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Basement membrane and the SIKVAV laminin-derived peptide promote tumor growth and metastases

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Summary

Laminin, the major glycoprotein component of basement membrane, promotes the malignant phenotype. Cells which are adherent to laminin are more malignant than the non-adherent cells and in certain tumor cells, the number of laminin receptors is positively correlated with malignancy. Laminin also increases collagenase IV activity, an enzyme demonstrated to be critical for tumor spread. A site on laminin, containing the amino acid sequence SIKVAV, has been identified which when injected intravenously with B16F10 melanoma cells, causes an increase in the number of colonies on the surface of the lungs. This peptide does not affect tumor cell arrest in the vasculature or the immune system. It does promote angiogenesis in various *in vitro* and *in vivo* models, thereby facilitating tumor cell survival.

When a complex mixture of laminin-enriched basement membrane components (Matrigel) is coinjected with tumor cells subcutaneously, tumor incidence and growth increases. Various tumor cell lines and primary isolates, which previously could not form tumors in mice, can be induced to grow rapidly in the presence of Matrigel. Slowly growing tumors or arrested tumors can also be induced to grow more quickly with additional injections of Matrigel. When an SIKVAV-containing synthetic peptide is coinjected with B16F10 tumor cells and Matrigel subcutaneously in mice, larger tumors are formed than that observed with either Matrigel or cells alone. Such studies define the role of laminin in tumor growth and spread and generate new models for studying therapeutic agents. Of particular interest is the ability to grow primary isolates which generally do not grow in mice.

Introduction

In order to metastasize, tumor cells must attach, degrade, and migrate through basement membrane, the thin extracellular matrix that underlies endothelial cells [1, 2]. Basement membrane is not an inert barrier that supports tissues, but rather it has profound effects on cell behavior [3–5]. Basement membranes are enriched in the glycoprotein laminin which facilitates tumor cell adhesion [6, 7] and promotes the malignant phenotype (Table 1). Small cell lung carcinoma cells grown in laminin are more resistant to cytotoxic drugs [8]. In addition, intravenous coinjection of laminin and B16F10 tumor cells results in an increase in the number of lung colonies [9]. Likewise, when a mixed population of tumor cells is allowed to adhere to laminin, the laminin-adherent cells are more malignant than either the non-laminin adherent cells or the parent population [10, 11]. This is not simply due to adherent cells being more malignant, since fibronectin-adherent cells do not show enhanced malignant potential. In addition, the number of laminin receptors correlates with the malignant potential of

various human and murine cell lines and with the Duke's staging of human colon carcinoma cells [12, 13], thus demonstrating a specific interaction of the tumor cells with laminin. Laminin promotes the activity of collagenase IV, a key enzyme involved in degradation of basement membrane and in tumor spread [14]. Together, these data demonstrate that the interaction of tumor cells with laminin is a critical event in malignancy. Identification of some of the mechanisms by which laminin enhances the malignant phenotype has led to new ideas on therapeutic approaches as well as to the ability to generate new animal models and cell lines for study. Many biologically active domains on laminin have been identified and others likely exist (reviewed in [15]). This review will concentrate on the use of a laminin-enriched basement membrane matrix (Matrigel), which promotes subcutaneous tumor growth and the incidence of tumor growth, and on active sites in laminin which promote metastases, angiogenesis, and collagenase IV activity. Such studies have also produced new animal models for tumor growth and for testing therapeutic agents.

Tumor cell-basement membrane interactions

Studies on the interactions of tumor cells with basement membrane have been facilitated by the availability of a complex mixture of basement membrane components (Matrigel) [3] and the purified components laminin and collagen IV [16, 17]. Matrigel is isolated from the Engelbreth-Holm-Schwarm (EHS) tumor and from placenta (Fig. 1) and has been found to contain all of the basement membrane components including laminin, entactin, collagen type IV, heparan sulfate proteoglycan, TGFβ, FGF, EGF, PDGF, and IL-1 [3, 18, 19]. Matrigel is a liquid at 4° C but reconstitutes into a gel at 24–37° C within minutes. Electron microscopy of polymerized Matrigel demonstrates lamina densa-like structures that resemble those observed in authentic basement membranes. Matrigel has been used as a culture substratum for both normal and malignant cells [4, 20]. Unlike non-transformed epithelial cells which cease multiplying and maintain a differentiated phenotype on Matrigel, many tumor cells grow rapidly and form branching and invasive colonies. In fact, the degree of branching morphology of tumor cells cultured on Matrigel has been used as an indicator of their invasive potential [20]. A related and more easily quantifiable assay employs a thin layer of Matrigel coated on filters placed in Boyden chambers. Here the ability of the tumor cells to attach and migrate through the matrix has been used to assess the invasiveness of tumor cells. Penetration through basement membrane can also be used to select for highly malignant subpopulations of tumor cells [20–29]. Such *in vitro* studies demonstrate a strong correlation between the invasiveness in Matrigel and the *in vivo* malignant phenotype of the cells.

Use of reconstituted basement membrane for tumor growth *in vivo*

Many mammalian tumor cell lines and human primary cells isolated from tumors are difficult to grow *in vivo*, even with large inocula in athymic mice [30]. Since tumor cells grow well in Matrigel *in vitro* [20], Matrigel was tested as a matrix for growing cells in mice. Initially small cell lung carcinoma cell (SCLC) lines mixed with Matrigel and coinjected in mice were found to promote rapid tumor growth [8]. When as few as 2.5×10^4 cells in a final volume of 0.5 ml/mouse were injected subcutaneously in athymic mice, tumors (2 cm) were observed within 45 days, whereas 10^6 SCLC cells injected alone did not yield tumors after 4 months. Since

Table 1. Effects of laminin on tumor cells

Increased activity
Adhesion
Growth
Migration
Metastases
Collagenase IV activity
Plasminogen activation
Drug resistance

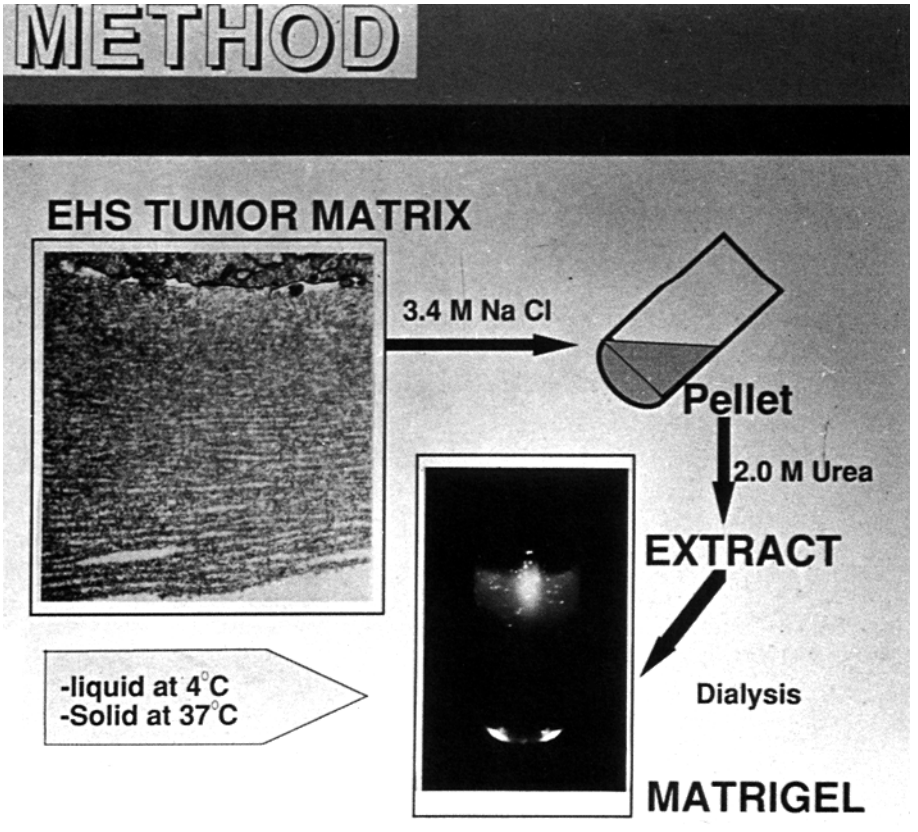

Fig. 1. Schematic representation of Matrigel preparation. EHS tumor tissue is first washed with high salt (3.4 M) to remove blood components and then the pellet is extracted with 2.0 M urea. The extract is dialyzed into physiological buffer where it is a liquid at 4° C and a gel at 37° C after a 20 min incubation.

this original observation, many other tumor-derived cell lines, primary isolates, and normal cells have been tested with this method (Fig. 2) [31, 32].

Indeed, Matrigel was found to promote the subcutaneous growth and incidence of both tumor cell lines and freshly-dissociated tumor cells (Table 2). Normal cells did not form tumors. In general, tumor growth was quite rapid. In cases such as B16F10 and KB cells, which can form tumors alone, the tumors were 5–10 fold larger when grown in the presence of Matrigel. Matrigel supported the growth in mice of human tumor biopsies which usually include normal stroma. Fibroblast growth was not enhanced by Matrigel, and thus Matrigel appears to specifically promote the proliferation of malignant cells but not of non-transformed cells when coinjected subcutaneously in mice. This alleviates the difficulties many investigators have found when trying to establish primary tumor isolates from human biopsies in culture.

The factors in Matrigel which promote tumor growth in mice are beginning to be defined. For example, the more concentrated the Matrigel, the better the rate of tumor growth [31]. Concentrations as low as 0.1 mg/mouse yield a two-fold increase in B16F10 melanoma tumor size over that observed in the absence of Matrigel, while a dose of 5 mg/mouse yields a 10-fold increase in tumor size. Cell contact with Matrigel is a requirement for enhanced tumor growth, since separate injection of cells or Matrigel on opposite sides of the mouse has no effect on tumor growth. Matrigel does not en-

Fig. 2. Appearance of mice after subcutaneous injection of HT-1080 cells in the absence and presence of Matrigel. Mice were injected with 5×10^5 cells in a final volume of 0.5 ml. After 5 weeks, the mice were photographed. Mice on the left received cells alone and those on the right received cells plus Matrigel (2.5 mg/mouse in a final volume of 0.5 ml).

hance tumor growth by functioning as an inert support. Collagen, which also forms a gel under physiological conditions, does not promote the growth of most tumor cells when injected subcutaneously (Table 3 and [31]).

None of the individual factors in Matrigel appears to support subcutaneous growth when tested alone. Coinjections with laminin did not stimulate small cell lung carcinoma tumor growth. Moreover, Matrigel from which growth factors (TGF-β, PDGF, FGF, EGF, and IL-1) have been removed or reduced still supports B16F10 tumor growth to the same extent as complete Matrigel. These data suggest that the extracellular matrix components present in Matrigel, such as collagen type IV and laminin, are important in the biological activity of Matrigel. The presence of laminin-derived synthetic peptides in Matrigel (see below) can increase tumor cell proliferation. Thus, it is likely that multiple tumor cell-matrix interactions, including interactions with laminin, are involved in the ability of tumor cells to grow *in vivo* when coinjected with Matrigel.

Laminin and active synthetic peptides

Laminin, the major component of Matrigel, has potent effects on tumor cells, including enhancing adhesion, proliferation, migration, plasminogen activation, cytotoxic drug resistance, collagenase IV production, and metastasis [6–15, 33–36]. The number of laminin receptors correlates with malignant potential [12, 13]. Laminin is a multidomain molecule composed of three chains designated A

(Mr = 400,000), B1 (Mr = 210,000), and B2 (Mr = 200,000) which are held together by disulfide bonds in a cruciform-like structure [2, 15]. A number of laminin homologues have been described which interact with the laminin chains to yield new isoforms. To date, one homologue of the B1 chain, designated S-laminin because it is found primarily at synapses, has been described [37]. At least three homologues of the A chain have been described and others likely exist [38–41].

Several active sites in laminin have been defined using antibodies, laminin fragments, and synthetic peptides. The P1 (pepsin digest) fragment encompasses the amino ends of all three chains which form the upper region of the cross [15]. This fragment of laminin, when coinjected intravenously with tumor cells into mice, reduces the number of melanoma cells which colonize the lung [11]. Three sequences, designated F9 (residues 641–660), PDSGR (residues 902–906), and CDPGYIGSR (residues 925–933), are localized within this domain on the B1 chain and block tumor colonization of lungs when coinjected with tumor cells [42–52].

The biological effects of the YIGSR peptide on tumor cells have been studied extensively. YIGSR is active for cell adhesion, migration, and inhibition of tumor metastases [42, 49, 53, 54] (Fig. 3). It is more active in the polymerized form and when coupled to polyethyleneglycol. YIGSR is effective with several different tumor cell types *in vivo* including B16F10, Lewis lung carcinoma, and B16BL6 cells. This peptide can block laminin-mediated cell adhesion in culture [42, 53] and was therefore initially thought to inhibit tumor metastases by inhibiting tumor arrest in the lungs. Studies with radiolabeled tumor cells, however, have demonstrated that YIGSR has no effect on tumor cell arrest (Table 4). Rather, a recent study using the chick chorioallantoic membrane assay suggests that a YIGSR-containing peptide blocks establishment of tumor cell metastases by inhibiting angiogenesis [48, 55, 56]. This possible mechanism of YIGSR inhibition of tumor metastases is consistent with the data which demonstrate that daily administration of YIGSR four days after the tumor cell injection reduces lung colonies by 80% [48]. The YIGSR peptide also blocks Matrigel-induced subcutaneous growth of human small cell lung carcinoma tumors in mice when coinjected with the tumor cells and Matri-

Table 2. Tumorigenity of cells coinjected with matrigel

Cells tested	Tumors formed		
	Without matrigel	With matrigel	Fold incr.
Murine			
B16F10 melanoma	+	+++	3–8X
NIH 3T3	–	+++	n.a.
T-EHS	+	+++	n.d.
Human			
SCLC (lung)	–	+++	n.a.
A253 (submandibular)	+	+++	6X
KB (epidermoid carcinoma)	+	+++	8X
Tsu-pr 1 (prostate)	–	+++	n.a.
Renal cell carcinoma	+	++	n.d.
Colon carcinoma	–	+++	n.a.
HT1080 fibrosarcoma	–	+++	n.a.
Normal cells			
Mesangial (human)	–	–	
Endothelial cells (human)	–	–	
Sertoli cells (rat)	–	–	
Chondrocytes (chick)	–	–	

n.a. = not applicable.
n.d. = not determined (tumors not measured).
– = no tumors observed.
+ = small tumors.
++ = large tumors (< 1 cm^3).
+++ = very large (> 1 cm^3).

Table 3. In vivo growth of human tumor cell lines in athymic mice

Cell/Treatments	Average tumor size in cm^3	(% Mice with tumors)
SCLC NCI–H187/0.75 mg Matrigel/mouse	11.8	(75%)
SCLC NCI–H187/0.75 mg Collagen/mouse	0	(0%)
SCLC NCI-H187 alone	0	(0%)
KB/1.0 mg Matrigel/mouse	18.6	(100%)
KB/1.0 mg Collagen/mouse	5.0	(100%)
KB alone	4.3	(100%)

5×10^5 cells were injected subcutaneously in a final volume of 0.5 ml. SCLC (small cell lung carcinoma) tumors were measured after 3 months, and KB (epidermoid carcinoma) tumors were measured after 1 month. A minimum of 4 mice were used for each group.

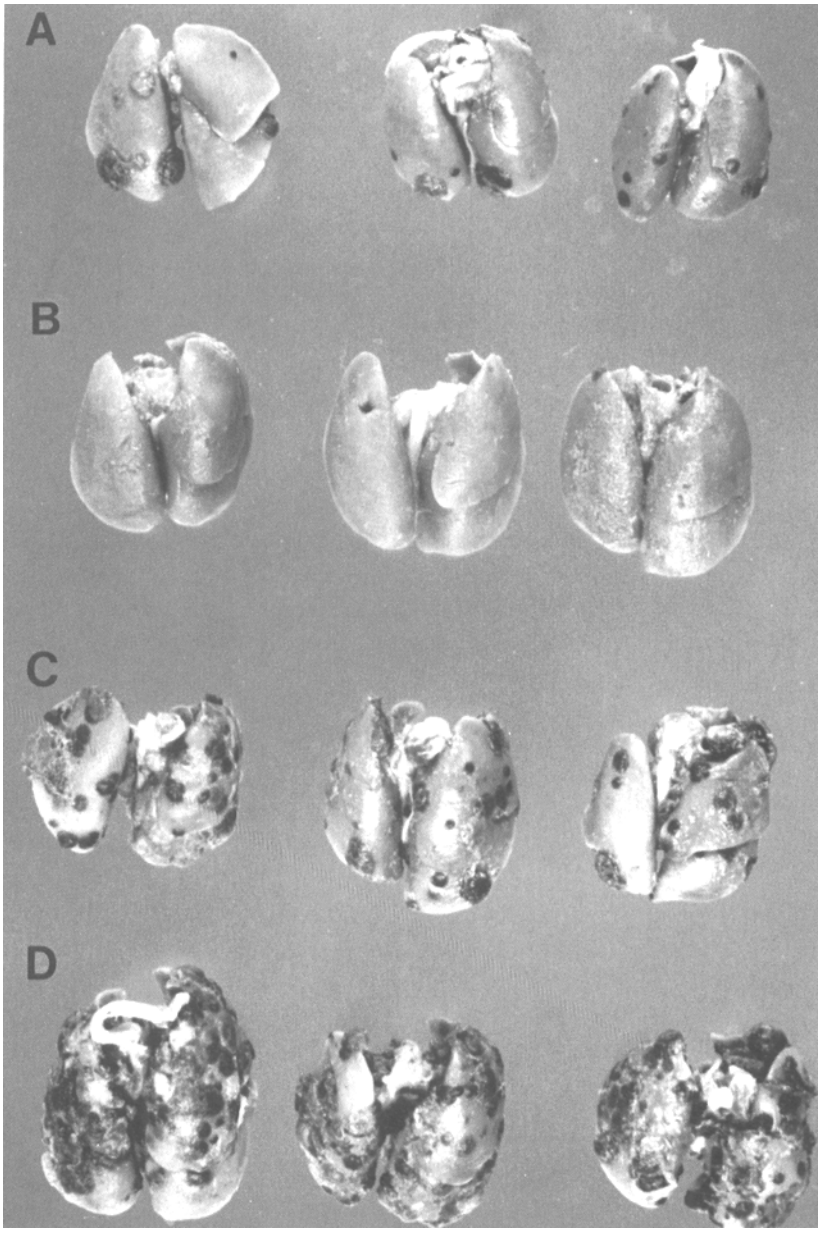

Fig. 3. Appearance of lungs after intravenous injection of B16F10 melanoma cells. Cells were injected in the (A) absence of peptide, or (B) in the presence of 1 mg/mouse of YIGSR, or (C) and (D) SIKVAV-containing peptides 0.5 and 1.0 mg respectively. Eight mice were injected in each group. Reprinted from [58] with permission.

gel [8]. These data demonstrate that YIGSR has a potent effect on blocking tumor growth and spread. Although tested with only a limited number of cells, it is possible that YIGSR may interact with a wide variety of cells and may be useful clinically.

Another site on laminin, containing the SIKVAV sequence (residues 2091 to 2108 on the A chain), has been found to be biologically active with a variety of tumor cells [57]. This peptide has many of the activities of the parent molecule, including

promoting tumor cell adhesion and migration, and increasing collagenase IV activity [57, 58]. When coinjected intravenously with B16F10 melanoma cells, a 2- to 5-fold increase in the number of lung colonies is observed. This peptide does not appear to affect tumor cell arrest (Table 4) in the lungs and does not require prolonged preincubation with the cells. In fact, the peptide can be injected intraperitoneally from 1 to 8 hours after the intravenous injection of the tumor cells and still promote colony formation on the lungs (Fig. 4). If injected one hour before the tumor cells, it is ineffective, suggesting that the SIKVAV-containing synthetic peptide does not act indirectly, such as by immune suppression. We sought to determine if mice immunized with SIKVAV would have a reduced number of lung colonies since antibodies to whole laminin reduced lung colonies [11]. Mice were immunized with the SIKVAV-containing peptide to determine if the growth of B16F10 colonies on the surface of the lungs after intravenous injection would be affected. A 2.5-fold decrease in the number of colonies on the surface of the lungs (Table 5) and an increase in the survival (data not shown) were observed in the mice immunized with SIKVAV-containing peptide compared to control mice immunized with a laminin B2 chain containing peptide. These data suggest that *in vivo*, the SIKVAV-containing site on laminin may be active in promoting tumor spread and/or growth.

The SIKVAV-containing peptide is strongly angiogenic in the chick chorioallantoic membrane assay and in an *in vitro* human umbilical vein capillarly-like tube forming assay [59]. It is also active in promoting collagenase IV activity and plasminogen activation [33, 58]. It may thus act to increase lung colonies either by promoting the ability of tumor cells to penetrate into tissues or once there to proliferate as a consequence of an increased

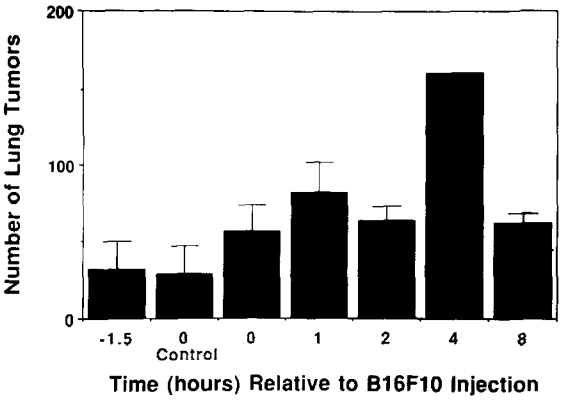

Fig. 4. Number of B16F10 melanoma colonies on the surface of the lungs of mice receiving IKVAV-containing peptide at various times after intravenous tumor cell injection. Mice received 1.5×10^4 tumor cells intravenously via the tail vein and the peptide was administered intraperitoneally either 1.5 hr before the tumor cells (−1.5), at the same time as the tumor cells (0) or at 1, 2, 4, or 8 h after the tumor cells. Control mice received injections of cells only. There were eight mice in each group. The mice were sacrificed at 3 weeks and the number of colonies on the surface of the lungs was counted by an observer unaware of the specimen treatment. The mean number of tumors/mouse ± standard error is plotted.

Table 4. B16F10 Tumor cell arrest in the presence of various coinjected peptides

Peptides	10 min	60 min
SIKVAV	11.8	4.8
YOSH E	10.8	5.5
CDPGYIGSR	15.7	4.5
Y(D-I)GSR	14.7	5.3

Data are expressed as mean cpm × 10^3. Mice were injected with 1 mg peptide/mouse and 5×10^5 cells. Peptides used were CSRARKQAASIKVAVSADR (SIKVAV), CTOLDNEVNGM LRQLEEAEN (YOSH E from the B2 chain), CDPGYIGSR, and Y(D-I)GSR (prepared by C. Schasteen from Monsanto with D-isoleucine). At 24 h, all mice had counts less than 1,000 and no differences were observed.

Table 5. Effects of active immunization against laminin sequences on experimental metastasis formation

Laminin sequences	Number of tumors ± standard error
Yosh E	10.67 ± 3.10
SIKVAV	4.00 ± 0.79

Mice were injected 1 mg/mouse with each peptide (Yosh E = CTOLDNEVNGMLRQLEEAEN from the B2 chain and CSRARKQAASIKVAVSADR from the A chain). Peptides were coupled to keyhole limpet hemocyanin. The initial immunization was injected with peptide plus complete Freund's adjuvant then followed 4 and 6 weeks later with peptide plus incomplete adjuvant. At 8 weeks, 5×10^5 cells were injected in the tail vein and the mice were sacrificed after 21 days. The number of tumors present on the lung surfaces was quantified by a blinded observer.

blood supply. The laminin-derived SIKVAV-containing peptide also promotes B16F10 tumor cell growth in the subcutaneous tumor cell-Matrigel model. Tumors formed in the presence of the SIKVAV-containing peptide and Matrigel are approximately three-fold larger than those observed with Matrigel and tumor cells alone (Kibbey, unpublished). Histologic examination of the tumors grown subcutaneously in the presence of the active peptide plus Matrigel reveal more blood vessels than those grown with Matrigel alone (Kibbey, unpublished). Thus, the SIKVAV-containing peptide can further tumor growth and perhaps be used to establish new models.

Conclusion

An extract of reconstituted basement membrane, termed Matrigel, increases both tumor incidence and growth when coinjected subcutaneously in mice. Subsequent tumor growth is rapid and has been achieved both with tumor cell lines and primary isolates from human tumors. The laminin in Matrigel is probably responsible in part for these effects, since laminin is active in cell culture in promoting the malignant phenotype. A synthetic laminin-derived peptide containing SIKVAV can promote the growth of Matrigel-induced tumors perhaps by promoting angiogenesis. Another synthetic laminin-derived peptide containing YIGSR blocks tumor growth and angiogenesis. These data demonstrate a new method for growing tumor cells in mice and define a model for testing specific therapeutics.

Key unanswered questions

We have demonstrated that Matrigel coinjection facilitates tumor cell growth. While this method can potentially generate useful *in vivo* models, what is observed to date is a phenomenon. The mechanisms of action have not been determined and modifications for greater growth, and perhaps metastasis, have not been described. These data may lead to a better understanding of the mechanisms of tumor growth and suggest new diagnostic or therapeutic approaches to human cancer.

1. What are the key components in Matrigel which are active in tumor growth promotion? Are any of them limiting in amount?
2. Laminin promotes cell proliferation but this activity has not yet been defined by a synthetic peptide. Are there any other sites on laminin which enhance tumor growth? Are there specific sites on laminin which increase drug resistance? Is there a role for the B2 chain of laminin?
3. What cell surface receptors are involved in tumor cell interactions with Matrigel?
4. What cellular events occur after binding to Matrigel?
5. Do the proteases produced by the tumor cells cleave laminin to generate cryptic domains with biological activity?
6. Many tumor cells are highly malignant and yet do not metastasize in the Matrigel implant model. Could a metastatic model be created by injecting tumor cells with Matrigel into an orthotopic location (e.g. renal cell carcinoma cells in the kidney)?

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