Int. J. Cancer: **77,** 632–639 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

LAMININ-α1-CHAIN SEQUENCE Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg (LQVQLSIR) ENHANCES MURINE MELANOMA CELL METASTASES

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We earlier screened overlapping synthetic peptides from the globular domain of the laminin α1 chain to identify active sites for cell attachment. We report here that one of the active cell-adhesion peptides, AG-73 (Arg-Lys-Arg-Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg-Thr; RKRLQVQLSIRT) causes B16-F10 murine melanoma cells to metastasize to the liver, a site not normally colonized by these cells. Increases in liver metastases and in lung colonization are observed in immune-deficient beige/nude/xid and in C57Bl/6 mice with this peptide. This metastatic activity was observed with i.v. and with i.p. peptide injections, regardless of tumor cell or of peptide-injection times. *In vitro,* the AG-73 peptide enhances tumor cell adhesion, migration, invasion, and gelatinase production, and blocks laminin-1-mediated cell migration. AG-73 was found to significantly inhibit cell adhesion to a proteolytic laminin-1 fragment, E3, containing the AG-73 sequence. Cell attachment to AG-73, the E3 fragment, and laminin-1 involved cation-dependent receptors. We report that a laminin peptide has the novel and unexpected activity of causing B16F10 melanoma cells, a lung selected cell line, to metastasize to the liver. The minimal active sequence of AG-73, LQVQLSIR, could be one of the most important biologically active sites of laminin-1, especially in promotion of the malignant phenotype. Activation of the malignant phenotype by this peptide provides a significant new model for understanding metastatic mechanisms. *Int. J. Cancer* 77:632–639, 1998.

Metastasis formation is a complex multistep process which includes cell invasion, migration, proliferation and protease activity. Specifically, tumor cells adhere to the basement membrane, degrade it and migrate through it in order to enter and exit the circulation (Liotta *et al.,* 1986). The basement membrane is a thin extracellular matrix underlying most epithelial as well as endothelial cells. Basement membrane is rich in the glycoprotein laminin, which has been shown to promote tumor-cell adhesion, migration, and also the malignant phenotype (Terranova *et al.,* 1982, 1984). I.v. injection of tumor cells cultured in the presence of laminin resulted in enhancement of the number of lung colonies (Barsky *et al.,* 1984). When a mixed population of tumor cells is selected based on adhesion to laminin, the laminin-adherent cells are more malignant than either the non-adherent or the parent cell population (Jun *et al.,* 1994; Kim *et al.,* 1995; Yamamura *et al.,* 1993). A related experiment using fibronectin-adherent cells did not show enhanced malignant potential (Terranova *et al.,* 1984). In addition, the expression of certain laminin receptors is positively correlated with the malignant behavior of cell lines and with the Dukes' stage of human colon carcinoma (Wewer *et al.,* 1986; Yow *et al.,* 1988; Mafune *et al.,* 1990).

Several active sites on laminin have been identified and tested as synthetic peptides, and these sites promote diverse biological activities (Kleinman *et al.,* 1993). A laminin-α1-chain (formerly named A chain)-derived synthetic peptide containing the amino-acid sequence Ile-Lys-Val-Ala-Val (IKVAV) mimics some of the activities of laminin in potentiating tumor-cell adhesion, migration and gelatinase production *in vitro,* and increased lung colonization *in vivo* (Kanemoto *et al.,* 1990). Increased lung metastases were observed even when the IKVAV peptide was injected several hours after the i.v. injection of melanoma cells. Another active site on laminin, Tyr-Ile-Gly-Ser-Arg (YIGSR) in the β1 chain (formerly named B1 chain), promotes tumor-cell adhesion and migration *in vitro,* but reduces melanoma lung colonization (Iwamoto *et al.,* 1987) and s.c. tumor growth (Sakamoto *et al.,* 1991). The YIGSR sequence induces apoptosis in a human cancer cell line (Kim *et al.,* 1994). Another active laminin-β1-chain peptide, Arg-Tyr-Val-Val-Leu-Pro-Arg (RYVVLPR), promotes fibrosarcoma-cell adhesion and spreading *in vitro,* but is less well characterized (Skubitz *et al.,* 1990).

We have established a method of screening protein sequences for cell-adhesion activity *in vitro* using synthetic peptides coupled to beads. We applied this screening method to the G domain (amino-acid residues 2111-3060) of the laminin α1 chain, which possesses several biological activities (Nomizu *et al.,* 1995). Among the 5 new active sites identified, one sequence, designated AG-73, showed the strongest cell-attachment activity. Here we report that this AG-73 peptide has the unique ability to promote development of liver metastases by B16F10 melanoma cells, which are selected for specific colonization to the lungs. Cells adhesion-selected to this peptide also form more liver metastases (Song *et al.,* 1997). The peptide also has several *in vitro* activities which may function to enhance tumor spread *in vivo.*

MATERIAL AND METHODS

Cell culture

B16-F10 cells (a gift of Dr. I.J. Fidler, Houston, TX) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY), non-essential amino acids and vitamins.

Synthetic peptides, E3 fragment, laminin-1 and matrigel

AG-73 peptide (RKRLQVQLSIRT) from the C-terminal globular domain of the murine laminin α1 chain (residues 2719-2730), AG-73S peptide (IRSQTLRLRVQK, scrambled amino-acid sequence of AG-73), AG-73T peptide (LQQRRSVLRTKI, scrambled amino-acid sequence of AG-73), IKVAV peptide (AASIK-

Abbreviations: AG-73, Arg-Lys-Arg-Leu-Gln-Val-Gln-Leu-Ser-Ile-Arg-Thr; bg/nd/xid, beige/nude/xid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECGF, endothelial cell growth factor; EHS, Engelbreth-Holm-Swarm; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; IKVAV peptide, Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg; PBS, phosphate-buffered saline; PVP, polyvinyl-pyrrolidone; SDS, sodium dodecyl sulfate; YIGSR peptide, Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg.

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Received 15 January 1998; Revised 18 March 1998

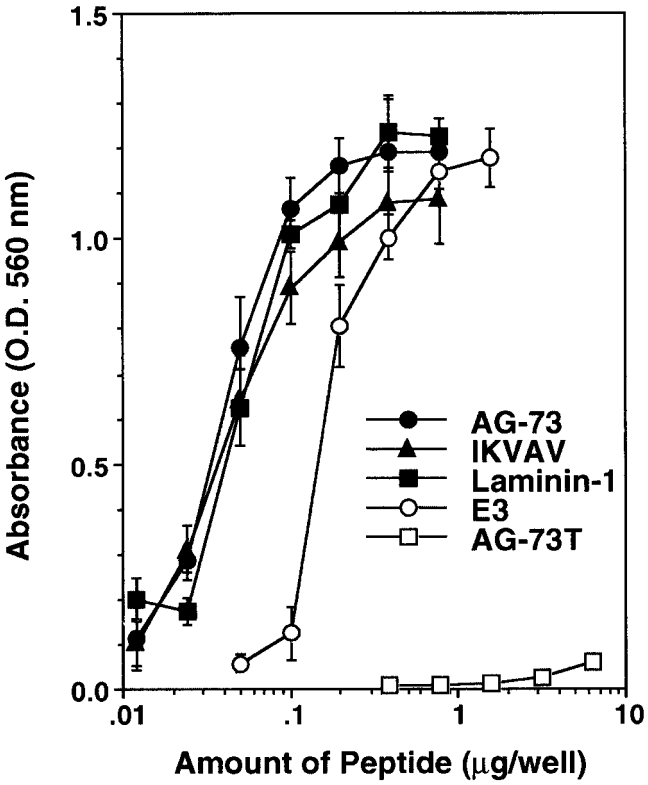

FIGURE 1 – Attachment of B16-F10 cells to synthetic peptides. Each well of 96-well plates was coated with various amounts of synthetic peptides, E3 fragment or laminin-1. B16-F10 cells (20,000) were added to each well, and attached cells were measured after a 30-min incubation, as described in ''Material and Methods''. Each peptide concentration was assayed in triplicate, and the experiments were repeated 3 times, with the results showing the same pattern. A representative result is shown with means and standard-deviation bars.

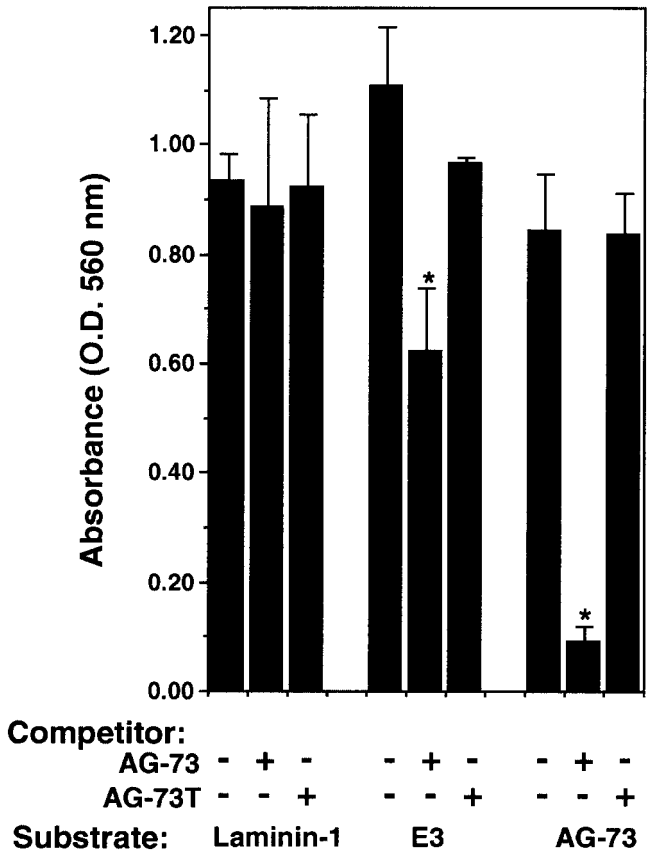

FIGURE 2 – Inhibition of B16-F10 mouse melanoma-cell attachment on laminin-1-, E3 fragment- and AG-73-coated plastic dishes by AG-73. A 96-well plate was coated with 0.1 μg/well of laminin-1, 0.5 μg/well of E3 or 0.05 μg/well of AG-73. Peptides (100 μg/ml) were added to the cell suspensions and the cells were added onto the plates. After a 15-min incubation, the attached cells were assessed by crystal-violet staining. Each value represents the mean of 5 separate determinations ± S.D. Duplicate experiments gave similar results. *The difference of absorbance, O.D. 560 nm, between AG-73-containing wells and control wells is significant ($p < 0.01$). Statistical values were obtained using Students' *t*-test.

VAVSADR) from the murine laminin α1 chain (residues 2097-2109), and YIGSR peptide (DPGYIGSR) from the murine β1 chain (residues 926-933) were synthesized by a t-butyloxycarbonyl-based solid-phase strategy (Nomizu *et al.*, 1995). Starting with a 4-methylbenzhydrylamine resin, the respective amino acids corresponding to the peptides were assembled in a stepwise manner with an Applied Biosystems (Foster City, CA) Peptide Synthesizer, Model 431A, using a single coupling protocol. De-protection and cleavage from the resin were achieved by treatment with anhydrous hydrogen fluoride, and the crude peptides were purified by reverse-phase high-performance liquid chromatography (using a Vydac 5C18 column and a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid). The purity of the peptides was confirmed by analytical high-performance liquid chromatography. The identity of the peptides was confirmed by amino-acid analysis and fast-atom-bombardment mass spectral analysis. Amino-acid analyses were performed at the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan. Mass spectra were measured in a glycerol matrix on a VG 7070E-HF double focusing mass spectrometer.

E3 fragment was obtained from Dr. P.D. Yurchenco, Robert Wood Medical School (Piscataway, NJ).

Mouse laminin-1 and Matrigel were prepared from the EHS tumor as described (Timpl *et al.*, 1979; Kleinman *et al.*, 1986).

Attachment assay

Cell attachment was assayed in round-bottom 96-well plastic plates (Immulon 2, Dynatech, Chantilly, VA) coated either with laminin-1 or with synthetic peptides. Various amounts of laminin-1 or peptides were dissolved in Milli-Q water, and 50 μl were added to each well, followed by drying overnight, for the peptides, or followed by 2-hr incubation, for laminin-1. Drying allows maximal binding of the peptides, whereas laminin-1 is best used after short-term coating. The wells were blocked by addition of 0.1 ml of 3% BSA in DMEM for 1 hr and washed twice with DMEM containing 0.1% BSA. B16-F10 melanoma cells were detached with Versene (0.02% EDTA in PBS) and re-suspended in DMEM containing 0.1% BSA. Cells (30,000 cells in 0.1 ml) were added to each well and incubated for 30 min at 37°C in 5% CO_2. After washing to remove the unattached cells, the attached cells were stained with 0.1 ml of a 0.2% crystal-violet aqueous solution in 20% methanol for 10 min. After washing and drying, 0.2 ml of 1% sodium dodecyl sulfate (SDS) was used to dissolve the cells, and the optical density at 560 nm was measured by a Titertek Multiskan.

In EDTA-inhibition experiments, the cells were first mixed with 3 mM EDTA and then 30,000 cells in 0.1 ml were plated in the wells and incubated for 30 min at 37°C in 5% CO_2. In AG-73-inhibition experiments, 100 μg/ml of AG-73 were added to the cells and pre-incubated for 15 min at room temperature. Then the cells were plated and incubated for 15 min at 37°C in 5% CO_2. Attached cells were assessed by measuring the optical density at 560 nm, as described above.

Chemotaxis assay

Polyvinylpyrrolidone (PVP)-free polycarbonate filters of 8-μm pore size (Nuclepore, Pleasanton, CA) were placed in Boyden blind-well chambers after placing the peptides, dissolved in 220 μl of DMEM with 0.1% BSA, into the lower chamber. B16-F10 cells detached with 0.02% EDTA in PBS were placed (200,000 per chamber) in the upper chamber. After incubation at 37°C for 5 hr in 5% CO_2, filters were stained with a 0.2% crystal-violet solution, and the cells on the upper surface were removed by scraping. The cells on the lower surface were counted under a microscope. Inhibition of migration to laminin-1 by the peptide was measured by placing 1 μg/ml of laminin-1 in the lower chamber as a chemo-attractant, and 200 μg/ml of AG-73 in the upper chamber.

Invasion assay

Invasiveness of B16-F10 cells in the presence of the peptides was tested using the standard *in vitro* invasion-assay method, with a minor modification in the Matrigel coating (Albini *et al.*, 1987). PVP-free polycarbonate filters of 8-μm pore size (Nuclepore) were coated with 1 μg of Matrigel and placed in Boyden blind-well chambers. NIH-3T3 conditioned media was used in the invasion assay because it is a strong general attractant for many cells, including B16-F10 cells. NIH-3T3 conditioned media, obtained by culturing cells for 18 hr in serum-free DMEM, were diluted 1:5 with DMEM containing 0.1% BSA, and were placed in the lower chamber. EDTA-detached B16-F10 cells (200,000/chamber) in DMEM containing 0.1% BSA were placed in the upper chamber. Either AG-73 or IKVAV peptide was added to both the upper and the lower chambers at a concentration of 100 μg/ml. After incubation for 5 hr at 37°C in 5% CO_2, the number of invaded cells was determined on triplicate filters.

Gelatin zymography

B16-F10 cells (2.5×10^6) were plated onto 150-mm culture dishes with complete media. After 24 hr, the media were replaced with serum-free DMEM containing various concentrations of the AG-73, AG-73S and AG-73T peptides. The supernatant was harvested after a 24-hr incubation at 37°C in 5% CO_2 and concentrated to 1/60. Equal aliquots of the supernatant from each culture condition were separated on a 10% polyacrylamide gel containing 0.2% gelatin. The gels were washed with 10 mM Tris-HCl (pH 7.4) containing 2.5% Triton-X for 30 min, followed by 2 changes of 10 mM Tris-HCl (pH 7.4) for 10 min. After incubation in 50 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$ and 1 mM $ZnCl_2$ at 37°C for 24 hr, Coomassie blue was added to visualize the digested gelatin bands.

In vivo *tumor metastasis and growth*

In all experiments, a minimum of 4 animals per data point was used. Each experiment was performed at least twice. For the experimental metastasis assay, 100,000 B16-F10 cells in 0.2 ml EMEM were injected via the tail vein into either syngeneic C57BL6/N mice or beige/nude/xid (bg/nd/xid) mice. The peptides were dissolved in Milli-Q water at a concentration of 5 mg/ml. I.v. injection of the indicated amount of peptide was performed by mixing the peptide solution with the cell suspension, and the

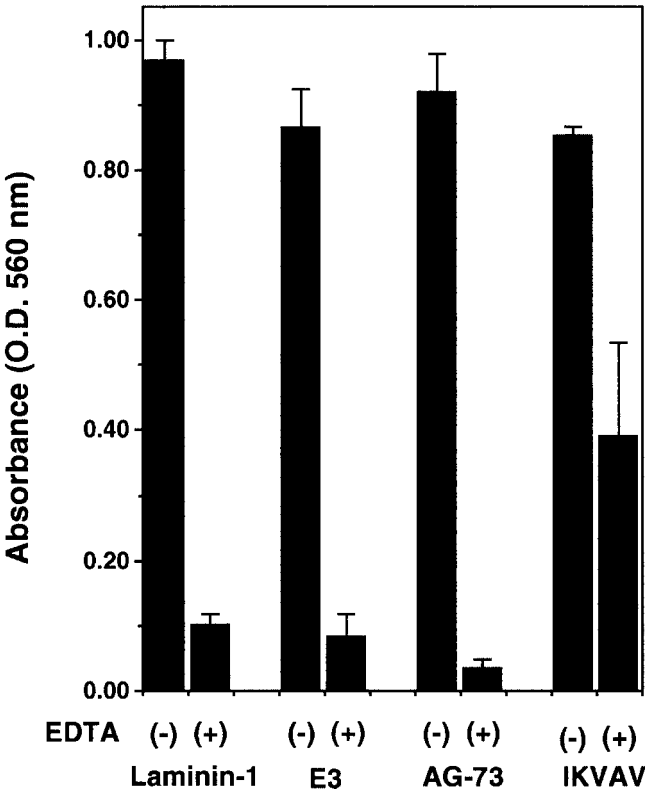

FIGURE 3 – Inhibition of B16-F10 mouse melanoma-cell attachment to laminin-1, E3 fragment, AG-73 and the IKVAV peptide by EDTA. A 96-well plate was coated with 0.1 μg/well of laminin-1, 1.0 μg/well of E3 or 0.5 μg/well of AG-73 and the IKVAV peptide. EDTA was added to the cell suspensions and the cells were added to the plates. After a 30-min incubation, the attached cells were assessed by crystal-violet staining. Each value represents the mean of 5 separate determinations ± S.D. Duplicate experiments gave similar results.

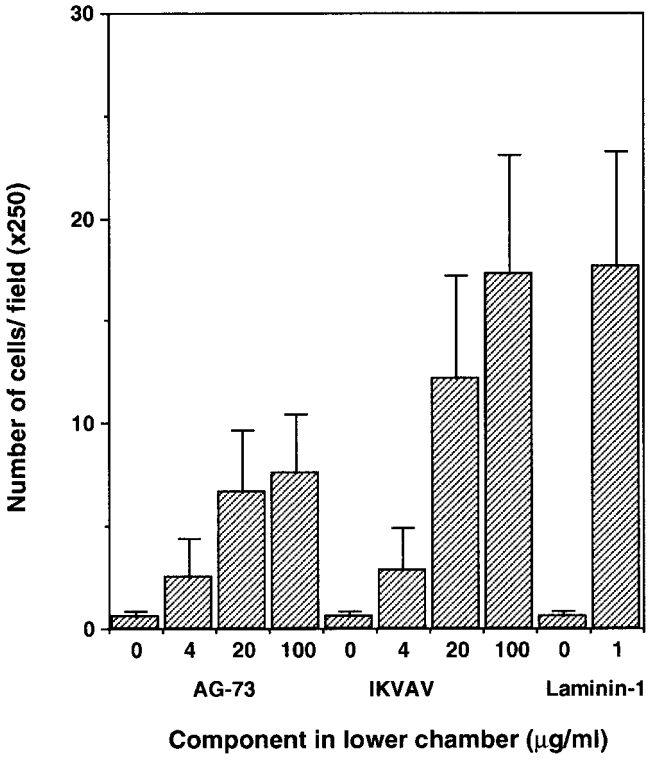

FIGURE 4 – Chemotactic activity of synthetic peptides. PVP-free filters of 8 mm pore size were placed in the Boyden chambers. Various concentrations of peptides were added in the lower chamber and 200,000 B16-F10 cells were placed in the upper chamber. The cells that migrated through the filters were counted after a 5-hr incubation. At least, 4 ×250 fields from each filter were counted, and each data point was based on triplicate filters. The experiments were repeated 3 times, with the results showing a similar pattern. Means and standard error bars are shown. Differences between IKVAV and AG-73 are not statistically significant ($p > 0.1$).

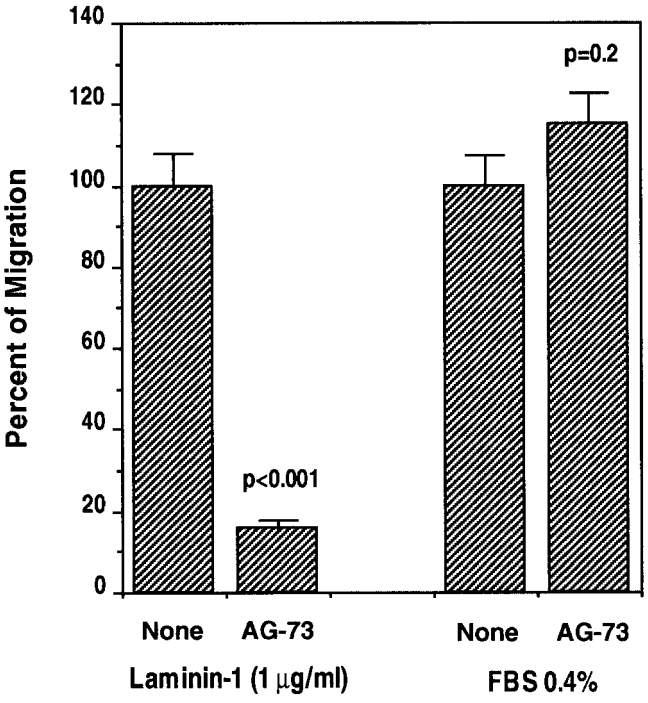

FIGURE 5 – Inhibition of migration to laminin-1 by AG-73. The method was similar to that described in Figure 4. The lower chamber contained 1 μg/ml of laminin-1 or 0.4% bovine serum as a chemo-attractant; the number of migrated cells in the absence of peptide was taken as 100%; 3 high-power fields from each filter were counted. The data are shown as means and standard errors.

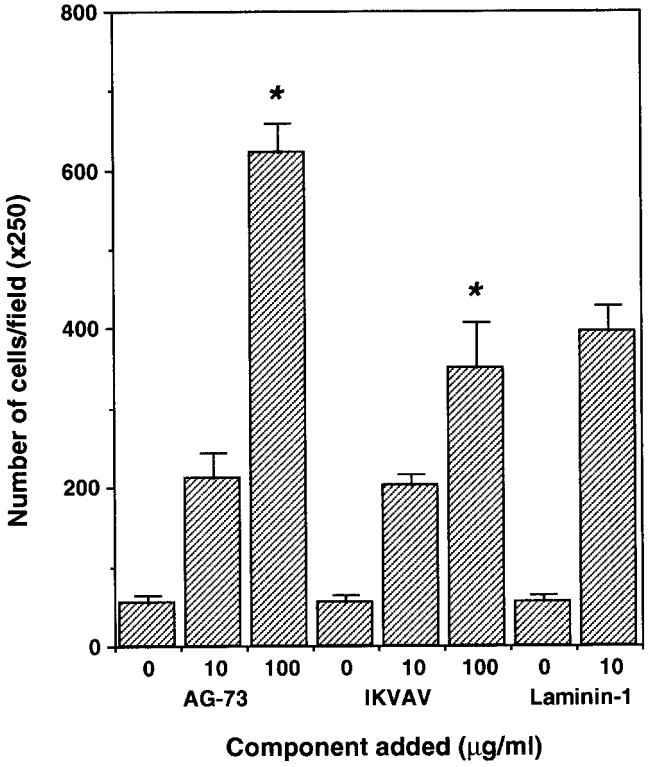

FIGURE 6 – B16-F10 cell invasion in the presence of AG-73 or of IKVAV peptide. PVP-free filters were coated with 1 μg of Matrigel and placed in Boyden blind wells. The bottom chamber contained one-fifth-strength NIH-3T3-cell-conditioned medium and 100 μg/ml of peptide, while the upper chamber contained 200,000 B16-F10 cells and the same concentration of peptide. The invaded cells were counted in triplicate filters, and the mean and standard errors are shown. The experiments were repeated twice, with the data showing the same pattern. (*Difference of invaded cells between marked points is significant. $p < 0.05$).

solution was injected within 1 min after mixing. For i.p. injection of the peptide, the peptide solution was injected immediately after the tail-vein injection. The animals were killed 15 days after injection, lungs and livers were removed, and the surface colonies were counted and analyzed by Student's *t*-test.

For the s.c.-tumor-growth assay, B16-F10 cells were suspended in cold Matrigel and the peptides were added to the cell-Matrigel suspension. The mice were injected s.c. in the right lower back with the cell-peptide-Matrigel mixture, which contained 500,000 cells and 0.1 mg of AG-73 peptide in 1 ml of Matrigel. Tumor sizes were measured every other day from day 7 to day 19 after the initial injection.

RESULTS

Cell attachment

We tested the AG-73 synthetic peptide (RKRLQVQLSIRT) for attachment activity with B16-F10 cells and compared it with another, reported active laminin peptide, IKVAV (AASIKVAVSADR). AG-73 was active for cell attachment similar to that of IKVAV and native laminin-1 (Fig. 1). Half-maximal cell adhesion occurred at less than 0.02 μg of AG-73. The E3 fragment, a proteolytic fragment from the C-terminus of the G domain containing the AG-73 sequence, also showed cell-attachment activity, but it appeared to be weaker than that of AG-73, possibly due to differences in molarity and/or conformation. The scrambled peptide, AG-73T, had no activity, even at the highest coating amount of 10 μg/well.

The inhibitory effects of the AG-73 peptide on B16-F10 cell attachment to laminin-1, the E-3 fragment and AG-73 were tested (Fig. 2). As a control, the scrambled peptide, AG-73T, was also tested. AG-73 did not affect cell attachment to laminin-1. AG-73 significantly inhibited cell attachment to the E3 fragment. AG-73 also strongly inhibited cell attachment on the AG-73-coated dishes, whereas AG-73T showed no effect on cell attachment to any of the substrates.

We also tested the effect of EDTA on cell attachment, to determine the role of cations (Fig. 3). EDTA completely inhibited cell attachment to laminin-1. Cell attachment to E3 and to AG-73 was also completely inhibited by 3 mM EDTA, whereas attachment to the IKVAV peptide was inhibited by only 50%. These results suggest that cell attachment to AG-73 and to E3 are mediated by (a) cation-dependent receptor(s).

In vitro *chemotaxis and invasion*

AG-73 peptide was tested for chemotactic activity, since B16-F10 cells have been found to migrate to laminin-1 (Iwamoto *et al.*, 1988). As reported by Kanemoto *et al.* (1990), the IKVAV peptide was active in promoting chemotaxis at 100 μg/ml, as shown by the ability of cells to migrate in the Boyden-chamber assay (Fig. 4). The AG-73 peptide was chemotactic, although it was less active than the IKVAV peptide. When AG-73 at a dose of 200 μg/ml was added to both upper and lower chambers, while laminin-1 (1 μg/ml) was added as a chemo-attractant in the lower chamber, migration of the cells was reduced to 15.9% of that observed with laminin-1 alone (Fig. 5). Addition of the AG-73 peptide did not inhibit migration when 0.4% bovine serum was used as a chemo-attractant, confirming the specificity of the peptide activity. These findings also strongly suggest that the AG-73 peptide exerts its activity through a receptor which also recognizes laminin-1.

AG-73, IKVAV and laminin-1 enhanced B16-F10 cell invasion *in vitro* through Matrigel, a basement membrane matrix (Fig. 6).

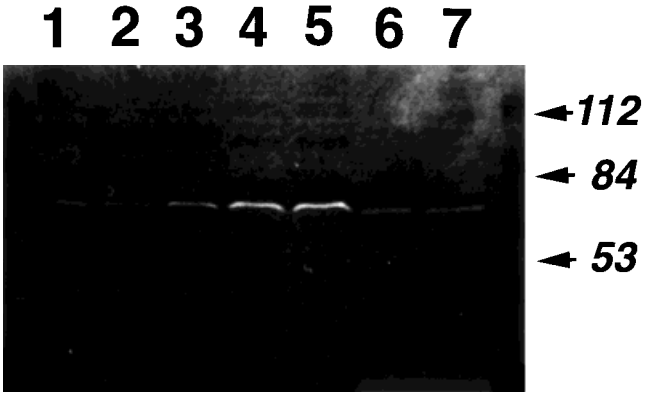

FIGURE 7 – Effect of the AG-73 peptide on activation of gelatinases. The conditioned media of B16-F10 cells incubated without peptide (lane 1) or with 25 μg/ml (lane 2), 50 μg/ml (lane 3), 75 μg/ml (lane 4) or 100 μg/ml (lane 5) of AG-73, or 100 μg/ml of AG-73T (lane 6) or 100 μg/ml of AG-73S (lane 7) were harvested and separated on 10% PAGE. Coomassie-blue staining revealed that the 92-kDa band was produced by the B16-F10 cells, and that the activity was enhanced by the AG-73 peptide, but not by control peptides. No other bands were affected by any of the peptides tested.

B16-F10 cells invaded 2-fold more to the AG-73 peptide (100 μg/ml) than to a similar amount of the IKVAV peptide. Invasion at lower peptide concentrations was similar for both peptides.

Gelatin zymography

Because gelatinases are important in the metastatic behavior of tumor cells, we measured gelatinase secretion by gelatin zymography (Fig. 7). AG-73 peptides increased 92-kDa gelatinase activity dose-dependently, while the control peptides, AG-73T and AG-73S, did not affect this activity. Addition of 50 μg/ml of the AG-73 peptide to B16-F10 cells doubled the amount of the activated form of gelatinase A. With 100 μg/ml of AG-73, the amount of the activated form of gelatinase A was increased approximately 5-fold over that observed with the control scrambled peptide. Similar results were obtained with HT-1080 fibrosarcoma and SW480 colon-cancer cells (data not shown). No change in the 72-kDa band was observed. Increased expression and activation of matrix metalloproteinases could explain, in part, the metastasis-promoting action of AG-73 (see below), and the increased invasiveness through the Matrigel.

In vivo *tumor metastasis and growth*

In preliminary experiments, we tested the effects of AG-73 on lung colonization in C57BL6/N mice injected i.v. with B16-F10 melanoma cells (200,000 cells/mouse). The mice were killed after 15 days and control animals (*i.e.*, no peptide) had a mean of 116.5

TABLE I – EFFECT OF AG-73 PEPTIDE ON LUNG AND LIVER COLONIZATION OF B16-F10 MELANOMA CELLS

Treatment	Lung colonies		Liver metastases	
	Number of animals with lung colonies/ Total animals	Mean number of lesions ± SE[5]	Number of animals with liver metastases/ Total animals	Mean number of lesions ± SE
C57 mice[1]				
No peptide	8/8	15.8 ± 3.5	0/8	0.0 ± 0.0
AG-73S, 2 mg i.p.	8/8	19.5 ± 7.5	1/8	0.3 ± 0.2
AG-73, 2 mg i.p.	8/8	63.8 ± 11.5[2]	8/8	33.3 ± 6.6[3]
Beige/nude/xid mice[4]				
No peptide	3/4	3.0 ± 1.6	0/4	0.0 ± 0.0
AG-73, 2 mg i.p.	6/6	11.7 ± 2.5	5/6	4.3 ± 0.9

[1]Effect of AG-73 or its scrambled control peptide (AG-73S) injected i.p. on promotion of metastatic lesions. Each mouse was injected with 100,000 B16-F10 cells via the tail vein and the peptide was immediately administered i.p. The numbers of colonies on the lungs and liver were counted 15 days later. AG-73 peptide increased lung colonization as well as liver metastases. The experiments were repeated with similar results.–[2]Significantly different from no-peptide control ($p < 0.001$) and from scrambled-peptide control ($p < 0.05$).–[3]Significantly different from no-peptide control ($p < 0.001$) and from scrambled-peptide control ($p < 0.001$).–[4]Effect of AG-73 peptide injected i.p. in immune-deficient mice. The beige/nude/xid mice were given the same amount of tumor cells and peptide as the C57 mice. AG-73 peptide promoted the development of hepatic and pulmonary metastatic lesions in this immunosuppressed mouse strain.–[5]SE, standard error.

TABLE II – OCCURRENCE OF PULMONARY AND HEPATIC METASTASES AFTER AG-73 PEPTIDE INJECTION

Treatment		Lung colonies		Liver metastasis	
At 0 hr	At 1 hr	Number of animals with lung colonies/ Total animals	Mean number of lesions ± SE	Number of animals with liver metastasis/ Total animals	Mean number of lesions ± SE
AG-73	B16-F10	6/6	55.5 ± 7.6[1]	4/6	2.5 ± 0.3
AG-73 + B16-F10	none	6/6	102.0 ± 20.9[2]	6/6	4.2 ± 0.8
B16-F10	AG-73	6/6	86.0 ± 20.8[3]	6/6	2.7 ± 0.7
B16-F10	none	14/14	27.2 ± 4.2	0/14	0.0 ± 0.0

AG-73 peptide (2 mg) was given i.p. either 1 hr before or 1 hr after tail-vein injection of the B16-F10 cells (100,000 cells per mouse). Although simultaneous administration showed the most potent effect on the enhancement of metastasis, pre- or post-injection of the peptide also promoted colonization in the lungs as well as metastasis in the liver.–[1]Significantly different from no-peptide control ($p < 0.005$).–[2]Significantly different from no-peptide control ($p < 0.001$).–[3]Significantly different from no-peptide control ($p < 0.005$). No difference between [1, 2 and 3] ($p > 0.05$). SE, standard error.

(±11.2) colonies in the lungs, while mice that received AG-73 peptide i.p. developed more lung colonies with means of 142.2 (±15.7) and 171.3 (±30.3) colonies per mouse receiving 1 mg and 2 mg peptide, respectively. I.v. injection of 1 mg of AG-73 yielded a mean number of lung colonies (136.3 ± 11.2) similar to that observed with i.p. injection. Unexpectedly, many of the mice treated with the AG-73 peptide developed hepatic metastases, whereas the control mice did not. This effect was most pronounced in mice receiving an i.p. peptide injection, in that 3 of the 5 mice receiving 1 mg i.p. (mean number of lesions ± standard error: 2.6 ± 1.2) and 5 of the 6 mice receiving 2 mg i.p. (16.3 ± 5.5) developed liver metastases. This effect of extrapulmonary metastases has not been reported with another metastasis-promoting laminin-derived peptide, IKVAV.

We next compared the scrambled peptide (AG-73S; IRSQTLRLRVQK) with the active AG-73 peptide using fewer tumor cells (100,000 per mouse) to facilitate the counting of the lung colonies. The animals given the AG-73 peptide i.p. had approximately 4 times more lung colonies than control animals (Table I). Scrambled peptide did not increase the number of lung colonies relative to the control. Hepatic metastases were observed in all animals (n = 8) receiving the AG-73 peptide, while no control animal and only one animal receiving scrambled peptide developed hepatic metastases.

To exclude a possible immune modulating effect of the AG-73 peptide, we used immune-deficient bg/nd/xid mice, which were injected with B16-F10 cells via the tail vein and i.p. with 2 mg of the AG-73 peptide. The number of lung colonies was increased 4-fold over that of the control. Of the 6 animals injected with AG-73, 5 developed liver metastases, whereas none of the control mice developed liver metastases (Table I). These data suggest that AG-73 did not compromise the immune system of the normal mice.

In order to evaluate the time of peptide injection on tumor growth, we injected the AG-73 peptide either 1 hr before, simultaneously, or 1 hr after tumor-cell administration in the tail vein (6 mice in each group). There was no significant difference between each injection condition, although the number of liver metastases was greatest in the simultaneous-injection group (Table II). As expected, none of the 14 control animals developed liver metastases.

We tested s.c. tumor growth in the presence of the AG-73 peptide, since the IKVAV peptide has been found to promote tumor growth (Kanemoto *et al.,* 1990). AG-73 peptide and a control scramble peptide AG-73T were each co-injected s.c., with B16-F10 melanoma cells, into mice with the basement membrane matrix Matrigel. AG-73 was able to significantly stimulate tumor growth over that observed in the absence of the peptide (Fig. 8). AG-73T peptide did not enhance tumor growth.

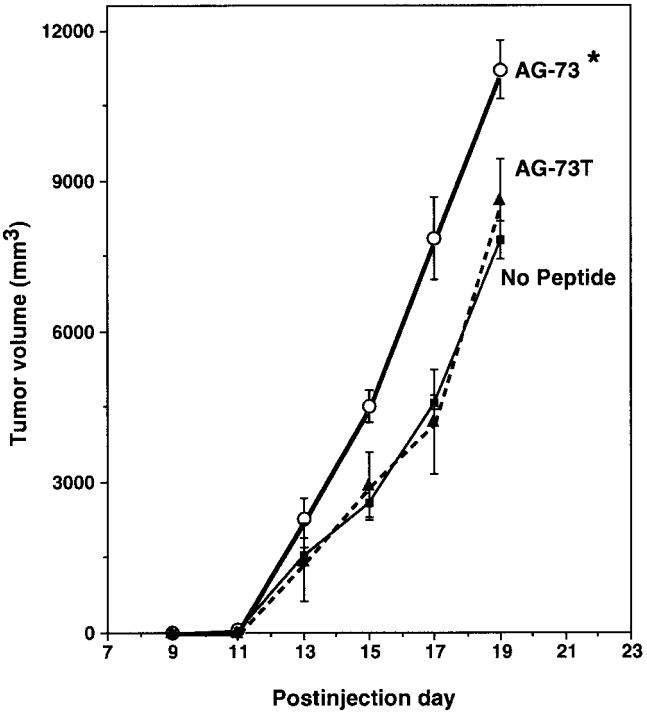

FIGURE 8 – Effect of the AG-73 peptide on s.c. melanoma tumor growth. Tumor measurements were made (length × width × height) with calipers, and the data are expressed as tumor volume (mm^3). The data are shown as means ± S.E. (n = 4-5). The experiments were repeated twice with 6 mice in each group for each experiment, with the results showing the same pattern. *Differences in tumor volume between AG-73-injected mice and control mice (either scrambled-peptide-injected or PBS-injected) are significant ($p < 0.05$) as determined by Student's *t*-test.

TABLE III – COMPARISON OF BIOLOGICAL ACTIVITY OF ACTIVE LAMININ PEPTIDES IN B16-F10 CELL EXPERIMENTS

Assay system	AG-73 (LQVQLSIR)	IKVAV	YIGSR
In vitro			
Attachment	++	++	+
Chemotaxis	+	++	+
Inhibition of migration	+	+	+
Invasion	++	+	−
In vivo			
Lung colonies	+	+	−
Liver metastasis	+	0	0
S.c. growth	+	+	−
Effectiveness after tumor-cell injection	+	+	−
Effectiveness before tumor-cell injection	+	0	0

++, more active when AG-73 and IKVAV are compared; +, active or increase; 0, inactive or no change; −, inhibitory or decrease.

DISCUSSION

Although several other active sites on laminin-1 have been identified, AG-73 is the first sequence found to promote hepatic metastases of normally lung-colonizing murine melanoma cells. AG-73 peptide has a number of activities that are related to the malignant phenotype. This peptide increases cell adhesion, migration, invasion, and gelatinase activity *in vitro,* as well as melanoma cell lung and liver colonization *in vivo.* It does not appear to increase metastasis by affecting the immune system, and has no effect on cell growth *in vitro.* The minimal active sequence, LQVQLSIR, (Nomizu *et al.,* 1995) has a unique action on tumor cells relative to other laminin-1-derived sequences described to date.

AG-73 peptide enhances the malignant phenotype, but appears to employ a different mechanism from the laminin-1-derived IKVAV peptide (Table III). AG-73 stimulated the growth of s.c. injected tumor cells (Table III) similar to that observed with the IKVAV peptide (Kanemoto *et al.,* 1990). The AG-73 peptide has no effect on angiogenesis, whereas the IKVAV peptide is active in several angiogenesis assays. The AG-73 peptide was active in promoting lung colonization and hepatic metastases when injected simultaneously with, or 1 hr before or 1 hr after the tumor-cell injections. In contrast, IKVAV is active in lung colonization only when injected at the same time as, or after, the administration of tumor cells (Sweeney *et al.,* 1991). We isolated AG-73-adhesion-selected melanoma cells and found that AG-73-adherent cells metastasize to the liver in the absence of peptide, suggesting a receptor-mediated event (Song *et al.,* 1997). In contrast, IKVAV-adherent-selected cells were not metastatic to the liver (Yamamura *et al.,* 1993). These findings, together with the unexpected induction of liver metastases, suggest that the AG-73 peptide acts via a

mechanism different from that of the IKVAV peptide. We have found that AG-73 promotes melanoma-cell invasion through immobilized extracellular matrix by increased degradation of the extracellular matrix. This increased degradation is accompanied by the peptide-mediated recruitment of seprase, a membrane-bound protease, in invadopodia in the malignant tumor cells (Nakahara *et al.,* 1998).

We have shown that AG-10 from the α1 G domain promoted cell attachment through α6β1 integrin, whereas AG-73 did not use α6β1 or α2β1 integrins for cell attachment (Nomizu *et al.,* 1995; Nakahara *et al.,* 1996). Using laminin-1 and AG-73 affinity chromatography with biotinylated surface proteins, we found that syndecan-1 is a strong ligand (data not shown). Syndecan-1 has been shown to interact with the E3 fragment of the laminin α1 G domain (Salmivirta *et al.,* 1996). Since heparin and heparan sulfate, but not other glycosaminoglycans, block cell binding to AG-73, we conclude that syndecan-1 is a surface ligand for AG-73. Furthermore, treatment of the cells with heparanase or heparitinase blocks binding to AG-73, whereas chondroitinase has no effect. Syndecan may function as a co-receptor with integrin (Colognato *et al.,* 1997), which may explain the observation of inhibition of adhesion to AG-73 by EDTA.

The mechanism for the metastases to the liver is not known. Although increased invasiveness and gelatinase secretion could play a role in enhancing the metastases observed with this peptide, these activities may not be alone in promoting liver metastases. The organ-specific metastasis could be caused either by a local effect stimulatory for tumor-cell growth or by preferential relocation of tumor cells. Increased angiogenesis or changes in function do not appear to be causative. An important experiment aimed at determining the mechanism was carried using AG-73-adhesion-selected cells (Song *et al.,* 1997). The cells were selected over 20 times for adhesion to AG-73 and then injected into mice. Liver metastases were observed in the absence of added peptide. These data suggest that the liver was not storing the peptide and then attracting the cells to it. Also, the peptide was probably not functioning to make the liver more receptive to tumor cells. YIGSR- and SIKVAV-adhesion-selected B16F10 melanoma cells are more tumorigenic, but do not metastasize to the liver, further suggesting a unique mechanism probably involving the tumor cell itself and activation by the peptide. Our current approach is to identify induced genes in B16F10 melanoma cells treated with the peptide, in the selected *vs.* the parent cells, and in liver *vs.* lung tumors using subtractive cDNA cloning.

Immune-system modulation does not appear to be a primary mechanism for increased metastasis, since immune-deficient mice showed no significant difference in metastases as compared with normal mice. We used bg/nd/xid mice, which show impaired lymphokine-activated-killer (LAK)-cell and B- and T-cell responses. The immune system, however, cannot be totally eliminated in these mice, so that it is possible that AG-73 impairs immune function, something not yet tested.

Since most of the biologically active sequences of laminin-1 contain an arginine or a lysine as a positively charged residue, these residues appear necessary for a ligand peptide to interact with cell-surface receptors. The minimum active sequence of AG-73 peptide (LQVQLSIR) in the mouse laminin α1 chain also contains an arginine. This sequence is conserved in the human laminin α1 chain (Haaparanta *et al.,* 1991), the human laminin α2 chain (Ehrig *et al.,* 1990), the mouse laminin α2 chain (Bernier *et al.,* 1994), and the *Drosophila* laminin α chain (Garrison *et al.,* 1991). Thus, AG-73 appears to be an important site for the function of the laminins.

There is evidence that the AG-73 sequence may be physiologically relevant in the intact molecule. AG-73 blocked cell attachment to the E3 fragment, which is the C-terminal portion of laminin α1 chain and contains the AG-73 sequence. This peptide does not block cell adhesion to laminin-1, possibly due to the presence of multiple cell-attachment sites on laminin-1. We show that the peptide can block soluble laminin-1-mediated melanoma-cell migration, suggesting that the conformation of laminin may be important in exposure of active sites. When HSG salivary-gland cells are exposed to the peptide in the presence of laminin-1, laminin-mediated acinar formation is blocked (Hoffman *et al.,* 1995). We have tried to generate antibodies in 2 rabbits with peptide attached to a lysine branch or to KLH, but no titer has been obtained, probably due to the conserved nature of this sequence. These data suggest that the AG-73 sequence is available on laminin-1 and that it has biological activity in various systems.

In summary, we have characterized the response of tumor cells to an active sequence (LQVQLSIR) in the G domain of the laminin α1 chain and have shown that this sequence is more potent than IKVAV, another active sequence, in many *in vitro*-assay systems related to malignant behavior. This peptide enhanced s.c. tumor growth and lung colonization. Furthermore, the AG-73 peptide unexpectedly caused B16-F10 melanoma cells to metastasize to the liver. This sequence is conserved during evolution and in various laminin α chains. The AG-73 sequence could be one of the most important ligand sites on laminin. Understanding the mechanism by which AG-73 promotes metastases may help determine ways to inhibit metastases. The AG-73 activation of malignancy provides a significant new model for understanding metastatic mechanisms.

REFERENCES

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J. and McEwan, R.N. A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res.,* **47,** 3239–3245 (1987).

Barsky, S.H., Rao, C.N., Williams, J.E. and Liotta, L.A., Laminin molecular domains which alter metastasis in a murine model. *J. clin. Invest.,* **74,** 843–848 (1984).

Bernier, S.M., Utani, A., Sugiyama, S., Doi, T., Polistina, C. and Yamada, Y., Cloning and expression of laminin α2 chain (M-chain) in the mouse. *Matrix Biol.,* **14,** 447–455 (1994).

Colognato, H., MacCarrick, M., O'Rear, J.J. and Yurchenco, P.D., The laminin α2-chain short arm mediates cell adhesion through both the α1β1 and α2β1 integrins. *J. biol. Chem.,* **272,** 29330–29336 (1997).

Ehrig, K., Leivo, I., Argraves, W.S., Ruoslahti, E. and Engvall, E., Merosin, a tissue-specific basement-membrane protein, is a laminin-like protein. *Proc. nat. Acad. Sci. (Wash.),* **87,** 3264–3268 (1990).

Garrison, K., MacKrell, A.J. and Fessler, J.H., Drosophila laminin A chain sequence, Interspecies comparison, and domain structure of a major carboxyl portion. *J. Biol. Chem.,* **266,** 22899–22904 (1991).

Haaparanta, T., Uitto, J., Ruoslahti, E. and Engvall, E., Molecular cloning of the cDNA encoding human laminin A chain. *Matrix,* **11,** 151–160 (1991).

Hoffman, M.P., Kibbey, M.C., Nomizu, M. and Kleinman, H.K., Laminin peptides promote acinar-like development of a human sub-mandibular cell line (HSG) *in vitro. J. Cell Biol.,* **6,** 169a (1995).

Iwamoto, Y., Graf, J, Sasaki, M., Kleinman, H.K., Greatorex, D.R., Martin, G.R., Robey, F.A. and Yamada, Y., A synthetic pentapeptide from the B1 chains of laminin is chemotactic for B16F10 melanoma cells. *J. cell. Physiol.,* **134,** 287–291 (1988).

Iwamoto, Y., Robey, F.A., Graf, J., Sasaki, M., Kleinman, H.K., Yamada, Y. and Martin, G.R., YIGSR, a pentapeptide from the B1 chain of laminin which inhibits tumor-cell metastasis. *Science,* **238,** 1132–1134 (1987).

Jun, S.H., Thompson, E.W., Gottardis, N., Torri, J., Yamamura, K., Kibbey, M.C., Kim, W.H. and Kleinman, H.K., Laminin-adhesion-selected primary human colon-cancer cells are more tumorigenic that the parental and non-adherent cells. *Int. J. Oncol.,* **4,** 55–60 (1994).

Kanemoto, T., Reich, R., Royce, L., Greatorex, D., Adler, S.H., Shiraishi, N., Martin, G.R., Yamada, Y. and Kleinman, H.K., Identification of an amino-acid sequence from the laminin A chain which stimulates metastases formation and collagenase-IV production. *Proc. nat. Acad. Sci. (Wash.),* **87,** 2279–2283 (1990).

KIM, W.H., JUN, S.H., KIBBEY, M.C., THOMPSON, E.W. and KLEINMAN, H.K., Expression of beta 1 integrin in laminin-adhesion-selected human colon-cancer cell lines of varying tumorigenicity. *Invasion Metast.,* **14,** 147–155 (1995).

KIM, W.H., SCHNAPER, H.W., NOMIZU, M., YAMADA, Y. and KLEINMAN, H.K., Apoptosis of human fibrosarcoma cells is induced by a multimeric synthetic tyr-ile-gly-ser-arg (YIGSR)-containing polypeptide from laminin. *Cancer Res.,* **54,** 5005–5010 (1994).

KLEINMAN, H.K., MCGARVEY, M.L., HASSELL, J.R., STAR, V.L., CANNON, F.B., LAURIE, G.W. and MARTIN, G.R., Basement-membrane complexes with biological activity. *Biochemistry,* **25,** 312–318 (1986).

KLEINMAN, H.K., WEEKS, B.S., SCHNAPER, H.W., KIBBEY, M.C., YAMAMURA, K. and GRANT, D.S., The laminins: a family of basement-membrane glycoproteins important in cell differentiation and tumor metastases. *Vit. Horm.,* **47,** 161–186 (1993).

LIOTTA, L.A., RAO, C.N. and WEWER, U.M., Biochemical interactions of tumor cells with the basement membrane. *Ann. Rev Biochem.,* **55,** 1037–1057 (1986).

MAFUNE, K., RAVIKUMAR, T.S., WONG, J.M.W., YOW, H., CHEN, L.B. and STEELE, G.D., JR., Expression of a Mr 32,000 laminin-binding protein RNA in human colon carcinoma correlates with disease progression. *Cancer Res.,* **50,** 3888–3891 (1990).

NAKAHARA, H., MUELLER, S.C., NOMIZU, M., YAMADA, Y., YEH, Y. and CHEN, W.-T., Activation of β1 integrin signaling stimulates typrosine phosphorylation of p190 RhoGAP and membrane-protrusive activities at invadopodia. *J. biol. Chem.,* **273,** 9–11 (1998).

NAKAHARA, H., NOMIZU, M., AKIYAMA, S.K., YAMADA, Y., YEH, Y. and CHEN, W.-T., A mechanism for the regulation of melanoma invasion. *J. biol. Chem.,* **271,** 27221–27224 (1996).

NOMIZU, M., KIM, W.H., YAMAMURA, K, UTANI A., SONG, S.-Y., OTAKA, A., ROLLER, P.P., KLEINMAN, H.K. and YAMADA, Y., Identification of cell-binding sites in the laminin-α1-chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J. biol. Chem.,* **270,** 20583–20590 (1995).

SAKAMOTO, N., IWAHANA, M., TANAKA, N.G. and OSADA, Y., Inhibition of angiogenesis and tumor growth by a synthetic peptide CDPGYIGSR-NH2. *Cancer Res.,* **51,** 903–906 (1991).

SALMIVIRTA, M., MALI, M., HEINO, J., HERMONEN, J. and JALKANEN, M., A novel laminin-binding form of syndecan-1 (cell-surface proteoglycan) produced by syndecan-1-cDNA-transfected NIH-3T3 cells. *Exp. Cell Res.,* **215,** 180–188 (1994).

SKUBITZ, A.P.N., MCCARTHY, J.B., ZHAO, Q., YI, X.-Y. and FURCHT, L.T., Definition of a sequence RYVVLPR within laminin peptide F9 that mediates fibrosarcoma adhesion and spreading. *Cancer Res.,* **50,** 7612–7622 (1990).

SONG, S.-Y., NOMIZU, M., YAMADA, Y. and KLEINMAN, H.K., Liver metastasis formation by laminin-1-peptide (LQVQLSIR)-adhesion-selected B16F10 melanoma cells. *Int. J. Cancer,* **71,** 436–441 (1997).

SWEENEY, T.M., KIBBEY, M.C., ZAIN, M., FRIDMAN, R. and KLEINMAN, H.K., Basement membrane and the laminin peptides containing IKVAV promote tumor growth and metastases. *Cancer Metast. Rev.,* **10,** 245–254 (1990).

TERRANOVA, V.P., LIOTTA, L.A., RUSSO, R.G. and MARTIN, G.R., Role of laminin in the attachment and metastasis of murine tumor cells. *Cancer Res.,* **42,** 2265–2269 (1982).

TERRANOVA, V.P., WILLIAMS, J.E., LIOTTA, L.A. and MARTIN, G.R., Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. *Science,* **226,** 982–985 (1984).

TIMPL, R., ROHDE, H., GEHRON-ROBEY, P., RENNARD, S.I., FOIDART, J.-M. and MARTIN, G.R., Laminin-a glycoprotein from basement membrane. *J. biol. Chem.,* **254,** 9933–9937 (1979).

WEWER, U.M., LIOTTA, L.A., JAYE, M., RICCA, G.A., DROHAN, W.N., CLAYSMITH, A.P., RAO, C.N., WIRTH, P., COLIGAN, J.E., ALBRECHTSEN, R., MUDRYJ, M. and SOBEL, M.E., Altered levels of laminin-receptor mRNA in various carcinoma cells that have different abilities to bind laminin. *Proc. nat. Acad. Sci. (Wash.),* **83,** 7137–7141 (1986).

YAMAMURA, K., KIBBEY, M.C. and KLEINMAN, H.K., Melanoma cells selected for adhesion to laminin peptides have different malignant properties. *Cancer Res.,* **53,** 423–428 (1993).

YOW, H.K., WONG, J.M., CHEN, H.S., LEE, C.G., DAVIS, S., STEELE, G.D. JR. and CHEN, L.B., Increased mRNA expression of a laminin-binding protein in human colon carcinoma. Complete sequence of a full-length cDNA encoding the protein. *Proc. nat. Acad. Sci. (Wash.),* **85,** 6394–6398 (1988).