Laminin Receptor Complementary DNA-deduced Synthetic Peptide Inhibits Cancer Cell Attachment to Endothelium

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ABSTRACT

Stable attachment of cancer cells to the endothelium is a key step in the formation of metastasis. In this study, we have investigated the possibility that interaction between laminin and its M, 67,000 high-affinity receptor (67 LR) could play a major role in this process. Scatchard analysis of laminin-binding studies showed that bovine aortic endothelial cells exhibit 46,000 high-affinity receptors that mediate, at least in part, the attachment of highly invasive melanoma cells. This endothelial cell-melanoma cell interaction was significantly inhibited by soluble laminin and by anti-laminin antibodies. Peptide G, an eicosapeptide derived from the cDNA elementary DNA sequence of the 67 LR precursor (IPCNKGAHVGLMWVMLAR) that specifically binds to laminin and presumably contains the active ligand-binding site of the receptor, specifically prevented attachment of the melanoma cells to both the bovine aortic endothelial cell monolayer and human umbilical vein endothelium. Thus, peptide G may selectively interfere with the metastatic cascade by inhibiting tumor cell attachment to endothelium via the laminin-67 LR pathway and is a potential new antimetastatic agent.

INTRODUCTION

Tumor metastasis is the major cause of morbidity and mortality in cancer patients (1). It is the result of a complex succession of events in which interactions between cancer cells and host tissues are determinant (2, 3). Stable attachment of tumor cells to the endothelium, the first step of extravasation, is particularly critical for the success of hematogenous metastasis (2-5). The identification of the specific mechanism(s) leading to the anchorage of metastatic cancer cells to the vasculature offers the potential for selectively interfering with the metastatic cascade. Previous reports have implicated the basement membrane component laminin in blood-borne metastasis formation (6-9). Precipitation of murine melanoma cells with laminin dramatically enhanced experimental metastasis (6). Highly metastatic fibrosarcoma cells expressed a higher affinity receptor, specifically prevented attachment of the melanoma cells to both the bovine aortic endothelial cell monolayer and human umbilical vein endothelium. Thus, peptide G may selectively interfere with the metastatic cascade by inhibiting tumor cell attachment to endothelium via the laminin-67 LR pathway and is a potential new antimetastatic agent.

MATERIALS AND METHODS

Reagents. Laminin was purified from the mouse Engelbreth Holm Swarm tumor as previously described (35). 125I (37 mCl/µg) was obtained from Amersham Corp. (Arlington Heights, IL) and Enzymobeads from Pierce (Rockford, IL). Polyclonal rabbit anti-laminin antibodies were obtained from BRL (Gaithersburg, MD). Synthetic Peptides and Their Corresponding Antibodies. Synthetic peptides corresponding to different regions of the 67 LR precursor as well as a scrambled sequence peptide were synthesized according to the predicted amino acid sequence of the cDNA clone on a Biosearch 9600 peptide synthesizer (Table 1). Purity of the peptides was verified by high-pressure liquid chromatography. The pi of each peptide was calculated by the IBI Puristel analysis software using the Henderson-Hasselbach equation. Their average hydrophobicity was calculated by the Helmwheel Program of PC/Gene. Anti-synthetic peptide antibodies 4160 and 4237, directed against extracellular domains of the 67 LR (Table 1), were raised in rabbits and affinity purified according to a protocol previously described (28). The specificity and sensitivity of each antibody was determined using an enzyme-linked immunosorbent assay as previously described (23). Cells and Umbilical Veins. Human melanoma A2058 cells were obtained from G. Todaro, National Cancer Institute and have been described (36). BAEC were kindly provided by the laboratory of Dr. J. Madri and were previously described (29, 30). The cells were grown in DMEM (Gibco) with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml), as described before (28). Umbilical veins were isolated from freshly delivered umbilical cords obtained from the Shady Grove Hospital (Rockville, MD). The veins were immediately flushed with DMEM and kept in the cold room until used. The maximum delay between delivery and the use of the umbilical vein was <3 h.

Laminin-binding Assay. To examine the presence of laminin-binding sites at the surface of BAEC, we measured the ability of iodinated...
laminin to bind to confluent BAEC monolayers grown in 96-well tissue culture clusters (Nunclon). To standardize the assay, confluent BAEC monolayers were prepared by plating 5 x 10^4 cells/well 2 days before the assay. Laminin was inoculated to a specific activity of 3-7 µCi/µg using Enzymobeads (Pierce, Rockford, IL) following the procedure recommended by the manufacturer. Triplicate wells of BAEC monolayers were incubated with increasing amounts of iodinated laminin for 90 min in PBS, pH 7.4, and 1% BSA, in the presence or the absence of a 200-fold excess of unlabeled laminin. After incubation, the wells were washed three times each with PBS containing 1% BSA, the cells were solubilized in 1% sodium dodecyl sulfate, and the radioactivity in each well was determined using a gamma counter. The apparent Kd was calculated using the computer-assisted program “Ligand” (37). The number of receptors/cell was determined by using the “Ligand” program and by counting the number of BAEC/well in triplicate wells prepared in the same way as was used for the binding assay.

Immunofluorescence. The presence of the 67 LR on the surface of BAEC was demonstrated on nonpermeabilized cells as described previously (22). Briefly, BAEC grown on glass coverslips were fixed in 3.7% formaldehyde in PBS. The cells were then washed five times with PBS and incubated for 30 min at room temperature with affinity-purified anti-laminin receptor synthetic peptide 4160 diluted 1:50 in PBS. Affinity-purified antibody 4160 (see Table 1) is directed against an eicosapeptide predicted from the cDNA sequence of the 67 LR precursor and has been previously characterized (23, 24). The cells were then washed five times in PBS, mounted, and examined under a Zeiss immunofluorescence microscope. In control experiments, the first antibody was replaced by normal rabbit IgG. Anti-actin antibody was used to assess the nonpermeabilized state of the cells (22). The presence of laminin on the surface of A2058 cells was examined using polyclonal anti-laminin antibodies (diluted 1:100 recommended by the manufacturer) on A2058 live cells in suspension prepared in 1% BSA as described before (38). In control experiments, cells were incubated with preimmune rabbit serum as the first antibody.

Attachment Assays. The interactions between human melanoma A2058 cells and endothelial cells were examined using a modification of a previously described in vitro model (32). BAEC cells were grown to confluence in 96-well plates as described above. A2058 cells were metabolically radiolabeled using [35S]methionine as described previously (28), gently detached with a rubber policeman, and resuspended to a concentration of 10^6 cells/ml in DMEM containing 0.1% BSA. The viability of the cells was assessed by the trypan blue exclusion test and was always found to be >95%. A2058 cells, 10^5/well, were then applied to BAEC confluent monolayers and incubated at 37°C for the given period of time. The unattached cells were removed by three gentle washes using DMEM containing 0.1% BSA. The attached cells were then lysed in 1% sodium dodecyl sulfate, and the radioactivity was quantified by liquid scintillation counting. The BAEC monolayers and the attachment of A2058 cells to the endothelial cells were analyzed with a Zeiss inverted microscope as well as by scanning electron microscopy. For the latter, samples were processed and examined as previously described (11).

The attachment assay described above was used to test the effect of laminin, anti-laminin antibodies, synthetic peptides deduced from the 67 LR precursor cDNA sequence, and their corresponding affinity-purified antibodies, on melanoma cell attachment to endothelium. To study laminin effects, increasing amounts of laminin were added simultaneously with A2058 cells to the BAEC monolayers for 20 min at 37°C. In other experiments, A2058 cells were preincubated with gentile rocking for 10 min at room temperature with serial dilutions of heat-inactivated anti-laminin antibodies, affinity-purified anti-laminin receptor synthetic peptide antibody 4160 or nonimmune serum and then incubated with BAEC monolayers for 20 min as described above. Finally, the potential inhibitory effect of peptide G, a 67 LR precursor eicosapeptide that contains the active binding domain of the 67 LR, was tested. A2058 cell suspensions were mixed with increasing amounts of peptide G, or control peptides, and incubated immediately, or after 30 min incubation at room temperature, with triplicate BAEC monolayers. The trypan blue exclusion test was used to exclude a direct toxic effect of the peptide on the A2058 cells. In some experiments, after 30 min incubation with peptide G at room temperature, A2058 cells were washed twice in DMEM containing 0.1% BSA before incubation with the BAEC monolayers. The attachment assays were carried out as described above.

The effect of peptide G or control peptides on the attachment of A2058 cells to ex vivo human umbilical vein endothelium was tested as follows. The freshly delivered vein was dissected and opened. Pieces of open umbilical vein with the endothelial surface facing up were placed in 24-well tissue culture clusters (Nuncron) containing a cushion of 1% agarose in DMEM. The umbilical vein endothelium was then incubated for 20 min at 37°C with a suspension of metabolically radiolabeled A2058 cells in DMEM containing 0.1% BSA. After incubation, the vein walls were washed three times in DMEM containing 0.1% BSA, and the bound radioactivity was measured by liquid scintillation and expressed as the amount of radioactivity/mm^2 of endothelium. Experiments were performed in triplicate.

RESULTS

BAEC Exhibit High-affinity Laminin-binding Sites. Laminin-binding assays, performed on confluent BAEC monolayers, indicated that BAEC express on their surface saturable binding sites for laminin (Fig. 1A). Scatchard analysis of the binding data performed using the computer-assisted program “Ligand” calculated that the apparent affinity of the laminin-binding sites was in the nM range (Kd 9 nM) (Fig. 1A, inset). The concentration of laminin that saturated these sites was calculated to be 7.45 x 10^-10 M which corresponds to 9.2 x 10^5 binding sites/well. Since the BAEC monolayer was composed of 200,000 endothelial cells/well, we calculated that each BAEC exhibits 46,000 laminin receptors on its surface. At a dilution of 1:100, antibody 4237, an affinity-purified polyclonal antibody raised against peptide G, partially (25%) inhibited the binding of laminin to BAEC. Antibody raised against control peptides had no inhibitory effect.

BAEC Express Cell Surface 67 LR. Affinity-purified antibody 4160 (Table 1) was used in immunofluorescence studies performed on BAEC cells grown on glass coverslips. The endothelial cell surface was stained with a fine granular pattern (Fig. 1B). Preincubation of antibody 4160 with its corresponding peptide completely abolished its ability to stain the BAEC (Fig. 1C).

Laminin and Anti-Laminin Antibodies Inhibit Attachment of A2058 Cells to BAEC Monolayers. The interactions between human melanoma A2058 cells and endothelial cells were analyzed by scanning electron microscopy (data not shown). Such studies confirmed that the BAEC formed confluent monolayers in the tissue culture wells. The endothelial cells came into close contact with each other. The melanoma cells attached to the endothelial cells within 10 min (data not shown). First, the cancer cells appeared to round and were covered with several retracted pseudopodia. Ten to 20 min after attachment, the cells emitted pseudopodia on the endothelial cells and spread onto the BAEC. This sequence of events was similar to previous observations of interactions between BAEC and murine B16 melanoma cells (33).

The potential modulatory effect of soluble laminin on the attachment of A2058 cells to BAEC monolayers was assessed by adding increasing concentrations of laminin with the melanoma cells to the BAEC monolayers. Fig. 2A shows that soluble laminin inhibited attachment of the melanoma cells to the...
Fig. 1. BAEC exhibit cell surface high-affinity laminin receptors. In A, confluent BAEC monolayers were incubated with increasing amounts of iodinated laminin and the amount of bound radioactive laminin was determined as described in "Materials and Methods." The specific binding (O) represents the difference between binding in the absence or the presence of a 200-fold excess of unlabeled laminin. For each experiment, results are presented as the average of triplicate determinations; counts in each experiment differed by <10%. In the inset, Scatchard plot regression of the binding data is shown. In B, indirect immunofluorescence was performed on nonpermeabilized BAEC using affinity-purified anti-laminin receptor synthetic peptide antibody as described in "Materials and Methods." The staining of the surface of the BAEC appears finely granular. In C, incubation of the antibody with its corresponding peptide abolished the staining. Bar, 10 μM.

Peptide G Inhibits Attachment of A2058 Cells to BAEC Monolayers. Peptide G, an eicosapeptide deduced from the 67 LR precursor cDNA sequence that contains the high-affinity binding site of the laminin receptor, was assayed as a potential inhibitor of cancer cell attachment to endothelium using the in vitro assay described above. Peptide G specifically inhibited, in a dose-dependent manner, the attachment of A2058 cells to confluent BAEC monolayers (Fig. 4). Neither peptide GX (Table 1), containing a scrambled peptide G sequence, nor peptides F and H, eicosapeptides corresponding to other extracellular domains of the 67 LR precursor, had such an inhibitory activity (Fig. 4).

Peptide G Inhibits Attachment of A2058 Cells to Human Umbilical Vein Endothelium. The effect of peptide G on the attachment of human melanoma cells to ex vivo human umbilical vein endothelium; a maximum effect (75% inhibition) was obtained at a laminin concentration of 200 μg/ml. Anti-laminin antibodies also interfered, in a dose-dependent manner, with the ability of A2058 cells to attach to BAEC monolayers (Fig. 2B). Neither preimmune rabbit serum (Fig. 2B) nor antibody 4160, which binds to the surface of A2058 cells inhibited the attachment of the melanoma cells to BAEC (data not shown). The anti-laminin antibodies specifically stained live A2058 cells, indicating that they are coated with laminin (Fig. 3).
INHIBITION OF CANCER CELL ATTACHMENT TO ENDOTHELIUM

Table 1 Laminin receptor synthetic peptides derived from the cDNA sequence and their corresponding antibodies

<table>
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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Position</th>
<th>pI</th>
<th>HP</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>TPGTFTNQIAAFREPRLLV</td>
<td>104–123</td>
<td>10.1</td>
<td>0.02</td>
<td>4160</td>
</tr>
<tr>
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<td>146–164</td>
<td>3.9</td>
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<tr>
<td>G</td>
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<td>11.1</td>
<td>0.19</td>
<td>4237</td>
</tr>
<tr>
<td>H</td>
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<td>216–234</td>
<td>4.3</td>
<td>0.02</td>
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<tr>
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<td>261–284</td>
<td>11.1</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>

* The numbers indicate the position of the first and the last amino acid of the designated peptide, numbered from the amino terminus of the laminin receptor sequence; peptide GX contains a scrambled sequence of peptide G.

\[ \text{pi} \] was calculated by the IBI pustell analysis software using the Henderson-Hasselbach equation.

\[ \text{Average hydrophobicity (HP)} \] was calculated by the Helwheel program of PC/Gene.

\* Affinity-purified antibodies (AB) raised against the corresponding synthetic peptide.

Fig. 2. Laminin and anti-laminin antibodies inhibit attachment of A2058 cells to BAEC monolayers. In A, human melanoma cells were incubated with BAEC monolayers in the presence of increasing amounts of laminin as described in “Materials and Methods.” In B, another set of experiments, A2058 cells were incubated with serial dilutions of anti-laminin polyclonal antibody (○) or preimmune serum (▲). Binding of the antibodies was revealed using fluoresceinated anti-rabbit IgG antibodies as described in “Materials and Methods.” Original magnification, × 100.

DISCUSSION

One of the most important events in blood-borne tumor metastasis is the attachment of circulating malignant cells to

Fig. 3. A2058 cells are coated with laminin. The presence of laminin on the surface of live A2058 cells in suspension was examined by indirect immunofluorescence using anti-laminin polyclonal antibody (○) or preimmune serum (▲). Binding of the antibodies was revealed using fluoresceinated anti-rabbit IgG antibodies as described in “Materials and Methods.” Original magnification, × 100.

Fig. 4. Peptide G inhibits attachment of melanoma cells to BAEC monolayers. Human melanoma cells and the indicated concentration of peptides G (○), GX (▲), F (▲), and H (△) were applied to BAEC monolayers for 20 min as described in “Materials and Methods.” For each experiment, results are presented as the percentage of control and are the average of triplicate determinations; counts in each experiment differed by <10%.

Fig. 5. Peptide G interfered with the attachment of A2058 cells to the human umbilical vein endothelium in a dose-dependent manner. Control peptide GX had no inhibitory activity (Fig. 5).

Fig. 6. Peptide G inhibits attachment of melanoma cells to BAEC monolayers.
the vascular endothelium (2–5, 11). The interactions of circulating malignant cells with endothelial cells have been extensively investigated (2, 5, 11, 32, 33, 39), mainly by using the endothelial monolayer in vitro model (5, 32, 33). These studies have shown that the most usual initial event in this interaction is the attachment of malignant cells to the apical surface of the endothelial cells through multiple adhesion mechanisms (5, 33). The cancer cells then induce endothelial cell retraction, a phenomenon that leads to the exposure of the subendothelial basement membrane. Cancer cells bind avidly to subendothelial basement membrane components including laminin, heparan sulfate proteoglycan, and type IV collagen (5). Thus, two different steps are involved in the initial interactions of cancer cells with the vasculature: (a) adhesion of cancer cells to the surface of the endothelial cells and (b) adhesion of the cancer cells to the subendothelial matrix. It is also possible that cancer cells may, in vivo, directly attach to naturally exposed subendothelial matrix. Nevertheless, in the experimental BAEC monolayer model used in the current study, the latter process does not appear to participate significantly in the initial attachment of cancer cells (33). In this report, we have confirmed that BAEC monolayers provide an appropriate model system to study the initial interactions between human A2058 melanoma cells and endothelium. Indeed, using scanning electron microscopy technique, we observed that the sequence of events in the attachment of melanoma cells to confluent BAEC monolayers was similar to those previously described for other cell lines (32, 33).

In this study, we have specifically investigated the mechanisms involved in the initial adhesion of cancer cells to the endothelial cell surface and have proposed the hypothesis that the 67 LR that are present on the surface of endothelial cells could play a critical role in the initial anchorage of laminin-coated cancer cells to the endothelium. This hypothesis requires (a) that the endothelial cells express unoccupied 67 LR on their surface and (b) that the cancer cells can be coated with laminin.

The 67 LR is a high-affinity receptor that has been previously identified on the surface of BAEC, in addition to other laminin receptors such as members of the integrin family (29, 30, 31). Binding studies performed on BAEC monolayers demonstrated that BAEC exhibit 46,000 saturable high-affinity laminin-binding sites/cell, with an apparent $K_d$ of 9 nM. This is similar to the affinity of laminin for the 67 LR found in tumor cells (13), suggesting that the laminin-binding sites present on BAEC are, at least in part, 67 LR. Affinity-purified antibodies raised against an extracellular domain of the 67 LR identified membrane-associated molecules on nonpermeabilized BAEC, confirming the presence of 67 LR on the BAEC surface. Furthermore, the binding of laminin to BAEC was partially (25%) inhibited by affinity-purified polyclonal antibody raised against a cDNA-derived 67 LR synthetic peptide containing the laminin-binding site. The low inhibitory activity may be explained by (a) the low titer of the antibody, due probably to the fact that the amino acid sequence used to immunize the rabbit is highly conserved between species and therefore poorly immunogenic (25, 28), (b) the low affinity of the anti-synthetic peptide antibody for the native receptor, and/or (c) by the presence of multiple laminin-binding proteins on the BAEC.

Peptide G is an eicosapeptide, predicted by the cDNA sequence of the 67 LR, that has been shown to contain a high-affinity laminin-binding site for laminin (34). To investigate the role of 67 LR present on endothelial cells in the attachment of laminin-coated melanoma cells, we tested the ability of peptide G to inhibit melanoma cell attachment to BAEC monolayers. As expected, peptide G specifically interfered with the attachment of A2058 cells to the endothelial cells in a dose-dependent manner. Neither peptide GX, containing a scrambled sequence of peptide G, nor other peptides, containing sequences adjacent to peptide G on the 67 LR, had modulatory activity. These experiments specifically implicate the 67 LR as playing a crucial role in the attachment of cancer cells to the endothelium. To our knowledge, this is the first demonstration that a synthetic extracellular matrix receptor peptide containing the ligand-binding site can interfere with ligand-receptor interactions.

The second requirement of our hypothesis was that cancer cells use laminin on their surface to interact with the BAEC 67 LR. Laminin, a high molecular weight basement membrane glycoprotein (35), has been implicated in the genesis of blood-borne metastases (6–9). A2058 cells are coated with laminin synthesized and secreted by the melanoma cells themselves (38). Using the BAEC monolayer system, we demonstrated that soluble laminin inhibits, in a dose-dependent manner, the attachment of A2058 cells to the endothelium. Presumably, the soluble laminin competes with the laminin on the melanoma cell surface for the laminin-binding sites present on the endothelial cells. The difference between the concentration of soluble laminin used to inhibit melanoma cell attachment to BAEC (100 μg/ml, Fig. 2) and the concentration needed to saturate BAEC laminin receptors (1 μg/ml, Fig. 1) is probably due to the fact that the two assays are exploring different biological events. In the former, soluble laminin competes with melanoma cell surface laminin for laminin-binding sites on BAEC, while in the latter, the direct interaction between soluble laminin and BAEC receptors is tested. When anti-laminin antibodies were added to the melanoma cells, they inhibited A2058 cell attachment to the endothelium. This effect was specific for laminin since another antibody that can bind to the A2058 cell surface had no inhibitory effect. We hypothesize that the laminin antibodies block the laminin on the melanoma cell surface and thus prevent melanoma cell interactions with the BAEC laminin-binding sites. These in vitro results confirm previous in vivo
experiments demonstrating the role of cancer cell surface laminin in cancer cell-endothelial cell interactions (6–12).

Our data have led us to conclude that the initial anchorage of laminin-coated melanoma cells to the endothelial cells in the BAEC monolayer model occurred, at least in part, through the 67 LR present on endothelial cells. This newly identified pathway is represented in Fig. 6C. According to this model, the mechanism by which peptide G inhibits the attachment of melanoma cells to the endothelium could be (a) competitive inhibition of the specific interaction of laminin on the melanoma cell with the 67 LR on the endothelial cells or (b) dissociation of laminin from the melanoma cell surface, preventing interaction with any and all types of laminin receptors on the endothelial surface. However, the latter alternative does not appear to be operative since laminin-binding studies have shown that peptide G does not inhibit the ability of laminin to bind to melanoma cells.4

It has been proposed, based on in vivo experimental data, that the role of laminin on the surface of circulating cancer cells is to mediate the attachment of cancer cells to type IV collagen molecules accessible in naturally exposed areas of the subendothelial matrix (6). This hypothetical pathway is represented in Fig. 6A. In addition, some investigators (40) have proposed that it is the increase of unoccupied laminin receptors on the cancer cell surface, and not the increase of cell surface laminin, that leads to the attachment of cancer cells to laminin molecules present in naturally exposed subendothelial basement membranes (Fig. 6B). These two mechanisms imply that the initial contact of circulating cancer cells with the vasculature is with naturally exposed subendothelial matrix and not with the endothelial cell surface itself. The exact pathway(s) relevant in vivo to the genesis of blood-borne metastasis has not been established to date. The three different scenarios presented in Fig. 6 are not mutually exclusive. A potential criticism of our study is that we have worked with a heterologous bovine/human model and that the results may be artifactual or irrelevant to human cancer. We therefore examined the effect of peptide G on the attachment of A2058 cells to ex vivo human umbilical vein endothelium. We have previously shown that anti-laminin antibodies inhibit attachment of cancer cells to ex vivo perfused human umbilical veins (11). In the current study, peptide G also specifically and significantly inhibited the attachment of human melanoma cells to human umbilical vein endothelium. These results clearly indicate that the 67 LR mediates, at least in part, the attachment of human cancer cells to human endothelium.

Several studies have suggested the existence of tissue-specific endothelial cell surface molecules involved in the preferential localization of specific metastatic cancer cells (40–44). We suggest that both tissue-specific and -non specific mechanisms exist for the anchorage of cancer cells to vascular endothelium. Such mechanisms must assure stable attachment of the cancer cell to the endothelium for a sufficient time to allow the next step of the metastatic cascade, namely, extravasation, to occur. The data presented in this study lead us to propose that the laminin/67 LR pathway is a nontissue-specific system that mediates strong attachment of cancer cells to endothelium.

Our study represents the first demonstration of the involvement of a specific laminin receptor in a crucial step of the metastatic cascade and identifies a potential mechanism for the attachment of cancer cells to endothelial cells. The relevance of this pathway in blood-borne metastasis will be established by testing the effect of peptide G on the metastatic potential of melanoma cells. Such experiments should reveal whether peptide G, as a potential inhibitor of this process, could be used as an antimetastatic agent to inhibit cancer cell arrest in the vasculature, preventing extravasation and the subsequent formation of metastatic foci.

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