cathepsin D at 5 μg/mL was added to 200 mL of plasma Fn at 1 mg/mL in 0.15 mol/L NaCl and 0.1 mol/L formate buffer, pH 3.7. Digestion at 30°C was monitored on 10% Laemmli sodium dodecyl sulfate (SDS) gels. After 3 hours, the digestion was quenched by addition of pepstatin (final concentration 1 μg/mL), and the solution applied to a 200-mL gelatin-Sepharose column equilibrated in 100 mmol/L phosphate buffer, pH 7. A 72-Kd

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Supported by Grant EY 05735 from the National Eye Institute and by VA Medical Research Funds.


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Blood, Vol 76, No 10 (November 15), 1990: pp 2117-2124

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fragment containing the amino-terminal 29-Kd segment and the 50-Kd gelatin-binding domain bound and was eluted with 3 mol/L urea with 0.1 mol/L phosphate, containing 1 U/mL Trasylol. This 72-Kd fragment contained a blocked amino terminus, and therefore is an amino-terminal fragment as shown previously. The nonadherent material contained a mixture of 160/150/120-Kd, 35-Kd, and smaller fragments. (Prolonged digestion produced mostly 120-Kd, 43-Kd, 35-Kd, and 20-Kd fragments). The nonadherent mixture was applied to a Sephacryl S-200 column in 100 mmol/L phosphate buffer, pH 7. The early peak contained a mixture of 160/150/120-Kd fragments which were not purified further for chemotaxis. These 160/150/120-Kd mixtures were earlier shown to be separable based on the affinity of the 150-Kd fragment for Hep, and the lack of Hep affinity of the 120-Kd fragment. The later peaks, which contained the 35-Kd and smaller fragments, were combined and applied to a Hep-Sepharose column equilibrated in 50 mmol/L NaCl with 50 mmol/L Tris, pH 7. The 35-Kd fragment bound and was eluted by addition of 50 mmol/L NaCl with 50 mmol/L Tris, pH 7. This fragment has been characterized and shown to contain the Hep II binding domain, by sulfhydryl content, amino-terminal sequence analysis, and affinity toward Hep. After the 72-Kd gelatin-binding fragment was dialyzed to remove Trasylol, thrombin (final concentration 1 U/mL) was added to generate the 29-Kd amino-terminal and 50-Kd gelatin-binding fragments. Digestion was monitored on 10% Laemmli SDS gels. After up to 2 days, the digest was applied to a gelatin-Sepharose column. The bound 50-Kd gelatin-binding fragment was eluted by addition of 3 mol/L urea with 0.1 mol/L phosphate buffer containing 1 U/mL Trasylol. The recovered 29-Kd fragment was previously shown to be an amino-terminal fragment by the presence of an amino-terminal blocked residue, disulfide content, and affinity toward Hep and fibrin. The 50-Kd fragment was previously shown to begin with residue 260 in the native sequence. After Fn was dialyzed to remove Trasylol, thrombin (final concentration 0.5 U/mL) was added to Fn in 0.1 mol/L phosphate buffer, pH 7, in order to generate the 190/170-Kd fragments. Digestion was monitored on 4% Laemmli SDS gels. After the native Fn had been converted to 190/170-Kd fragments, Trasylol (2 U/mL) was added to inhibit thrombin. The digest was applied to a gelatin-Sepharose column to isolate only gelatin-adherent material containing the 50-Kd gelatin-binding segment. Unbound material contained mostly the amino-terminal 29-Kd fragment, which was recovered and purified further by Sephacryl S-200 chromatography. Gelatin-adherent material was eluted with 3 mol/L Tris with 0.1 mol/L phosphate buffer, containing 1 U/mL Trasylol. The bound material was dialyzed against 50 mmol/L Tris with 0.1 mmol/L NaCl, pH 7, and applied to a Hep-Sepharose column. Hep-bound material was eluted with 500 mmol/L NaCl with 50 mmol/L Tris, pH 7, containing 1 U/mL Trasylol. Finally, the material was applied to a Sephacryl S-200 column to remove any lower molecular weight material. With this procedure, the isolated 190/170-Kd fragments were defined as those that retained the gelatin-binding and the Hep II domains. Sequence analysis confirmed that these fragments began with residue 260 of the native sequence, as does the 50-Kd segment. Therefore, these fragments lacked the 29-Kd segment but continued through the Hep II binding domain as shown. All preparations used in this study were stored at -70°C and derived from stock preparations of these previously characterized, homogeneous fragments (Fig 1), shown previously to possess biologic activity for binding the respective ligand(s). Samples (50 μg) were applied in a 5% to 20% Laemmli linear gradient acrylamide gel. The concentrations of Fn and its fragments were established from absorbance at 280 nm, using the extinction coefficients for 1 mg/mL solutions. Aprotinin (1 U/mL), EDTA (1 mmol/L), phenylmethylsulfonylfluoride (1 mmol/L), and Trasylol (1-2 U/mL) were re-moved by dialysis using Spectropor (molecular weight cutoff 12,000 to 14,000, Scientific Products, McGaw Hill, IL) just before use in chemotaxis experiments. Fragments stored at -70°C did not show additional proteolysis for up to 2 years (Homandberg, unpublished data, May 1989).

**Preparation of synthetic fibronectin fragments.** Peptides were synthesized by a solid-phase method using an Applied Biosystems Automatic Peptide synthesizer (Foster City, CA), and purified by gel filtration and reverse phase high-performance liquid chromatography. Amino acid composition and sequence were confirmed using an Applied Biosystems Automatic Amino Acid Analyzer and Sequencer. K1 (residues 240 to 265) and K2 (residues 253 to 276) are located on the amino-terminal side of the thrombin cleavage site between the 29- and 50-Kd segments of the 72-Kd fragment. K3 (residues 277 to 300) contains the cleavage site.

**Cell preparations.** Peripheral blood cells were isolated from heparinized venous blood of healthy volunteers by Dextran T-500 sedimentation, followed by Ficoll-Hypaque density separation (Pharmacia Fine Chemicals, Piscataway, NJ). The protocol was approved by the Institutional Review Board, University of Tennessee, Memphis, in accord with an approved assurance filed with the Department of Health and Human Services. Mononuclear cells (about 25% MOs) at the interface were washed and resuspended at 1.5 x 10^6 MOs/mL in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 2% bovine serum albumin (BSA, Sigma Chemical Co), pH 7.2. PMNs (>98%) in the pellet were freed of erythrocytes by hypotonic saline lysis, washed, and resuspended at 2.2 x 10^6 PMNs/mL in the same medium, pH 7.4.

**Chemotaxis assays.** For MO chemotaxis, a 5.0-μm pore size polyvinylpyrrolidone-treated polycarbonate filter separated the mi-
crowells of the chemotaxis chamber (Neuro Probe, Bethesda, MD). The top wells received 50 μL of the cell suspension, and the bottom wells 30 μL of test suspension. After chambers were incubated for 90 minutes at 37°C in humidified air, filters were removed, fixed, and stained.17 Chemotactic activity was recorded as the mean number of MOs per field (1,000 x SEM) that migrated to the lower surface of triplicate filters in five microscopic fields. The SEM was <15% of the mean in each experiment. Because of day-to-day variability in MO response, these data were calculated for statistical comparisons as percent relative migration, with the response to ZAS for each donor normalized to 100% by the following formula:

\[
\text{% Migration} = \frac{\text{Experimental} - \text{negative control}}{\text{Positive control} - \text{negative control}} \times 100,
\]

where negative control was medium and positive control was ZAS. For experiments testing chemotactic activity of synthetic Fn fragments for human MOs, blindwell chambers were used, using MOs at 4.5 × 10^6/mL and 3-μm pore size polycarbonate filters. Data were expressed as the chemotactic index, defined as the quotient of MO migration toward an experimental sample divided by MO migration toward medium.11

For some experiments, complexes of 72-Kd fragments with gelatin were made by first heating to 60°C type I porcine gelatin at 5 mg/mL with 100 mmol/L NaCl, pH 7.0. This gelatin (5 mg/mL) was added to a 72-Kd solution at 1 mg/mL, equivalent to a 1:1 ratio of components. This stable complex was used without further purification. To generate reduced and alkylated fragments, solutions of 29- or 72-Kd Fn fragments at 1 mg/mL in 7 mol/L guanidine HCl with 0.1 mol/L borate buffer, pH 8.0, were reduced with 0.1 mol/L dithiothreitol at 37°C, and reacted with 0.12 mol/L iodoacetate for 30 minutes at 37°C. The solution was then dialyzed against phosphate-buffered saline. These reduced and alkylated fragments were added to the solutions used to fill the bottom wells. In some experiments, Hep (final concentration 6 U/μL) was added to the solutions used to fill the bottom wells. PMN chemotaxis was quantified by the leading front technique,11 defined as the maximum distance (in μm) that two or more cells migrated into the filter in five microscopic fields (400×). A 5.0-μm pore size Polyscience nitrocellulose filter (Neuro Probe) was used, and chambers were incubated for 35 minutes. Chemotactic activity was recorded as the mean distance ± SEM of triplicate samples. The SEM was <15% of the mean.

**Enzyme-linked immunosorbent assay (ELISA).** Polystyrene 96-well plates (16 mm²) were coated overnight with the 160/150/120-Kd cell-binding fragment and blocked with 5% BSA. The 29-, 72-, or 160/150/120-Kd Fn fragments were incubated with 1:5,000 to 1:60,000 dilutions of ascitic fluid containing a monoclonal antibody specific for the 110-Kd cell-binding domain fragment22 for 90 minutes at 37°C. Samples were added to the wells and incubated for 2 hours at ambient temperature with horseradish peroxidase–conjugated goat antimouse IgG. Samples were read on a Bio-Tek Microplate Autoreader (Winooski, VT). Blank coated wells were treated with all but the Fn fragment–antibody mixture.

**Statistical methods.** Statistical differences were assessed by Student’s two-tailed t test for paired data.

**RESULTS**

**Purity of Fn fragment preparations.** Plasma Fn showed <15% degradation to >200 Kd fragments (Fig 1). The 190/170-Kd and the 160/150/120-Kd Fn fragments were incubated with 1:5,000 to 1:60,000 dilutions of ascitic fluid containing a monoclonal antibody specific for the 110-Kd cell-binding domain fragment22 for 90 minutes at 37°C. Samples were added to the wells and incubated for 2 hours at ambient temperature with horseradish peroxidase–conjugated goat antimouse IgG. Samples were read on a Bio-Tek Microplate Autoreader (Winooski, VT). Blank coated wells were treated with all but the Fn fragment–antibody mixture.

**Experimental methods.** Statistical differences were assessed by Student's two-tailed t test for paired data.

**Fig 2.** Chemotactic activity of Fn and Fn fragments for MOs. Data for the 35Kd fragment were similar to that of Fn. For 29- and 72-Kd fragments at 1.0 to 2.0 μmol/L, P < .02 compared with buffer, and was not significantly different compared with ZAS. All other values were significantly different from 5% ZAS but not from buffer. *n = 1.
Table 1. Gelatin Effects on 72-Kd Fragment Activity

<table>
<thead>
<tr>
<th>Bottom Well</th>
<th>MO + Fn Fragment (μmol/L)</th>
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<tr>
<td>72 Kd</td>
<td>1.0 μM*</td>
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<tr>
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</tr>
<tr>
<td>1.0</td>
<td>37</td>
</tr>
<tr>
<td>1.5</td>
<td>77</td>
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<tr>
<td>2.0</td>
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<tr>
<td>72 Kd gelatin</td>
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<tr>
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<td>1.5</td>
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<tr>
<td>2.0</td>
<td>72</td>
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</tbody>
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Migration toward 5% ZAS was 326 ± 9, and toward buffer 49 ± 3. Data are mean MO/field (×1,000) ± SEM where the SEM ≤ 3. Representative of two experiments.

*Concentration of 72-Kd or gelatin 72-Kd fragment in the bottom well.

Table 2. Checkerboard Analysis of Amino Terminal Fn Fragments

<table>
<thead>
<tr>
<th>Bottom Well</th>
<th>MO + Fn Fragment (μmol/L)</th>
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<tbody>
<tr>
<td>72 Kd*</td>
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</tr>
<tr>
<td>0</td>
<td>31</td>
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<tr>
<td>1.0</td>
<td>37</td>
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<tr>
<td>1.5</td>
<td>77</td>
</tr>
<tr>
<td>2.0</td>
<td>72</td>
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<tr>
<td>29 Kd*</td>
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</tr>
<tr>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>1.0</td>
<td>60</td>
</tr>
<tr>
<td>1.5</td>
<td>85</td>
</tr>
<tr>
<td>2.0</td>
<td>79</td>
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</table>

Migration toward 5% ZAS was 94 ± 1 in the 72-Kd checkerboard and 158 ± 1 in the 29-Kd experiment. Mean MO/field (×1,000) ± SEM. SEM ≤ 3.

About one third as potent. Migration toward the 72-Kd fragment was unchanged by equimolar amounts of the fibroblast attachment sequence Arg-Gly-Asp-Ser, but complexes of gelatin and 72-Kd fragments decreased chemotaxis by 28% to 30%, compared with migration toward the 72-Kd fragment alone (n = 2; Table 1). When disulfide bonds in the 29- and 72-Kd fragments were reduced and reformation blocked with carboxymethyl groups, chemotaxis was unaffected (data not shown). Treatment of the 29- and 72-Kd fragments with Hep decreased chemotaxis by 8% to 12% and 0%, respectively, but similar treatment of 5% ZAS with Hep blocked chemotaxis by 50%. MO migration toward Hep-treated buffer was unaffected. In the same concentration range, migration toward the 50-Kd (n = 4), 190/170-Kd (n = 2), 35-Kd (n = 8), and 160/150/120-Kd (n = 6) fragments, and intact Fn (n = 3) was not statistically significant, compared with ZAS (Fig 2).

To determine whether the activity of the 29- and 72-Kd fragments was chemotactic or chemokinetic, varying concentrations of K1 and K2, peptides located on the amino-terminal side of the cleavage site (n = 3). Migration toward K3, which contains the cleavage site, was biphasic (n ≥ 3) (Fig 3). Checkerboard analysis confirmed true chemotaxis in the 1 to 100 μmol/L range of K3 (Table 3). The three synthetic Fn peptides were not chemotactic for human PMNs (data not shown). These data provide further evidence of a structural domain within the amino-terminal 29-Kd fragment that is chemotactic for human MOs.

Clark et al reported that the 120-Kd cell-binding domain fragment at 0.1 μmol/L was selectively chemotactic for human MOs. The 29- and 72-Kd Fn fragments used in these experiments showed no presence of larger fragments by SDS-PAGE (Fig 1). To test for minute contamination, these same fragments were tested for presence of the 160/150/120-Kd fragment at the 33% inhibition level, ie, <0.009 μmol/L 160/150/120-Kd fragment is present in the amino-terminal fragments (n = 3). Thus, the cell-binding fragment cannot be responsible for the observed chemotaxis in the amino-terminal fragments.

PMN chemotaxis. Fn has been shown to mediate PMN adherence,12 and to act as the necessary cofactor for hyaluronic acid-induced PMN migration.7 Neither Fn alone at physiologic concentrations nor its fragments were chemotactic for PMNs.14 In our assay, PMNs did not migrate toward Fn (n = 5) or the 29-Kd (n = 8), 50-Kd (n = 4), 72-Kd (n = 6), 160/150/120-Kd (n = 3), and 35-Kd (n = 7) fragments at 0.05 to 2.0 μmol/L (Fig 5). Checkerboard
CHEMOTACTIC N-TERMINAL FIBRONECTIN FRAGMENTS

Inhibition ELISA using wells coated with the 160/150/120-Kd cell-binding domain fragment. A 1:40,000 dilution of the monoclonal antibody specific for the 110-Kd cell-binding domain was used. Representative of three experiments. (— □ —), 140 Kd; (— □ —), 29 Kd; (— □ —), 72 Kd.

Fig 4.

analysis did not confirm chemotaxis or chemokinesis of the 72-Kd fragment (data not shown).

DISCUSSION

Intact Fn is a substrate for multiple plasma proteinases, such as thrombin and plasmin, for leukocyte neutral proteinases, and likely for tissue proteinases, most of which are probably present at inflammatory sites. Limited proteolysis of Fn by these enzymes produces fragments with biologic properties not necessarily contained in the parent molecule. Such unmasking of activities leads, for example, to increased inhibitory activity toward endothelial cells, increased affinity toward Hep, tumor growth-enhancing activity, fibroblast mitogenesis, opsonin-independent phagocytosis, and chemotaxis of MOs.

Yonemasu et al reported that intact Fn was chemotactic at >0.1 μmol/L for PMNs and at >0.0001 μmol/L for MOs. Terranova et al found that Fn at 0.006 to 0.1 μmol/L was not chemotactic for PMNs. Norris et al showed that mixtures of Fn fragments, especially low molecular weight fractions, were chemotactic for MOs, but not for PMNs or lymphocytes, whereas intact Fn was inactive. They observed a bimodal response, with a chemokinetic effect at high concentrations of fragments. The mixtures were formed during plasma Fn isolation in the absence of proteinase inhibitors, and were thus presumed to be due to a serine proteinase contamination.

Subsequently, these investigators reported that the 120-Kd fibroblastic cell-binding fragment at 0.1 μmol/L was chemotactic for MOs. This 120-Kd fragment was released, using thermolysin at pH 7, from the chemotactically inactive 150/160-Kd fragment that contains the 120-Kd sequence and the Hep II domain. Our results with the 160/150/120-Kd fibroblast-binding fragment at 0.05 to 2.0 μmol/L, which was generated at pH 3.7 from cathepsin D and thrombin digests of Fn, are in contrast to those of Clark et al. We found that only the 29- and 72-Kd fragments of Fn were selective chemoattractants for human MOs but not PMNs, whereas intact Fn and its 50-, 35-, and 160/150/120-Kd fragments were inactive for both cell types. The chemotactic activity of the synthetic amino-terminal Fn fragment K3, which contains the thrombin cleavage site between the 29- and 50-Kd subfragments of the amino-terminal 72-Kd segment, is additional evidence favoring the chemotactic activity of the 29- and 72-Kd fragments generated enzymatically from native Fn. We cannot explain definitively the differences noted, but suggest three possible explanations. First, it is possible that our heterogeneous cathepsin D- and thrombin-generated 160/150/120-Kd preparation contained more of the thermolysin-generated 150/160-Kd fragment with cryptic chemotactic activity than the chemotactically active thermolysin-generated 120-Kd fragment. The amino-terminal fragments used here contained <1% of the cathepsin D- and thrombin-generated 160/150/120-Kd fragment at a concentration well below the reported peak chemotactic response of the thermolysin-generated 120-Kd fragment (0.1 μmol/L). Second, the amino-terminal fragments may contain sequence homology to the thermolysin-generated 120-Kd segment. Using a computer program (Dr Jerome Seyer, University of Tennessee, Memphis), it was determined that two areas of Fn contain about 67% homology to the amino-terminal fragments, but neither was within or near the cell-binding domain.

Third, we cannot exclude that these 150/160- and 160/150/120-Kd fragments, generated by different proteinases and at different pH values, may have different properties because of conformation of the protein sequence. Enzymatic cleavage near the carboxyl-terminal region at different sites

Fig 5.

Chemotactic activity of Fn and Fn fragments for PMNs. All values were significantly different from 5% ZAS but not from buffer.
may liberate regions with different activities.\textsuperscript{10,15,28} For example, the thermolysin-generated 120-Kd cell-binding fragment contained a cryptic site for MO chemotaxis that was expressed only upon enzymatic cleavage of Fn or larger Fn fragments.\textsuperscript{15} Similarly, the 120-Kd \( \alpha \)-chymotrypsin-generated cell-binding domain of Fn inhibited adhesion of 3T3 cells more effectively than intact Fn,\textsuperscript{28} an example of a cryptic site for a glycosaminoglycan (GAG)-binding domain. Using a series of fragments from thermolysin digests of Fn, these investigators showed that Fn fragments containing the cell-binding domain, but differing in size, did not all demonstrate a GAG-binding domain. They concluded that this cryptic GAG-binding domain must be located close to the cell-binding domain, and is extremely sensitive to the conformation of the protein sequence that lies between the cell-binding and carboxyl-terminal Hep-binding domains of Fn. The domain within the thermolysin-generated 120-Kd cell-binding fragment that is responsible for MO chemotaxis may be expressed in a similar fashion. Preliminary work in our lab with an \( \alpha \)-chymotrypsin-generated 120-Kd Fn fragment that contained the cell-binding domain (Calbiochem, San Diego, CA) showed increased migration (mean migration = 55\%) of human MOs toward a 0.1 \( \mu \)mol/L concentration of the fragment, and a trend toward increased migration (mean migration = 39\%) at 2 \( \mu \)mol/L (n \( \geq \) 3). Thus, our heterogeneous cathepsin D- and \( \alpha \)-chymotrypsin-generated 160/150/120-Kd fragment may express activities different from those of the thermolysin-induced 120-Kd fragment, perhaps because of conformational changes due to differing protein sequence. Further work with a series of enzymatically generated Fn fragments of different size and thus protein sequence, or with synthetic Fn fragments would be required to answer this question definitively.

Other undetected trace contaminants from plasma Fn preparation could be responsible for the observed chemotactic activity. For example, the potent chemottractant C5a could be present. Norris et al showed that anti-C5 antibodies failed to inhibit chemotactic activity of mixtures of Fn fragments, whereas anti-Fn antibodies almost completely abolished chemotaxis toward mixtures of Fn fragments but not toward ZAS.\textsuperscript{14} Furthermore, human serum albumin failed to inhibit MO migration.\textsuperscript{14} The 29- and 72-Kd fragments used in this study were >98\% homogeneous electrophoretically, as shown previously.\textsuperscript{3,9,10} Although it is possible that a low concentration of a contaminant was responsible for the activity of the 29- and 72-Kd fragments, it is highly unlikely that such a contaminant would be found only in these two Fn fragments, and not in the other inactive fragment preparations (Fig 2).

Little is known about Fn-cell receptor interactions. Other cells have been shown to migrate toward micromolar concentrations of intact Fn, including rabbit corneal epithelial cells in situ,\textsuperscript{29} 16 epithelial and mesenchymal cell types,\textsuperscript{36} metastatic tumor cells,\textsuperscript{31} and fibroblasts,\textsuperscript{27,25} but the role of receptors is not known. The tetrapeptide sequence Arg-Gly-Asp-Ser, derived from the fibroblast-binding domain of Fn, competes with Fn in binding to fibroblasts\textsuperscript{26,37} and platelets,\textsuperscript{28} but the cellular receptor for this attachment site is difficult to elucidate because of low binding affinity.\textsuperscript{38,39} The Fn receptors on MOs mediate adherence\textsuperscript{40} and enhance phagocytosis via the Fn and C3b receptors,\textsuperscript{5,7} but their relation to chemotaxis is not known.

Mixtures of small-molecular weight, chemotactically active Fn fragments can be generated from intact Fn and its larger fragments during injury.\textsuperscript{14,40} Our work supports the hypothesis\textsuperscript{14} that proteases released at sites of inflammation can induce MO recruitment through production of chemotactic Fn fragments at physiologic concentrations. Of the fragments tested, we have found that the amino-terminal 29-Kd fragment, its precursor 72-Kd fragment, and a related synthetic peptide are effective chemoattractants.

We conclude that this additional chemotactic domain of Fn is within the 29-Kd segment because (a) the 50-Kd fragment, also contained within the 72-Kd segment, is inactive; and (b) the 190/170-Kd fragment that lacks the 29-Kd segment is inactive. However, the 72-Kd fragment is more active than the 29-Kd segment. This suggests that other sequences near the amino terminus are active, or affect the locomotory response of MOs in another fashion. For example, proteolytic cleavage that produces the 29-Kd segment may occur within a part of the chemotactic domain, and thus generates a less active 29-Kd fragment. The chemotactic activity of the synthetic amino-terminal Fn fragment K3, which contains the thrombin cleavage site, supports this hypothesis. Alternatively, the digestion may occur outside the domain, but may permit a conformational change that masks part of the chemotactic domain.\textsuperscript{28} The data on the remaining fragments are consistent with either explanation, but the data with the reduced 29- and 72-Kd Fn fragments suggest that conformation in this structural domain is not critical to MO chemotaxis. The 160/150/120-Kd fragment produced at pH 3.7 is inactive, as well as the 190/170-Kd fragment that contains the 160/150/120-Kd segment. The 35-Kd segment located near or at the carboxyl terminus of the 190/170- and 160/150/120-Kd fragments, respectively, is also inactive.

Fn is present in connective tissue matrix and could be degraded during inflammation by plasma, tissue, and leukocyte proteases to yield selective chemotactic signals for MOs. Thus, specific Fn fragments could regulate various aspects of leukocyte behavior, facilitating MO accumulation at sites of inflammation and injury, enhancing clearance of debris by PMNs and MOs, and promoting tissue remodeling by MOs. The 29-Kd fragment also inhibits endothelial cell growth.\textsuperscript{5,4} We speculate that the 29-Kd fragment inhibits endothelial cell proliferation for a time period sufficient for clearance of debris at the injury site by simultaneously recruited MOs. A similar paradoxical role may exist for transforming growth factor-\( \beta \), which is released from alpha granules of platelets within a few minutes of vessel injury.\textsuperscript{41,44}

ACKNOWLEDGMENT

The authors thank Robert W. Lightfoot, Jr, MD, Arnold E. Postlethwaite, MD, and Robert O. Endres, PhD, for helpful discussions, and Rachael Elizabeth Jackson and James Hallman for technical assistance.
CHEMOTACTIC N-TERMINAL FIBRONECTIN FRAGMENTS

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The amino-terminal 29- and 72-Kd fragments of fibronectin mediate selective monocyte recruitment

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