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Identification of an amino acid sequence from the laminin A chain that stimulates metastasis and collagenase IV production

(tumor/basement membrane/protease/extracellular matrix)

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ABSTRACT **Tumor cells attach, degrade, and migrate through basement membranes as they metastasize. Laminin, a major glycoprotein of basement membranes, promotes the metastatic activity of tumor cells by stimulating the attachment and migration of the cells and their secretion of collagenase IV. We have identified a synthetic peptide of 19 amino acids (Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg) from the sequence of the A chain of laminin that increases experimental metastases of the lungs by murine melanoma cells. The peptide is active when injected either intravenously or intraperitoneally. The peptide increased collagenase IV activity, a key enzyme in the breakdown of basement membranes, to the same extent as laminin. This peptide represents an active site on laminin for promotion of the metastatic phenotype and generates a probe for studying the regulation of malignant activities.**

Laminin (M_r = 800,000) is the major glycoprotein of basement membranes, the thin extracellular matrix that underlies epithelial cells and surrounds nerve, muscle, and fat cells (1–3). Laminin is composed of three chains designated A (M_r = 400,000), B1 (M_r = 210,000), and B2 (M_r = 200,000) that are held together by disulfide bonds. All three chains have been cloned and sequenced (4–7). Laminin promotes cellular adhesion, growth, migration, and differentiation of cells as well as neurite outgrowth and collagenase IV production (for review, see refs. 3 and 8).

Much progress has been made in identifying the active sites of laminin by using antibodies, proteolytic fragments, and synthetic peptides (9–13). A site of five amino acids, Tyr-Ile-Gly-Ser-Arg-NH_2, on the B1 chain has been identified that promotes cell adhesion, migration, and binding to the 32/67-kDa laminin-binding protein (14, 15) that may function as a receptor (16–18). This peptide also inhibits the colonization of the lung by melanoma cells in mice (19) and blocks neural crest outgrowth from the notochord (20). This peptide lacks the other activities attributed to laminin, such as cell growth and neurite outgrowth. Another active site, designated F9, on the B1 chain has been localized near the amino terminus (21). This site binds to heparin and can promote cell adhesion. On the A chain, an active Arg-Gly-Asp (RGD)-containing sequence has been identified (K. Tashiro, G. C. Sephel, D.G., M. Sasaki, G.R.M., H.K.K., and Y.Y., unpublished results). This sequence in fibronectin has been shown to be a cell recognition site and it is an active site on other adhesion molecules (22). A fourth site of 19 amino acids (designated PA22-2) on the A chain at the end of the long arm has also been identified that can promote cell adhesion and neurite outgrowth (23, 24). Within this peptide, the sequence Ile-Lys-Val-Ala-Val was found to be required for biological activity. These different active sites appear distinct in their activities and in their sequences and likely involve recognition by various cell types through different cellular receptors.

In vitro, laminin has been found to interact preferentially with tumor cells and to enhance the metastatic phenotype. Tumor cells adhere well to laminin and the adherent cells are more tumorigenic than either the unattached cells or the parent population (25–29). Culturing tumor cells in the presence of laminin results in at least a 3-fold increase in tumor formation upon subsequent injection into mice (28, 29). The number of 32/67-kDa laminin receptors is also positively correlated with the malignant phenotype (16). In addition, laminin induces tumor cells to produce collagenase IV, the enzyme active in degrading basement membranes (30). Inhibition of this enzyme activity with specific collagenase IV inhibitors reduces the formation of tumors in the lungs of mice injected with B16F10 melanoma cells (31). These studies demonstrate that interaction of tumor cells with the basement membrane actually potentiates the malignant phenotype.

In the present study, we tested synthetic peptides from the amino acid sequence of the laminin A chain. We report here that this site, PA22-2 (Cys-Ser-Arg-Ala-Arg-Lys-Glu-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg), which is located in the E8 domain just above the carboxyl-terminal globule promotes tumor cell metastasis and induces collagenase IV activity.

MATERIALS AND METHODS

Synthetic Peptides and Laminin. Synthetic peptides were synthesized using an automated model 431A synthesizer (Applied Biosystems) as described (14). All peptides were synthesized in the amide form and, where indicated, cyclized peptides were prepared (32). The composition and purity of the peptides were determined by amino acid analyses and HPLC. All peptides were filtered to remove endotoxin and subsequently tested for endotoxin levels.

PA22-2 with tyrosine at the amino terminus was radioiodinated by using Iodo-Gen reagent (Pierce) (33). This peptide (0.5 μg) and $Na^{125}I$ (2 mCi; 1 Ci = 37 GBq) were added to a reaction vessel treated with Iodo-Gen and incubated at room temperature for 15 min. After incubation, the reaction was stopped by adding NaI. The iodinated peptide was separated from free $Na^{125}I$ and other reagents by gel filtration on a NAP 10 column (Pharmacia). The specific activity of this peptide was 2900 Ci/mmol. Mice were injected with a radiolabeled peptide in a final volume of 0.1 ml. After 2, 5, 10, 20, 30, 40, or 60 min, the animals were sacrificed, 300 μl of blood was drawn from the heart, and radioactivity in the blood was measured.

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Laminin was prepared from the EHS (Engelbreth–Holm–Swarm) tumor as described (1).

Cell Culture. HT-1080 cells, a human fibrosarcoma line, were obtained from American Type Culture Collection and were maintained as described (14). B16F10 melanoma cells (a generous gift of I. J. Fidler, M. D. Anderson Hospital, Houston) were cultured in Eagle's minimal essential medium (EMEM) containing 5% (vol/vol) fetal calf serum, glutamine, vitamins, amino acids, and antibiotics (19). Before each assay (34), the cells were rinsed once with calcium- and magnesium-free Dulbecco's phosphate-buffered saline containing 1.0 mM EDTA and then incubated in the same buffer until the cells released from the dish. The cells were subsequently centrifuged and resuspended in the EMEM medium lacking serum. C57BL/6 mice aged 4–6 weeks were injected in the tail vein with the indicated number of cells with or without the peptide in a final volume of 0.2 ml of EMEM. At least eight mice were used for each experimental group. When peptides were present with the injected cells, they were mixed with the tumor cells just prior to injection. Intraperitoneal injections (0.5 ml per mouse) of peptide in EMEM were carried out immediately after tail vein injections or 45 min later.

Collagenase Assay. Collagenase IV activity produced by the cells was measured using a solid-phase radioassay (31). Briefly, collagen IV extracted from the Engelbreth–Holm–Swarm tumor (35) was iodinated by the Bolten–Hunter method, and a solution of the labeled collagen (10,000–20,000 cpm) was applied to microtiter plates (Removawell; Dynatech) and allowed to bind overnight. Serum-free medium that had been exposed to the cells for 6 hr was added to the wells of the microtiter plates for 24 hr at 37°C, and the amount of labeled collagen released from the solid phase in the presence of serine protease inhibitors was measured. Each peptide was tested at three concentrations in duplicate. Each experiment was repeated twice. PA22-2 and smaller peptides contained within (including Cys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg-NH_2) were tested.

RESULTS

In previous studies, we found that a synthetic peptide from the sequence of the B1 chain of laminin reduced the number of metastases in mice injected with melanoma cells (19). Similar studies were undertaken here but with peptides from the A chain of laminin. The effect of the laminin A chain synthetic peptide, designated PA22-2, with the B1 chain peptide, Tyr-Ile-Gly-Ser-Arg, on lung colonization using B16F10 melanoma cells showed strikingly different effects (Fig. 1). Numerous melanotic colonies developed on the surface of the lungs 2 or 3 weeks after the injection of syngeneic mice with malignant melanoma cells. Injection of B16F10 cells in the presence of cyclic Tyr-Ile-Gly-Ser-Arg (1 mg per mouse) caused greater than an 80% reduction in the number of colonies, as reported (19, 36). In contrast, the laminin A chain peptide, PA22-2, increased the number of lung colonies (Fig. 1 and Table 1). The increase was proportional to the amount of peptide injected with the cells. At high doses of peptide, so many lesions had formed that they appeared fused and it became difficult to quantitate the number of lung colonies. Various amounts of peptide were tested on mice receiving two amounts of cells. When we injected 5×10^4 cells per mouse, all of the mice in each group had lung tumors and there was a 2-fold increase in the number of tumors found with the peptide-injected mice (Table 1). When only 1.5×10^4 cells plus 0.1 mg of peptide were injected per mouse, only half of the mice had tumor colonies on the lung. With increased amounts of PA22-2 peptide (0.5 and 1.0 mg per mouse), an approximately 5-fold increase in lung tumors was detected.

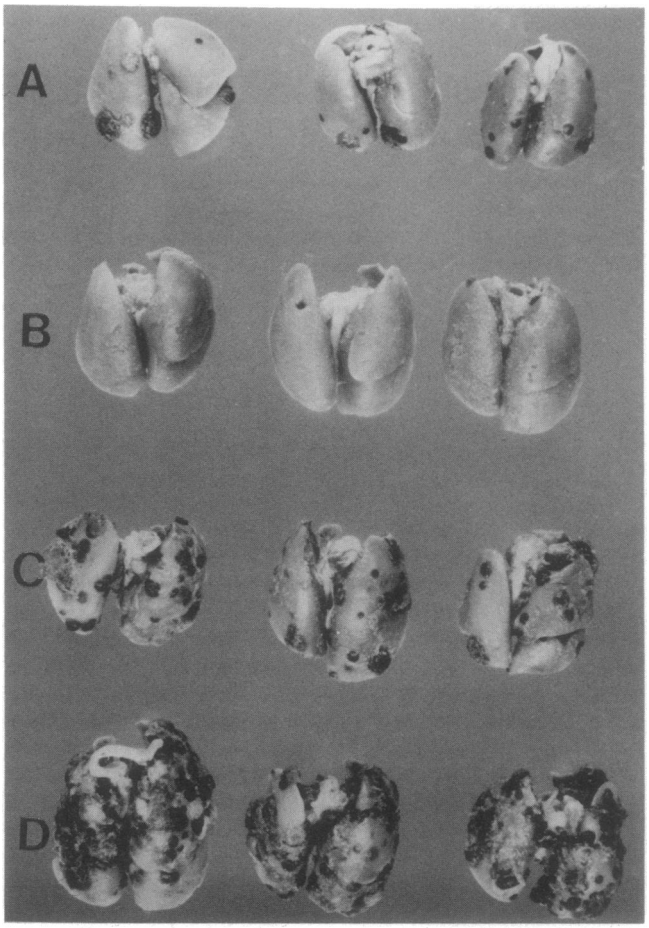

FIG. 1. Effect of laminin synthetic peptides on melanoma lung colonization. Peptide was mixed with 1×10^5 cells in a final volume of 0.2 ml just prior to injection into the tail vein of mice. Rows: A, control; B, animals receiving 1.0 mg of Tyr-Ile-Gly-Ser-Arg-NH_2; C, animals receiving 0.5 mg of PA22-2; D, animals receiving 1.0 mg of PA22-2. Eight mice were used in each group and the mice were sacrificed at 3 weeks. The number of tumors on each lung were counted and the results are as follows: control averaged 22 tumors per mouse (range 15–35 tumors), Tyr-Ile-Gly-Ser-Arg-NH_2 averaged 4 tumors per mouse (range 0–12 tumors), 0.5 mg of PA22-2 averaged 35 tumors per mouse (range 21–53 tumors), and 1 mg of PA22-2 averaged 42 tumors per mouse (range 20–85 tumors).

The PA22-2 peptide also increased the number of metastatic lesions when injected intraperitoneally either immediately or 45 min after tail vein injection of the B16F10 melanoma cells (Table 2). Immediate intraperitoneal injec-

Table 1. Effects of various concentrations of PA22-2 peptide on the colonization of lungs by B16F10 melanoma cells

Cells injected, no.	Peptide, mg	No. mice with tumors/ total no. mice	No. tumors per mouse
15,000	0	4/10	0.9 (0–5)
	0.1	4/8	2.6 (0–8)
	0.5	8/10	3.0 (0–7)
	1.0	8/10	4.7 (0–10)
50,000	0	7/7	9.0 (2–46)
	0.1	7/7	12.4 (1–27)
	0.5	5/5	12.7 (2–50)
	1.0	7/7	18.9 (9–25)

Numbers in parentheses are the range of the number of tumors per mouse. Mice injected with 50,000 cells in the presence of cyclic Tyr-Ile-Gly-Ser-Arg (1 mg per mouse) had an average of 1.8 tumors per mouse with 4 out of 7 mice having tumors.

Table 2. Comparison of intraperitoneal and intravenous peptide injection on colonization of murine lungs by B16F10 melanoma cells

Treatment	No. mice with tumors/ total no. mice	No. tumors per mouse
Control	5/10	1.3 (0–4)
PA22-2 i.v.	7/8	6.8 (0–14)
PA22-2 i.p.	6/8	2.6 (0–8)
PA22-2 i.p. 45 min after cells	7/8	5.5 (0–15)

Approximately 2×10^4 cells in 0.2 ml was injected per mouse. After 23 days, the mice were sacrificed and the number of tumors on the surface of the lungs was quantitated. Many of the mice treated with the peptide had tumors in the muscle lining of the chest cavity. Numbers in parentheses are the range of the number of tumors per mouse.

tion of peptide resulted in a lesser stimulation of metastasis formation than did the intravenous injection (2.6 vs. 6.8 tumors per mouse). Injection of peptide intraperitoneally 45 min after injection of the tumor cells yielded a greater number of tumors (5.5 tumors per mouse) over that observed with cotemperal injection (2.6 tumors per mouse) but was still less than that observed with the intravenous injection (6.8 tumors per mouse). We conclude that the cells do not require a prolonged incubation with the peptide to be activated. The higher levels of peptide that the cells are exposed to by coinjection intravenously may be responsible for the greater number of metastases.

We also determined the half-life of the PA22-2 peptide in the circulation. Fifty percent of the labeled peptide is cleared from the blood at 10–20 min, further indicating that the cells do not require prolonged incubation with the peptide. This clearance time is comparable to that (8–12 min) observed with fibronectin-derived synthetic peptides (37).

No difference was noted in the size of the lung colonies in the peptide-treated animals and in the controls, but there was a shorter survival of animals receiving both the PA22-2 peptide plus the melanoma cells than in the animals receiving either cells alone or cells plus cyclic Tyr-Ile-Gly-Ser-Arg (Fig. 2). After injection of the tumor cells, 50% of the control animals were dead by day 23 and the rest died by day 25 after injection of the tumor cells. Half of the Tyr-Ile-Gly-Ser-Arg-treated animals were dead by day 25 and the remainder survived more than 2 months, at which time they were

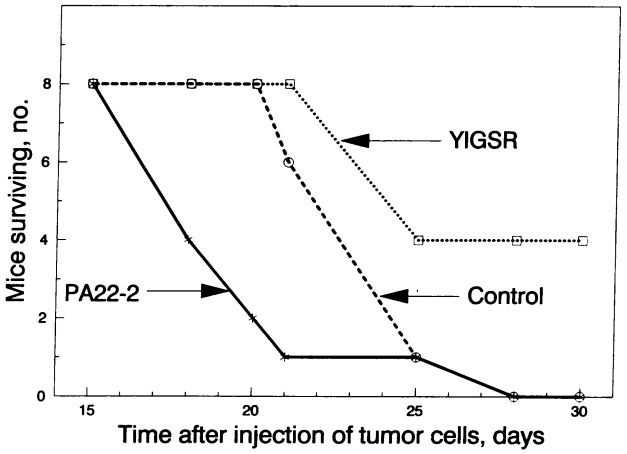

FIG. 2. Effect of laminin peptides on mouse survival after injection of B16F10 melanoma cells. All animals were injected by way of the tail vein with 1.0 mg of the indicated peptide and 1×10^5 cells in a final volume of 0.2 ml. The number of animal deaths were recorded several times per week. The four Tyr-Ile-Gly-Ser-Arg (YIGSR)-injected mice lived for more than 2 months and then were sacrificed.

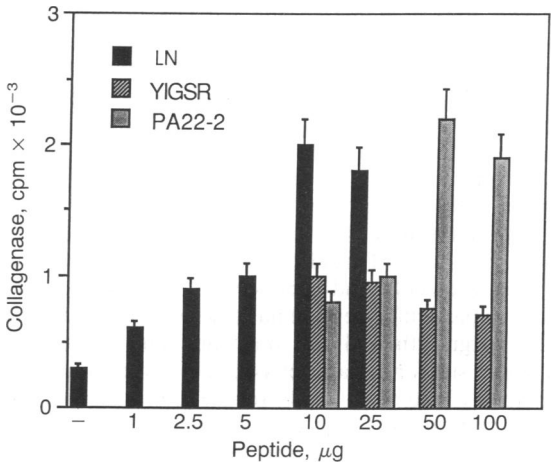

FIG. 3. Effect of laminin peptides on collagenase IV activity. B16F10 melanoma cells (2×10^4 cells per 16-mm-diameter dish) were allowed to attach to the peptide-coated surfaces in medium lacking serum. After 6 hr the medium was collected and assayed for enzyme activity. Similar data were also obtained with HT-1080 cells (data not shown). LN, Laminin; YIGSR, Tyr-Ile-Gly-Ser-Arg.

sacrificed and appeared to have no lung tumors. In contrast, the A chain peptide-treated animals died sooner than either the control or the Tyr-Ile-Gly-Ser-Arg-treated animals, with 50% of the animals dead on day 18. We also noted that one-third of the animals treated with the A chain peptide had extra pulmonary tumors mostly located in the muscle wall of the chest cavity (data not shown).

Since collagenase IV is elevated by laminin and is involved in the invasion of the cells through basement membrane barriers, we examined whether the A chain peptide influenced the production of this enzyme. As reported (30), laminin caused a dose-dependent increase in collagenase IV activity in B16F10 (Fig. 3) and in HT-1080 cells (data not shown) with the maximum reached at 10 μg per dish (Fig. 3). The Tyr-Ile-Gly-Ser-Arg-NH_2 peptide had some activity (three times background) in increasing collagenase IV activity. The A chain peptide also caused a dose-dependent increase (up to 7 times background) in both cell types. Enzyme activity reached the same level as that observed with laminin although much more peptide was required. Since cycloheximide treatment of the cells blocked the increase in collagenase IV activity, it is likely that laminin and the peptide act to increase the synthesis of the enzyme (data not shown). By using a zymogram assay, collagenase IV activity could not be detected unless the cells were treated with PA22-2 (data not shown). Both laminin and the peptide were active whether presented to the cells as a dried substratum or in the medium, but, when present in the medium, the activity was less by one order of magnitude. Various smaller overlapping peptides contained within the PA22-2 sequence were tested to determine the smallest active peptide required for collagenase activation. None of these peptides including those containing the Ile-Lys-Val-Ala-Val sequence, which has been found to be active for cell adhesion and migration (23, 24), were active, indicating that the entire sequence is required (data not shown).

DISCUSSION

Laminin has been implicated in the metastasis of malignant tumor cells by helping cells attach and localize to basement membranes as well as by stimulating cellular motility (25–27). In addition, some studies suggest that tumor cells are more malignant when cultured with laminin and that laminin stimulates the production of collagenase IV, which the cells use

to breech basement membrane barriers (28–30). Thus, it seemed possible that there is a domain in laminin that induces the malignant phenotype in tumor cells. In related studies, we have found that a synthetic peptide from the B1 chain of laminin, Tyr-Ile-Gly-Ser-Arg-NH_2, is a potent antagonist able to reduce the number of metastases when coinjected with melanoma cells (19, 36). When the PA22-2 peptide, a synthetic peptide from the A chain of laminin, was studied, we found that it increased the number of lung lesions formed by melanoma cells. This effect was dose-dependent and was observed when cells and peptide were injected into different sites. This particular peptide has also been found to promote neurite outgrowth and cell migration and to serve as an attachment site for certain cells (23, 24). Also this peptide can compete with laminin itself for cell attachment. Smaller peptides containing the Ile-Lys-Val-Ala-Val were all found to be active in cell adhesion and those lacking this sequence were inactive, suggesting this sequence as the important site. Thus, this sequence appears to include a major biologically active site in laminin.

Metastasis is a complex process involving the dissemination of tumor cells through the body, avoidance of host surveillance, and invasion and growth of the cells in a second site (25). The number of metastases that occur after intravenous injection of cells is dependent on each process and can be varied at each step. Because laminin induces an increased production of collagenase IV by tumor cells (30), we also tested the effect of the peptide on collagenase IV production. Indeed, the peptide did stimulate collagenase IV production to the same levels observed with laminin. However, considerably higher levels of peptide were required possibly due to inaccessibility of the active site of the peptide bound to the plastic. We did not find any smaller peptides derived from the PA22-2 sequence including those with the active (adhesion) Ile-Lys-Val-Ala-Val sequence able to induce collagenase IV activity. This peptide did not alter the rate of growth of the tumors when cells and peptide were injected subcutaneously, nor did the peptide cause an aggregation of the cells in suspension. Therefore, due to the known importance of collagenase IV in the metastatic process, the stimulation of collagenase IV production as well as the affect of this peptide on cell motility could account for the increased malignancies in the presence of the peptide.

The peptide was effective when injected intraperitoneally 45 min after the intravenous injection of the tumor cells. It is known from *in vivo* studies using labeled cells that at 45 min the cells are still attached to the blood vessel wall and have not yet penetrated the tissue (38). In addition, *in vitro* assays using amnion or a reconstituted basement membrane generally require 6–24 hr for the tumor cells to penetrate (39, 40). Although the peptide is rapidly cleared from the circulation (50% removed by 20 min), it is still very effective. The peptide likely has a strong and rapid affinity for the cells. The time of 45 min after injection may be a critical time at which the cells have attached and the collagenase enzyme is just beginning to be activated. The presence of a peptide that promotes collagenase activity and cell migration at this time may thus favor an increase in tumor cell penetration into the tissue.

The ability to stimulate the metastatic activity of the tumor cells by a simple synthetic peptide provides a probe into the process of metastases. Probably, specific cell receptors exist for this peptide and analogues of the peptide may compete for this site and be inhibitors of metastasis.

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