Fibronectin Enhancement of Directed Migration of B16 Melanoma Cells

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ABSTRACT

The migration of B16 melanoma cells into porous nitrocellulose filters fitted between the upper and lower compartments of blindwell chemotactic chambers was examined by light microscopy. Human plasma fibronectin placed in the lower compartment of such chambers enhanced in a time-, temperature-, and dose-dependent manner the directed migration of B16 cells into the filter. Fibronectin placed either in the upper compartment alone or in equal concentrations in both compartments did not result in a significant increase in B16 cell migration, indicating that a positive gradient of fibronectin is required. Pretreatment of filters with fibronectin to establish a gradient of bound fibronectin also stimulated the directed migration of B16 cells. The response to fibronectin appeared to be specific, since other plasma proteins and reduced fibronectin or trypsin-digested fibronectin failed to enhance the migration over base-line values. These results suggest that a specific haptotactic-chemotactic response to fibronectin was responsible for enhanced B16 cell migration.

INTRODUCTION

Directed cell migration appears to be an important process in many diverse systems including tissue organization in developing embryos, wound healing, extravasation of WBCs, neovascularization, and invasive behavior of tumor cells. The molecular mechanism of cell locomotion is highly complex and still poorly understood. The cellular processes involved in locomotory behavior include the cytoskeleton, surface membrane topography, cellular metabolism, energy production, and cell-cell and cell-substratum interactions. A well-defined molecule that appears to affect cell shape, cell attachment, and cell motility is fibronectin. This molecule is a high-molecular weight glycoprotein that is normally found in a high-molecular weight form in plasma where it is known as cold-insoluble globulin. In the past few years, there have been significant indications that fibronectin can function as a chemotactic agent in mediating the directed migration of normal cells. The exact mechanism by which fibronectin acts as a chemotactic agent is unknown. Many transformed cells, in contrast to their normal counterparts, are devoid of surface fibronectin and the addition of fibronectin to such transformed cultures has been shown to affect a variety of cell behavior patterns including adhesiveness, cell spreading, random locomotion, surface membrane ruffling, and restoration of organized cytoskeleton. We have examined the migration of malignant B16 melanoma cells in modified chemotactic chambers and tested the effect of exogenous fibronectin on the directed locomotory behavior of the entire cell population as it migrated into a porous nitrocellulose filter.

MATERIALS AND METHODS

Cell Culture. B16 melanoma cells were maintained in sealed 75-sq mm tissue culture flasks in a 37°, 10% CO2 incubator in minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum. Culture passages were performed with 0.25% trypsin with 0.05% EDTA. Cells were harvested for migration experiments with 5 mM EDTA in PBS deficient in Ca2+ and Mg2+ unless otherwise indicated.

Cell Migration Assay. A 12-μm pore, 180-μm thick nitrocellulose filter was prewetted with PBS and placed into chemotactic chambers (Neuroprobe Institute of Biological Research, Bethesda, MD). Human plasma fibronectin or other indicated proteins were added (0.2 ml; 300 μg/ml in minimal essential medium or PBS) to the lower compartment. A suspension of B16 cells (0.5 ml; 5 × 10⁵ cells/ml) was inoculated into the upper compartment. The chambers were incubated at 37° in a 10% CO2 atmosphere for 5 hr unless otherwise indicated. After incubation, filters were removed and fixed with 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, for 30 min, rinsed with phosphate buffer, and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer. The filters were then rinsed with 0.85% NaCl solution (saline) and dehydrated with ethanol, cleared with propylene oxide, and embedded in Epon-812. These blocks were then polymerized in a 60° oven for at least 24 hr, and transverse sections (≈1.5 μm thick) were cut with an LKB microtome and stained with 0.1% toluidine blue in sodium borate.

The extent of B16 cell migration into the filter was measured with a ×40 objective and ×10 ocular-micrometer. Five random fields/thick section of filter were studied. In general, a total of approximately 100 cells was counted for each variable in any given experiment. The number of cells migrating 10-μm increments of depth into the filter was divided by the total number of cells in the filter, and a percentage was calculated. The percentage values were shown to be an accurate representation of the total B16 cell population in control experiments using [3H]thymidine-labeled cells and determining that the number of cells in the filter represented 80 to 100% of the total number of cells inoculated into the upper chamber. A second means of analysis used was the locomotion index, derived from the product of the number of cells that migrated specific distances multiplied by those distances. The sum of these products was then divided by the total number of cells, thus providing an approximate average depth of migration. All experimental conditions were performed in triplicate, and Student's t test was used to determine the statistical significance of results.

Treatment of Fibronectin. Human fibronectin was obtained from Dr. M. Mosesson and Dr. D. Amarani (Mt. Sinai Medical Center, Wilwaukee, Wisconsin). Recipient of Grant BC163 from the American Cancer Society and SUNY Downstate Medical Center, Brooklyn, New York 11203.

1 Recipient of Grant AI16480 from NIH.

2 To whom requests for reprints should be addressed, at State University of New York, Downstate Medical Center Box 44, 450 Clarkson Avenue, Brooklyn, NY 11203. Recipient of Grants BC1853 from the American Cancer Society and CA16740 from NIH.

Received July 22, 1983; accepted December 1, 1983.

APRIL 1984 1657
RESULTS

Fibronectin-dependent B16 Cell Migration. When a suspension of B16 melanoma cells is placed in the upper compartment of a chemotactic chamber, the directed migration of these cells into a 12-μm-pore nitrocellulose filter is stimulated by the presence of fibronectin in the lower compartment. The time course and extent of B16 cell migration when either buffer (control) or human plasma fibronectin is present in the lower compartment of a chemotactic chamber is shown in Chart 1. After 1 and 2.5 hr of incubation, 27 and 34%, respectively, of the B16 cells in the fibronectin-containing chambers have migrated 20 μm or more into the filter, while only 3 and 7%, respectively, of the cells in the control chambers have migrated this distance. More than 90% of the cells in the control chambers remain at the origin of the filter or within a cell diameter’s distance (10 μm) of the origin.

After 5 hr of incubation, 92% of the B16 cells in the control chambers are found within the first 20 μm of the filter, and only 8% of the cells have migrated 30 μm. In contrast, more than 30% of the cells incubated in fibronectin-containing chambers have migrated between 30 and 60 μm by 5 hr. Migration under all conditions slows down between 5 and 10 hr. From the data in Chart 1, a locomotion index can be calculated (29) which is a reflection of the entire migrating population. The locomotion indices, presented in Chart 1 (insets), also indicate that B16 cell migration is increased significantly by fibronectin. The increase in locomotion index is especially pronounced at early times (1 and 2.5 hr) when most of the control cells have not yet moved into the filter.

A light micrograph further illustrating the effect of fibronectin is shown in Fig. 1. After 5 hr, very few B16 cells have migrated more than 20 to 30 μm from the surface of the filter when only buffer is in the lower compartment of the chemotactic chamber (Fig. 1a). However, when fibronectin is present in the lower compartment, B16 cells can be seen to have migrated considerably farther into the filter (Fig. 1b). Electron microscopy shows that migrating B16 cells are deformable, adapting to the shape of the pores in the nitrocellulose filters. A melanoma cell at one stage during the locomotory process is pictured in Fig. 2. The tumor cell shows morphological orientation with a leading pseudopod free of cell organelles. At this stage, most of the organelles are located behind the nucleus. This polarization of organelles is characteristic of other motile cells (13, 51).

In order to examine the specificity of this response, modified fibronectin and a number of other human plasma proteins were tested for their effect on B16 cell migration. The data in Table 1 indicate that fibronectin, or fibronectin which had been dialyzed to remove any potential low-molecular-weight chemotactic peptides, elicited an enhancement in cell migration as evidenced by the increased number of cells that migrated 40 μm or more into the filter. Fibrinogen or immunoglobulin at identical protein concentrations did not significantly alter the pattern of B16 cell migration from that of the control (no protein). The specificity of the response is further indicated by the absence of enhanced migration when incubation is carried out with trypsin-digested fibronectin or with fibronectin the secondary protein structure of...
Fibronectin and B16 Cell Migration

A solution of fibronectin at the indicated concentrations was placed in the lower compartment of a chemotactic chamber. B16 cells (0.5 ml; 5 x 10^⁶ cells/ml) were inoculated into the upper compartment. The chemotactic chambers were incubated at 37° for 5 hr. Cell migration was analyzed as described in Table 1 and "Materials and Methods."

<table>
<thead>
<tr>
<th>Fibronectin concentration in lower chamber (µg/ml)</th>
<th>Cell migration into filter (% of cell population)</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>50</td>
<td>14</td>
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<tr>
<td>300</td>
<td>14</td>
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<tr>
<td>700</td>
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* Percentage of cells that remained at or near the surface of the filter (0 to 20 µm) or migrated the indicated distance into the filter.

which was altered by disulfide bond reduction.

Table 2 shows the effect of fibronectin concentration on a 5-hr B16 cell migration experiment. The number of cells that migrated 40 µm or more into the filter increased proportionally with increasing concentration of fibronectin in the lower compartment. In response to fibronectin (300 µg/ml), 20% of the cells migrated 40 µm or more into the filter, while no B16 cells migrated this far in the absence of fibronectin. The pattern of cells migrating in response to fibronectin (50 µg/ml) was variable, and sometimes only slightly enhanced over the buffer control. Concentrations of fibronectin below 50 µg/ml (0.1, 1.0, and 10 µg/ml) did not elicit any increase in migration over control (data not shown). A 700 µg/ml concentration of fibronectin increased the migration of B16 cells (Table 2), but often the locomotion index of cells responding to fibronectin is only slightly increased over that observed in response to 300 µg/ml. Thus, the concentration of fibronectin found to be the most active and consistent in effect was 300 µg/ml (0.7 µM), the concentration found in human plasma (31). This concentration was used in all further experiments, unless otherwise noted.

A wide range of B16 cell concentrations (5 x 10⁴ to 2 x 10⁶) was tested in the upper compartment of chemotactic chambers in the presence and absence of fibronectin (300 µg/ml) in the lower compartment. A concentration of 5 x 10⁶ cells/ml was shown to be optimal (data not shown). Concentrations below 5 x 10⁶ cells/ml did not provide an adequate number of observable, migrating cells in each filter section to be of statistical significance. At concentrations of 1 x 10⁶ cells/ml and above, the number of cells at or near the surface of the filter was so great that accurate morphological distinctions between individual cells could not be made. Thus, an inoculum of 5 x 10⁶ cells/ml (0.5 ml) was used for all migration assays.

The effect of temperature on B16 cell migration was examined by placing chemotactic chambers in vessels equilibrated with 10% CO₂ and incubating them at 5°, 26°, and 37° for 5 hr. As shown in Chart 2, at 5°, there is little difference between the locomotion indices of cells migrating in response to buffer and those responding to fibronectin. At 26°, a significant increase in the locomotion index of cells responding to fibronectin is observed, and this increase was even greater at 37°. Similar results were obtained when chambers were incubated at these 3 different temperatures and analyzed following a 1-hr incubation (results not shown).

Nature of the Fibronectin Response. Fibronectin may have a direct effect on B16 cells by diffusing through the filter into the upper compartment and binding to the B16 cell surface. Such cellular-bound fibronectin might simply enhance the migratory ability of the B16 cells. In order to examine this possibility, cultures of B16 cells were pretreated with fibronectin (300 µg/ml) in serum-free medium for the standard incubation time of 5 hr at 37°. The cultures were washed, and the cells were harvested and added to assay chambers which contained buffer or fibronectin in the lower compartment. The chambers were then incubated for 5 hr, and migration was analyzed in the standard manner. The results showed no significant difference between the migration pattern of cells pretreated with fibronectin and those pretreated with serum-free medium in the absence of fibronectin (data not shown).

Fibronectin may be stimulating B16 migration as a chemokinetic agent by increasing random locomotion, or as a chemotactic agent by increasing directed locomotion. This aspect was examined by varying the position of fibronectin in the upper and lower compartments of assay chambers. The results (Table 3) indicate that enhanced B16 cell migration occurs when a positive gradient of fibronectin is established across the filter, with fibronectin in the lower compartment and no fibronectin in the upper compartment. A gradient in the opposite direction, or equal concentrations of fibronectin above and below the filter, had only minimal effect on B16 cell migration as compared to controls (no fibronectin in either chamber). These results indicate that fibronectin is behaving predominantly as a chemotactic agent, increasing

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Table 2

<table>
<thead>
<tr>
<th>Fibronectin concentration in lower chamber (µg/ml)</th>
<th>Cell migration into filter (% of cell population)</th>
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<tbody>
<tr>
<td>0</td>
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<td>700</td>
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* Percentage of cells that remained at or near the surface of the filter (0 to 20 µm) or migrated the indicated distance into the filter.

Table 3

<table>
<thead>
<tr>
<th>Protein in upper chamber</th>
<th>Protein in lower chamber</th>
<th>Cell migration into filter (% of cell population)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>Fibronectin</td>
<td>25</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Fibronectin</td>
<td>36</td>
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* Percentage of cells that remained at or near the surface of the filter (0 to 20 µm) or migrated the indicated distance into the filter.
directed migration of B16 cells in response to a fibronectin concentration gradient.

It was possible that fibronectin was causing enhanced migration by simply increasing either the rate or extent of B16 cell attachment to the surface of the filter in those chambers that contained fibronectin in the lower compartment. To examine this possibility, B16 cells were allowed to attach to a filter in the absence of fibronectin. Following a 1-hr attachment period, the filters were washed free of nonadherent cells and placed in a second set of chemotaxis chambers containing either buffer or fibronectin in the lower compartment. A 5-hr incubation was carried out, and the filters were analyzed for cell migration. The results (Table 4) demonstrate that fibronectin still caused enhanced migration of B16 cells when the attachment phase was standardized by using filters preattached with equal numbers of B16 cells.

Fibronectin may exert its directed cell locomotory effect either as a soluble chemotactic agent, diffusing upward through the filter toward the migratory cells, or as a haptotactic agent bound to the filter and mediating enhanced cell-substratum interaction along a concentration gradient of substrate-bound fibronectin. This was examined by pretreatment of the substratum (nitrocellulose filter) with fibronectin. Filters were incubated in the absence of cells in chemotaxis chambers containing fibronectin above and/or below the filter in order to preestablish a fibronectin gradient within the filter. The filters were extensively washed to remove any unbound fibronectin within the filter pores. The washed filters were placed into a second set of chemotactic chambers. B16 cell migration across the pretreated filters was carried out in chambers containing only buffer in the upper and lower compartments. A standard migration assay was performed in parallel using untreated filters and a solution of fibronectin in the lower chamber. The results (Table 5) indicate that filters treated with fibronectin (0/300) significantly increased B16 cell migration as compared to filters that had been preincubated with only buffer in both chambers (0/0). The extent of B16 cell migration in a fibronectin-pretreated filter was equal to or greater than that observed when fibronectin was added directly to a lower chamber in the standard migration assay across an untreated filter. The pretreatment of filters in a homogeneous environment of fibronectin (300/300) caused a small increase in migration, but not as great as that observed when filters were pretreated to establish a positive gradient of fibronectin (0/300).

These results suggest that fibronectin can serve as a haptotactic agent in stimulating B16 cell migration, and that a gradient of substrate-bound fibronectin is more effective than a homogeneous coating of fibronectin.

**DISCUSSION**

The effect of fibronectin on cellular behavior has been extensively documented. Initial reports published a decade ago by Hynes (23) demonstrated the presence of this molecule on the surface of fibroblasts and its near absence on virally transformed cells. Subsequently, numerous investigations focused on the role of fibronectin in mediating the social behavior of various cell types (24, 48). The influence of fibronectin on cell adhesion, cell shape, and cytoskeletal organization has now been clearly established. More recently, fibronectin has been implicated in cell locomotion and morphogenetic movement in embryonic and adult tissues (5, 8, 14, 18, 21, 33, 36), suggesting a nearly ubiquitous role for this molecule in tissue and cellular organization. The present study, in which chemotaxis chambers and thick, porous nitrocellulose filters have been used, has allowed for direct observation of malignant cell migration and the influence of fibronectin on such migration. The enhancement of directional migration of B16 melanoma cells caused by native fibronectin appears to be specific, since structurally modified fibronectin and other plasma proteins failed to elicit such a response (Table 1). The assay system used permits migratory responses to be observed in a relatively short period of time, from 1 to 5 hr (Fig. 1). This eliminates the involvement of cell doubling time and differential cell proliferation and also reduces the potentially deleterious effect of prolonged incubation of cells in serum-free medium. These latter aspects must be taken into account when commonly used 12- to 48-hr cell migration assays are performed (9, 45, 50). In addition, observations at early times (1 hr), when 60 to 65% of the cells in the fibronectin-containing chambers have migrated away from the surface of the filter as compared to only 20% of the control cells (Fig. 1), suggest that a significant portion of the B16 cell inoculum, rather than a minor subpopulation, responds to the presence of fibronectin.

The use of relatively thick filters (100 to 200 μm) permits either single cells (Fig. 2) or the entire population of cells to be observed (Fig. 1) as they migrate with time into and along the pores of the
nitrocellulose filter. The most distinct advantage of the present assay system, however, is the manipulation of both the removable filters and the separate upper and lower chambers. Results obtained by such manipulations have indicated that: (a) a gradient of fibronectin is the major effector of enhanced B16 cell-directed migration (Table 3); (b) fibronectin is affecting the actual movement of the cells, and does so in a manner independent of the cell’s initial adhesion and attachment to the filter (Table 4); and (c) the fibronectin molecule appears to be acting as a haptotactic agent in mediating the directed locomotion of the malignant cells as they migrate along a positive gradient of substratum-bound fibronectin (Table 5).

The exact mechanism and the physiological significance of enhanced B16 cell migration in response to fibronectin are not elucidated by the present study. Nevertheless, the nature of the specific response to fibronectin does permit some speculation concerning malignant cell migration. It appears that locomotion of many cell types, normal and malignant, is due to the continuous making and breaking of specific cell contacts with the substratum (16, 19). These cell contacts involve cytoskeletal components of the cell along with ecto- and transmembrane elements which mediate the contact between substratum and the locomotory machinery of the cell (10, 15, 41). A concentration gradient of specific substratum adhesion molecule(s) may be the driving force for the making and subsequent breaking of the cell contracts, resulting in a continuous, unidirectional mode of locomotion along a path of preferential adhesion. Such a concept was originally proposed by Carter (11) to explain the directionality of malignant cell movement in tumor invasion. It was hypothesized that movement towards surfaces of greater adhesion is a general phenomenon applicable to all cells which are dependent on contact with a surface for their motility (11). In the very simple in vitro model proposed herein, fibronectin bound to the nitrocellulose filter might provide the adhesive traction necessary for enhanced B16 cell migration as the cells initially attach and pull themselves along a gradient of bound fibronectin. In the absence of a gradient, when equal concentrations of fibronectin are placed above and below the filter (Tables 3 and 5), very little net directional movement occurs in response to the homogeneous environment of fibronectin.

The movement of different cells along a substratum may depend on the relative strength of traction and contractility of individual cell types. Harris et al. (19, 20) have shown that such cellular traction, defined as tangential shear force, is strongest in fibroblasts and weakest in the most mobile and invasive cells. Schor et al. (40) also have demonstrated that fibronectin bound to collagen gels inhibits the migration of hamster fibroblasts but increases the random migration of hamster melanoma cells. It is suggested that such variation in migratory behavior is due to a differential response to fibronectin and also possibly to a differential production of endogenous fibronectin by the different cell types. Thus, the diminished adhesiveness and enhanced motility often observed with malignant cells in vivo and in vitro may be due to quantitative, rather than qualitative, differences in the malignant cell’s traction with its encountered substratum.

Although it has been shown in a number of studies that fibronectin enhances the directed migration of various normal cells (5, 8, 14, 18, 21, 33, 36) and, in a few studies, the random migration of transformed cells (1, 40), the present study has demonstrated for the first time (see “Note Added in Proof”) that unidirectional migration of a malignant cell can occur along a haptotactic gradient of substratum-bound fibronectin. The physiological relevance of this observation, however, at present is unknown, since the existence of such fibronectin gradients in vivo may be difficult to demonstrate and quantitate. Nevertheless, a metastasizing tumor cell might encounter such a gradient when it interacts with the endothelial lining of a blood vessel. Fibronectin is present in high concentrations in the subendothelial basal lamina in contrast to the low levels found on the endothelial cell apical surface (7, 25), and this might represent a natural gradient. It has been observed that tumor cells, including B16 melanoma, stimulate retraction of endothelial cells and preferentially move and adhere to the underlying subendothelial basal lamina matrix in vitro (25, 34). It is also known that B16 cells and other malignant cells release specific enzymes that can solubilize and degrade fibronectin in such matrices, and cells of high invasive and malignant potential do this more effectively (26, 28, 34). As the invading cells enzymatically remove fibronectin, they may create a gradient of fibronectin in their path. The tumor cells could continue to degrade fibronectin at cell-substratum contact points and migrate along the basal lamina and possibly into surrounding tissues where local concentrations of matrix fibronectin might be higher. Thus, the ability of B16 cells to respond to a haptotactic gradient of fibronectin may be an important motivating factor in their invasive and metastatic behavior.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Peter B. Armstrong for his suggestions and critical reading of the manuscript.

Note Added in Proof

A similar response of B16 melanoma cells to a haptotactic gradient of fibronectin has been observed by McCarthy and Furcht (J. Cell Biol., in press, 1984 (communicated by L. Furcht)).

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Fig. 1. Light micrograph of B16 cells migrating into nitrocellulose filters. B16 cell migration into filters was carried out for 5 hr exactly as described in Chart 1. a, filter from a chemotactic chamber which contained buffer in the lower compartment. Very few cells have migrated more than 20 μm. b, filter from a chemotactic chamber which contained fibronectin in the lower compartment. Extensive migration away from the surface of the filter has occurred, and some cells have migrated as much as 60 μm. (× 450).

Fig. 2. Electron micrograph of B16 cell. Electron microscopic examination of B16 cells migrating through a 12-μm-pore nitrocellulose filter in response to fibronectin (300 μg/ml) revealed a morphologically oriented cell with a leading pseudopod free of organelles. At this particular stage in the locomotory process, the organelles are located behind the nucleus (× 5500; bar, 1 μm).
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