

INDUCTION OF FIBROBLAST CHEMOTAXIS BY FIBRONECTIN
Localization of the Chemotactic Region to a 140,000-Molecular Weight
Non-Gelatin-binding Fragment*

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Fibronectin, a class of adhesive, high-molecular-weight glycoproteins, is present in connective tissue, primitive mesenchyme, basement membranes, amniotic fluid, cerebrospinal fluid, and plasma (1, 2). A variety of cells synthesize fibronectin; however, the amount of fibronectin present on the surface of transformed or neoplastic cells is greatly reduced (1-3). The biological properties of fibronectin are numerous. It is known that fibronectin promotes cellular aggregation, cell-substratum adhesiveness, partially restores transformed fibroblasts to a more normal appearance, promotes locomotion of certain types of cells, facilitates reticuloendothelial system clearance of colloids, and specifically binds to macromolecules such as types I, II, and III collagens, fibrin, fibrinogen, heparin, and actin (1-9). Plasma and cellular fibronectin are structurally similar, share immunological identity, and have many common biological properties (2).

Fibronectin is a substrate for plasma transglutaminase, thrombin plasmin, cathepsin D, leukocyte elastase, trypsin, chymotrypsin, and subtilisin (3, 10-13). It is composed of two almost identical, polypeptide chains with a 220,000 mol wt (2). Limited proteolysis of fibronectin yields fragments that retain some of the biologic activities ascribed to the native molecule (12-15). For example, cathepsin D cleavage produces a major 72,000-mol wt fragment that possesses gelatin-binding activity and a major 137,000-mol wt fragment that lacks gelatin-binding activity (12, 14).

Mechanisms that control the organization, distribution, and migration of connective tissue fibroblasts in vivo under pathologic and normal physiologic conditions are poorly understood. An assay that measures chemotactic (directional) migration of human fibroblasts in vitro has been developed in our laboratory (16). By employing this assay, it has been possible to isolate and identify specific substances that are capable of effecting chemotactic migration of fibroblasts (16-19). In the present study, we have observed that plasma and cellular fibronectin induce chemotactic migration of fibroblasts in vitro. The region of the fibronectin molecule responsible for its

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chemotactic activity is contained in a major 140,000-mol wt fragment generated by cathepsin D cleavage. This chemotactic fragment lacks gelatin-binding activity.

Materials and Methods

Purification of Fibronectin. Fibronectin was purified from normal human citrated plasma and supernates obtained from cultures of human lung fibroblasts by the technique of affinity chromatography employing a gelatin-agarose column (9). The affinity column was prepared by coupling gelatin obtained from type I rat skin collagen to cyanogen bromide-activated Sepharose 4B particles (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). After the column was washed extensively with phosphate-buffered saline plus 0.2 M KBr, adsorbed fibronectin was eluted from the column with 1 M KBr in 0.05 M Tris-HCl buffer (pH 5.3) as originally described (20, 21). Fibronectin recovered from the column was dialyzed against Tris-HCl buffer (pH 8.3) and was stored either at -70°C or in the lyophilized state at -20°C in a dessicator. The purity of fibronectin was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Protein stainings of the gels indicated >98% purity.

Fibroblast Chemotaxis. Fibroblast chemotaxis was measured as previously described (16). Indicator cells used in the assay were human foreskin fibroblasts harvested from monolayer cultures by trypsinization. Serum-free suspensions of harvested fibroblasts were placed in the upper compartments of blind-well chemotaxis chambers equipped with porous ($8\ \mu\text{m}$) gelatin-treated polycarbonate filters. Test substances were added to the compartment below the filter and, in some experiments, also to the upper compartment of each chamber. After a 150-min incubation at 37°C , fibroblast chemotaxis was quantitated by counting the number of fibroblasts that migrated through the pores to the lower surface of each filter in 20 oil immersion fields (OIF). All samples were tested in triplicate. Final chemotactic activity was expressed as the mean \pm SEM of the replicates.

Monocytes and Neutrophil Chemotaxis. Chemotaxis of human peripheral blood monocytes and neutrophils was measured as previously described (22).

Isolation of Gelatin-binding and Non-Gelatin-binding Fragments of Fibronectin. Plasma fibronectin was digested with cathepsin D (Sigma Chemical Co., St. Louis, Mo.) at pH 3.5 and 30°C in 4 h as previously described (12, 14, 23). The reaction was terminated by the addition of a 10-fold molar excess of pepstatin A (Peptide Research Institute, Osaka, Japan). The reaction products were brought to neutral pH by addition of 1.5 M Tris, pH 8.8, and applied to a gelatin-Sepharose column as previously described (12, 14). Non-gelatin-binding fragments were collected in the fall-through, and gelatin-binding fragments were eluted from the column with 6 M urea as previously described (12, 14) at 25°C . Chromatography of non-gelatin-binding fragments and plasma fibronectin was achieved on a $1.0 \times 100\text{-cm}$ column of Sephadex G-200 equilibrated with 0.015 M glycylglycine/0.14 M NaCl at pH 7.2. Protein content of column fractions was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.).

Results

Chemotactic Property of Fibronectin. Plasma fibronectin stimulated the migration of fibroblasts in a dose-dependent manner, with maximal migration occurring at a concentration of $1.2\ \mu\text{g}/\text{ml}$ (Fig. 1). However, plasma fibronectin did not induce migration of human neutrophils or monocytes at concentrations ranging from 0.4 to $100\ \mu\text{g}/\text{ml}$ (data not shown).

Zigmond-Hirsch checkerboard analysis revealed that fibronectin-induced migration of fibroblasts when it was present in higher concentration in the lower compartment of the chemotaxis chambers (Table I). These data suggest that fibronectin induces fibroblast migration by acting as a chemoattractant (24).

To confirm that fibroblasts were migrating in response to a gradient of fibronectin and not to other substances that could be present in small amounts in fibronectin preparations, fibronectin was chromatographed on Sephadex G-200. Chemotactic activity co-eluted with fibronectin (data not shown).

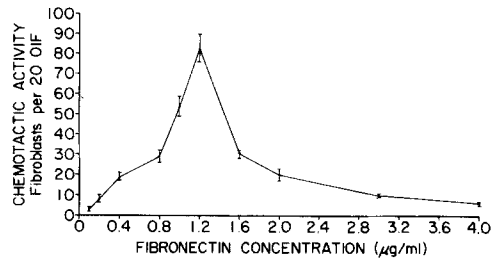


FIG. 1

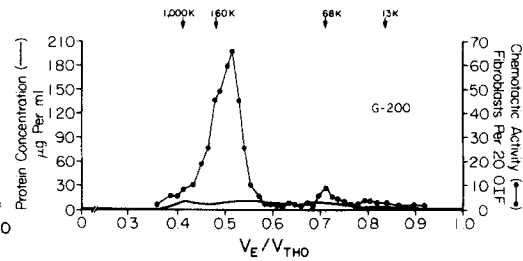


FIG. 2

FIG. 1. Plasma fibronectin was dissolved in MEM and assayed at the concentrations indicated for its ability to induce fibroblast migration.

FIG. 2. Non-gelatin-binding fragments were derived from cathepsin D cleavage of plasma fibronectin and were purified by affinity chromatography as described in Materials and Methods. Isolated non-gelatin-binding fragments (1 mg) were chromatographed on a Sephadex G-200 column as described in Materials and Methods, and column fraction were diluted 50% with MEM and tested for chemotactic activity. V_E , Volume of eluent; V_{THO} , volume of tritiated water.

TABLE I
Effect of Varying Concentration Gradients of Fibronectin on Fibroblast Migration*

Fibronectin concentration in upper compartment $\mu\text{g/ml}$	Fibronectin concentration in lower compartment ($\mu\text{g/ml}$)				
	1.2	1.0	0.8	0.6	0
1.2	3 ± 1	4 ± 1	3 ± 1	3 ± 1	3 ± 1
1.0	16 ± 2	4 ± 1	5 ± 1	6 ± 1	6 ± 1
0.8	31 ± 1	17 ± 2	5 ± 1	6 ± 1	5 ± 1
0.6	39 ± 5	29 ± 4	21 ± 3	6 ± 1	5 ± 1
0	78 ± 3	61 ± 7	44 ± 5	32 ± 5	4 ± 1

* Different concentrations of plasma fibronectin were added to the upper and lower compartments of the chemotaxis chambers, and the number of fibroblasts migrating to the lower surface of each filter was quantitated.

TABLE II
Induction of Fibroblast Migration by Cell-Derived Fibronectin*

Fibronectin concentration $\mu\text{g/ml}$	Chemotactic activity <i>Fibroblasts/20 OIF</i>
50.0	4 ± 1
5.0	4 ± 1
2.50	13 ± 1
1.25	61 ± 5
0.75	32 ± 4
0.25	11 ± 1
MEM	3 ± 1

* Cell-derived fibronectin was dissolved in minimum essential medium (MEM) and tested for chemotactic activity.

It was of interest to determine whether fibronectin of cellular origin was as effective as plasma-derived fibronectin in inducing fibroblast migration. Migration of fibroblasts was observed at concentrations of cell-derived fibronectin between 0.75 and 2.5 $\mu\text{g/ml}$, similar to that obtained with plasma-derived fibronectin (Table II and Fig. 1).

Fibronectin Fragments. It was of interest to determine whether gelatin-binding or non-gelatin-binding fragments resulting from cleavage of fibronectin by cathepsin D retained chemotactic activity. Non-gelatin-binding fragments were chemotactic at

TABLE III
*Migration of Fibroblasts to Gelatin-binding and Non-Gelatin-binding
 Fragments of Fibronectin*

Fragment concentration <i>µg/ml</i>	Chemotactic activity	
	Gelatin-binding	Non-gelatin-binding
	<i>Fibroblasts/20 OIF</i>	
50.0	5 ± 1	21 ± 1
20.0	3 ± 1	47 ± 5
10.0	5 ± 2	66 ± 5
5.0	4 ± 1	51 ± 5
2.5	4 ± 1	17 ± 2
1.6	3 ± 1	6 ± 1
0.8	3 ± 2	4 ± 1

* Plasma fibronectin was cleaved by cathepsin D, and gelatin-binding and non-gelatin-binding fragments were isolated by affinity chromatography as described in Materials and Methods and tested in the same experiment for chemotactic activity.

concentrations ranging from 2.5 to 50 $\mu\text{g/ml}$, with maximal response occurring at 10 $\mu\text{g/ml}$ (Table III). Fibroblasts did not migrate in response to gelatin-binding fragments (Table III).

To determine the approximate size of the active fragment, non-gelatin-binding fragments were chromatographed on Sephadex G-200, and column fractions were tested for chemotactic activity (Fig. 2). A single peak of chemotactic activity eluted from the column just after the 160,000-mol wt marker (Fig. 2).

Discussion

Fibronectin of plasma or cellular origin is chemotactic for human dermal fibroblasts *in vitro*. After cleavage of fibronectin by cathepsin D, chemotactic activity is found in a non-gelatin-binding fragment having an estimated molecular weight of 140,000. Gelatin-binding fragments do not possess chemotactic activity.

Although the mechanism by which fibronectin causes the chemotactic migration of fibroblasts is not apparent from this study, these data strongly suggest that fibronectin does not produce its effect by acting as a simple ligand between cell surface receptor and the gelatin-coated polycarbonate filters. Additional data that do not support a ligand role for fibronectin in its induction of fibroblast chemotaxis are the findings that (a) the amount of gelatin deposited on each surface of the gelatin-treated polycarbonate filter used in the chemotaxis assay is <4 ng, (b) preincubation of gelatin-treated filters with fibronectin (4 $\mu\text{g/ml}$) for 150 min at 37°C, followed by rinsing in distilled water does not alter the chemotactic response of fibroblasts to fibronectin, and (c) fibroblasts also respond chemotactically to fibronectin when the polycarbonate filters are treated with the synthetic peptide glycyl-prolyl-L-hydroxyproline (A. Postlethwaite. Unpublished observations.).

The concentration of fibronectin in plasma is ~ 300 $\mu\text{g/ml}$ (2). In this study, fibroblasts were responsive to lower concentrations (0.4–2 $\mu\text{g/ml}$) of native fibronectin, however, higher concentrations of cathepsin D-generated non-gelatin-binding fragments were required to effect fibroblast migration. Higher concentrations (>2 $\mu\text{g/ml}$) of native fibronectin elicited no migration. A similar bell-shaped dose-response curve has been observed in the chemotaxis of neutrophils to formylmethionyl peptides (25). The diminished response to large amounts of chemoattractant may result from rapid diffusion of the chemoattractant to the upper cell compartment, with a concomitant

saturation of available receptors on plasma membranes on the responding cells. It is also possible that fibroblasts *in vivo* respond optimally to higher concentrations of fibronectin than they do *in vitro* under the artificial conditions of the assay system employed in this study.

Fibronectin and/or its biologically active fragments may play a significant role in effecting fibroblast migration *in vivo*. The role of fibronectin as a fibroblast chemoattractant should be considered in relation to other chemotactic factors for fibroblasts we have previously described (16-19). Specific chemotactic lymphokines have been characterized for human and guinea pig fibroblasts (16, 17). These lymphokines could be released at sites of cell-mediated immune reactions *in vivo*. A fragment from human C5 is also chemotactic for fibroblasts, suggesting that the complement system when activated could provide a specific chemoattractant for fibroblasts (18). In addition, collagen and collagen-derived peptides are chemotactic for fibroblasts (19). Because collagen and fibronectin are both present in the connective tissue matrix, their degradation by specific proteinases during the course of inflammatory reactions of all types could generate additional chemotactic signals for fibroblasts. Thus, collagen-derived peptides and biologically active fibronectin peptides could serve as important amplifiers of fibroblast chemotaxis and could greatly facilitate the accumulation of fibroblasts at sites of connective tissue injury and inflammation. During embryonic development and morphogenesis, the chemotactic property of fibronectin and derived peptides could function to direct migration of fibroblasts to effect specific organization of connective tissue.

Summary

Plasma and cell-derived fibronectin are potent chemoattractants for human dermal fibroblasts *in vitro*. The chemotactic property of fibronectin resides in a major 140,000-mol wt non-gelatin-binding fragment of the native molecule. Human monocytes and neutrophils do not recognize fibronectin as a chemotactic stimulus. These findings suggest that fibronectin and perhaps certain fragments of fibronectin may function *in vivo* as a specific chemoattractant for fibroblasts and could, therefore, induce directional migration of fibroblasts to sites of tissue injury, remodeling or morphogenesis.

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