Type V Collagen Inhibits Migratory Response of Mouse B16F10 Melanoma Cells to Soluble and Substratum-Bound Fibronectin

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Abstract: The role of extracellular matrix components in the migration of cancer cells was investigated by means of a modified Boyden's chamber method. Mouse B16F10 melanoma cells demonstrated migratory responses to fibronectin and laminin in solution, but not to types I, III, IV or V collagens or elastin-derived peptide in solution. Mouse B16F10 melanoma cells demonstrated migratory response to substratum-bound fibronectin (haptotaxis) in a dose-related manner as well as to soluble fibronectin (chemotaxis). Type V collagen inhibited the migratory response of mouse B16F10 melanoma cells to soluble and substratum-bound fibronectin completely, although it did not inhibit cell attachment. Types I and III collagens and elastin-derived peptide caused partial inhibition of the migration of cells to soluble fibronectin. Type IV collagen had no inhibitory activity with either soluble or substratum-bound fibronectin. These results suggest that the migration of mouse B16F10 melanoma cells is influenced with the composition of the extracellular matrix components surrounding the cells and that the effect of extracellular matrix components differs whether the migratory response is chemotaxis or haptotaxis.

Keywords: mouse B16F10 melanoma cell, chemotaxis, haptotaxis, fibronectin, type V collagen

INTRODUCTION

The migration of tumor cells to components in extracellular matrices plays an important role in the early stage of cancer cell metastasis. Extracellular matrix components including fibronectin1, laminin1, collagen2 and elastin-derived peptide, VGVAPG3 are known to induce the adhesion and migration of cancer cells in vitro. Proteolytic fragments and synthetic peptides with the sequences of these matrix components have also been reported to inhibit experimental4 and spontaneous metastasis5 of tumor cells in vivo. However, it is unknown whether or not the effects of such matrix components on migratory behavior of cancer cells can be influenced with other matrix components that are localized in the vicinity of cancer cells.

In the present study we first examined the in vitro migratory response of mouse B16F10 melanoma cells to extracellular matrix components including fibronectin, laminin, various types of collagens, and elastin in solution. We found that fibronectin and laminin in solution enhance the migratory response of mouse B16F10 melanoma cells. However, the mechanisms of the migratory response of tumor cells to extracellular matrix components has been reported to differ depending on whether the extracellular matrix molecules are in solution or insoluble7. Next, we assessed the migratory response of mouse B16F10 melanoma cells to soluble (chemotaxis) and substratum-bound fibronectin (haptotaxis), and examined the effect of various types of collagens and elastin in solution on cellular chemotaxis and haptotaxis to fibronectin. Chemotaxis and haptotaxis of mouse B16F10 melanoma cells to fibronectin were significantly inhibited with type V collagen. The importance of matrix components and their interactions in cancer cell migration in vivo is discussed.

MATERIALS AND METHODS

Cell cultures: Mouse B16F10 melanoma cells, generously provided by Professor T. Turuo, Institute of Molecular and Cellular Biosciences, University of Tokyo, were cultured in
modified Eagle's medium (MEM) (Gibco Laboratories, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco Laboratories). For migration assays, subconfluent cultures were harvested with 5 mM EDTA in Dulbecco's phosphate-buffered saline (PBS) deficient in Ca²⁺ and Mg²⁺ and washed with MEM containing 10% FCS.

Preparation of protein substrates: Type I collagen was prepared from human bone and types III and V collagens were isolated from human placenta according to the previously reported method. Type IV collagen from human placenta was generously provided by Professor A. Ooshima, Department of Pathology, Wakayama Prefectural Medical University. Collagens were dissolved in 0.1 M acetic acid at a concentration of 1 mg/ml and diluted with MEM. Human fibronectin and laminin were purchased from Collaborative Research Laboratories, Inc. Bedford, MA. Elastin derived peptide (EDP) was prepared from human aorta according to the method previously reported.

Migration assay: Migration assays were performed in triplicate using a modified Boyden’s chamber with nucleopore polycarbonate filters (8 μm pore size, Costar Co. Pleasanton, CA). In the chemotactic migration assays, 0.5 ml aliquots of B16F10 melanoma cells in suspension (5 x 10⁵/ml in MEM) were placed into the upper wells of the chambers after 0.9 ml aliquots of attractants diluted in MEM (fibronectin, laminin, types I, III, IV or V collagens or EDP) were placed into the lower wells. For haptotactic migration assays, the filters were precoated with fibronectin on the lower surface at 37°C in a humidified incubator with a 5% CO₂ atmosphere for three hours. Negative control filters were precoated with MEM alone. After incubation, the filters were washed three times with PBS and dried at room temperature. MEM was placed in the lower wells of the chambers and then 0.5 ml of cells in suspension were placed into the upper wells prepared with precoated filters. The chambers were incubated at 37°C in a humidified incubator with a 5% CO₂ atmosphere for three hours. After incubation, the upper surface of the filter was washed with PBS. The filters were removed from the chambers and stained with hematoxylin eosin (H. E.). Chemotactic activity was quantified by counting the number of migrating cells on the lower surface of the filter in 10 randomly selected high power fields (HPF) (×400, Magnification). These experiments were repeated three times. Data are presented as the means plus or minus the standard errors for three experiments.

Inhibition assay: In the chemotactic migration assays, 0.45 ml of fibronectin at a concentration of 12 μg/ml was mixed with an equal volume of types I, III, IV, or denatured type V collagens, EDP in MEM at 20 μg/ml or MEM alone as a control. The protein mixtures were placed into the lower wells of the chambers. In the haptotactic migration assays, the filters were precoated with 6 μg/ml of fibronectin on the lower surface. Reagents at a concentration of 10 μg/ml or MEM as a control were placed into the lower wells of the chambers prepared with fibronectin-coated filters. B16F10 melanoma cells in suspension (0.5 ml, 5 x 10⁵/ml in MEM) were placed into the upper wells and the chambers were incubated as above. Percent migratory activity was quantified as: (number of cells migrating in the presence of inhibitor/number of cells migrating in the absence of inhibitor) x 100. Data are presented as the means plus or minus the standard errors for three experiments. Statistical significance of the data was evaluated with Student’s t-test.

Attachment assay: Inhibition assays were performed as above. The upper surface of the filter was gently washed twice with PBS, and the filters were fixed and stained with H. E. Only visual examination of the upper surface was performed.

RESULTS

1. Migratory response of mouse B16F10 melanoma cells to fibronectin and laminin in solution

Among the seven matrix components tested, only fibronectin and laminin in solution (6 μg/ml) enhanced the migratory response of mouse B16F10 melanoma cells (240 ± 49, 841 ± 120). Types I, III, IV, and V collagens and EDP in solution (10 μg/ml) had no such activity (Table 1).

2. Inhibitory effect of type V collagen on the migratory response of mouse B16F10 melanoma cells to soluble and substratum-bound fibronectin

The soluble fibronectin-induced migratory response of mouse B16F10 melanoma cells was dependent on the concentration of fibronectin...
Table 1: Migratory response of mouse B16F10 melanoma cells in the upper wells to matrix components in the lower wells. Values are means ± standard errors for three experiments.

<table>
<thead>
<tr>
<th>Reagents (lower well)</th>
<th>Actual Migratory Activity (cells/10HPF) mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN (6)</td>
<td>240 ± 49</td>
</tr>
<tr>
<td>LN (6)</td>
<td>841 ± 120</td>
</tr>
<tr>
<td>Type I collagen (10)</td>
<td>0</td>
</tr>
<tr>
<td>Type III collagen (10)</td>
<td>0</td>
</tr>
<tr>
<td>Type IV collagen (10)</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Type V collagen (10)</td>
<td>0</td>
</tr>
<tr>
<td>EDP (10)</td>
<td>0</td>
</tr>
</tbody>
</table>

( ) : concentration, µg/ml

from 3 µg/ml to 12.5 µg/ml (Fig. 1a). From checker-board analysis, the mode of cell migration by soluble fibronectin was found to be mainly chemotactic (data not shown). Mouse B16F10 melanoma cells demonstrated a migratory response to substratum-bound fibronectin in a dose-related manner from 3 µg/ml to 12.5 µg/ml as found with soluble fibronectin (haptotaxis) (Fig. 1b). The concentration-response curves for chemotaxis and haptotaxis were nearly similar.

The effect of types I, III, IV, V, and denatured type V collagens and EDP in solution (10 µg/ml) on the chemotaxis and haptotaxis of B16F10 melanoma cells to fibronectin was examined (Fig. 2, 3). When these matrix molecules in solution were mixed with soluble fibronectin and put into the lower wells of the chambers (Fig. 2), type V collagen was found to inhibit significantly the migratory response of cells to soluble fibronectin (2 ± 1%, p < 0.001). Types I and III collagens and EDP also inhibited the migratory response to lesser extents (45 ± 11%, 45 ± 11%, 45 ± 11% for types I, III, and EDP, respectively).

Fig. 1: Concentration curves of the migratory response of mouse B16F10 melanoma cells to soluble (a) and substratum-bound fibronectin (b). Activity is expressed as the number of migrating cells counted in 10 high power fields (× 400). Values are means ± standard errors for three experiments.

Fig. 2: Inhibitory effect of various types of collagens and EDP in solution on the soluble fibronectin-induced migratory response of mouse B16F10 melanoma cells. Soluble fibronectin, 6 µg/ml, was mixed with various matrix components at 10 µg/ml and the mixtures were placed into the lower wells of a Boyden’s chamber apparatus. Migration assays were performed. Percent migratory activity is expressed as percent migration relative to the migratory activity of soluble fibronectin. Values are means ± standard errors for three experiments.

* type V: control; p < 0.001, ** types I and III, EDP: control; p < 0.01
cell migration to soluble and substratum-bound fibronectin was inhibited with type V collagen. There was no decrease in the number of cells attached to the upper surface of the filter (Fig. 4).

During incubation of mouse B16F10 melanoma cells with various matrix components at 10 μg/ml, migration was significantly inhibited in the presence of fibronecin at a concentration of 6 μg/ml. Migration assays were performed. Percent migratory activity is expressed as percent migration relative to the migratory activity of substratum-bound fibronectin. Values are means ± standard errors for three experiments: *Type V: control; p<0.001.

80±3%, 26±10%; p<0.01). However, type IV and denatured type V collagens had no such inhibitory activity. When the matrix molecules in solution were put into the lower wells of chambers prepared with the filters precoated with fibronectin (Fig. 3), only type V collagen significantly inhibited the migratory response of cells to substratum-bound fibronectin (1±0%, p<0.001). Types I, III, IV, and denatured type V collagen and EDP showed no inhibitory activity.

Direct incubation of type V collagen and EDP with cells in the upper wells did not produce any significant inhibition of the migratory response to soluble fibronectin (108±1%, 119±1%) (Table 2).

The effect of type V collagen on the attachment of mouse B16F10 melanoma cells to the upper surface of the filter was examined. While cell migration to soluble and substratum-bound fibronectin was inhibited with type V collagen, there was no decrease in the number of cells attached to the upper surface of the filter (Fig. 4).

Direct incubation of type V collagen and EDP with cells or soluble fibronectin on the migratory activity of soluble fibronectin in the lower wells. Soluble EDP and type V collagen were put into the upper wells with the cell suspensions or into the lower wells with soluble fibronectin. Migratory activity is expressed as percent migration relative to the migratory activity of soluble fibronectin. Values are means ± standard errors for three experiments.

**DISCUSSION**

Mouse B16F10 melanoma cells are highly metastatic to the lung. In the lung, where B16F10 melanoma cells migrate and proliferate, the cells encounter extracellular matrix components including fibronectin, laminin, collagens, and elastin. In the present study, we have examined the effect of these matrix molecules, which are the major architectural components of the lung, on the migratory response of mouse B16F10 melanoma cells in vitro using a modified Boyden's chamber. Among the matrix molecules examined, fibronectin and laminin in solution significantly enhanced the migratory response of type V collagen and EDP solution did not enhance cell migration (Table 1). Mundy et al. reported that types I, III, IV, and V collagens and EDP in solution did not enhance cell migration (Table 1). Mundy et al. reported that types I, III, and V collagens were chemotactic for Walker 256 rat carcinosarcoma cells. Blood et al. reported that elastin-derived peptide, VGVAPG, was chemotactic for the M27 clone of murine Lewis lung carcinoma. The present results, however, indicate that types I, III and V collagens and EDP are not chemotactic for mouse B16F10 melanoma cells. The migratory response of different kinds of tumor cells to collagens and elastin therefore appears to be different.

The migratory responses of tumor cells to extracellular matrix molecules have been report-

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**Table 2** Effect of direct incubation of type V collagen and EDP with cells or soluble fibronectin on the migratory activity of soluble fibronectin in the lower wells.

<table>
<thead>
<tr>
<th>Cells and reagents in Boyden's chamber</th>
<th>Upper well</th>
<th>Lower well</th>
<th>Activity(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells in MEM</td>
<td>FN (6)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>cells in Type V (10)</td>
<td>FN (6)</td>
<td>108±1</td>
<td></td>
</tr>
<tr>
<td>cells in MEM</td>
<td>FN (6) + Type V (10)</td>
<td>2±1</td>
<td></td>
</tr>
<tr>
<td>cells in EDP (10)</td>
<td>FN (6)</td>
<td>119±1</td>
<td></td>
</tr>
<tr>
<td>cells in MEM</td>
<td>FN (6) + EDP (10)</td>
<td>26±10</td>
<td></td>
</tr>
</tbody>
</table>

( ) : concentration, μg/ml

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Fig. 4 Effect of type V collagen on the attachment of mouse B16F10 melanoma cells to the upper surface of filter prepared with fibronectin. While the migratory response of mouse B16F10 melanoma cells to soluble and substratum-bound fibronectin was inhibited with type V collagen (a), there was no decrease in the number of cells adhering to the upper surface of the filter (b). ×25
ed to differ depending on whether the molecules are in solution or insoluble\(^2\). A2058 human melanoma cells demonstrate chemotaxis and haptotaxis to fibronectin, lammin, and type IV collagen. However, some differences between the concentration-response curves for chemotaxis and haptotaxis have been reported, and the effect of pertussis toxin and cholera toxin on the chemotaxis of A2058 cells has been reported to differ from the effect on haptotaxis\(^7\). We therefore examined the migratory response of mouse B16F10 melanoma cells to soluble fibronectin (chemotaxis) and substrate-bound fibronectin (haptotaxis), and the effect of other matrix molecules on these chemotactic and haptotactic responses. The patterns of chemotaxis and haptotaxis of mouse B16F10 melanoma cells to fibronectin were dose-related from 3 \(\mu\)g/ml to 12.5 \(\mu\)g/ml and nearly the same (Fig. 1). Type V collagen significantly inhibited chemotaxis and haptotaxis of mouse B16F10 melanoma cells to fibronectin (2 \(\pm\)1%, 1 \(\pm\)0%, \(p<0.001\)) (Fig. 2, 3). Type I and type III collagens and EDP inhibited only chemotaxis (45 \(\pm\)11%, 80 \(\pm\)3%, 26 \(\pm\)10%; \(p<0.01\)) (Fig. 2). Type IV collagen had no inhibitory, synergistic, or additive effects on chemotaxis or haptotaxis to fibronectin.

The mechanisms by which type V collagen inhibits the migratory response of cells to soluble and solid fibronectin are unknown. The fact that direct incubation of cells with type V collagen did not inhibit cell migration suggests that the inhibitory effect of type V collagen may be due to an interaction between type V collagen and fibronectin. So far, three cell adhesion sites of fibronectin, the cell binding domain containing RGDS\(^9\), CS1 and CS5 in the type III connecting segment region (III CS)\(^10\), and peptide 1 and peptide 2 in the heparin binding domain just adjacent to III CS on the amino-terminal side\(^11\) have been reported. The cell binding domain containing RGDS and the III CS binding domain have been found to bind \(\alpha_\beta\beta\) integrin receptors, respectively, and the heparin binding domain interacts with cell surface proteoglycans\(^12\). Of these adhesion domains, a 75 kD tryptic fragment of fibronectin containing RGDS has been reported to promote the adhesion, spread, and migration of B16F10 melanoma cells\(^13\). However, a 33 kD heparin binding fragment containing CS1, peptide 1, and peptide 2 enhances adhesion, but does not induce B16F10 melanoma cell migration\(^13\). The fact that type V collagen inhibits cell migration, but not cell adhesion, indicates that type V collagen interferes with the binding of cell surface to the 75 kD tryptic fragment. The fact that denatured type V collagen does not inhibit cell migration suggests that the tertiary structure of type V collagen is needed for this inhibition. It is reported that the behavior of the cells induced by some matrix molecules is modulated by other matrix molecules. Chondroitin sulfate proteoglycan inhibits the migration of neural crest cells on fibronectin\(^14\), while an immobilized large chondroitin sulfate proteoglycan from chick embryo fibroblasts, PG-M, inhibits cell adhesion to various substrates\(^15\). It therefore appears possible that cancer cell migration may be modulated by type V collagen in vivo.

Type V collagen exists in vivo in various connective tissues as fine fibrils located either alone or between types I and III collagens, and connects the basal lamina to interstitial collagen fibers\(^16\). Type V collagen has been reported to bind to insulin\(^17\) and heparin\(^18\) and has been suggested that it acts as a reservoir for insulin and contributes to the growth control of animal cells\(^17\). The present study suggests that type V collagen located between the basal lamina and the interstitium inhibits cells from migrating from vessels and retains them. The cells therefore grow between the basal lamina and the interstitium.

Types I and III collagens and EDP partially inhibit chemotaxis of mouse B16F10 melanoma cells to fibronectin, but do not inhibit haptotaxis. The mechanisms for this are unknown, but there may be some differences between the two types of migratory response.

Our results suggest that not only cell-matrix interactions but also matrix-matrix interactions may influence the extent of extravasation and tissue invasion by certain cancer cell types.

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REFERENCES


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