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Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines

(tumor/extracellular matrix/adhesion/chemotherapy)

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ABSTRACT **Small cell lung cancer (SCLC) is a fatal malignancy due to its propensity to metastasize widely and to reoccur after chemotherapy in a drug-resistant form. While most SCLC cell lines are anchorage independent for growth, laminin induced the attachment of five of six SCLC cell lines tested (NCI-N417, NCI-H345, NCI-H146, NCI-H187, NCI-H510, and NCI-H209). NCI-N417 SCLC cells adopted a flattened morphology on laminin, and a classic SCLC cell line (NCI-H345) demonstrated a neuron-like appearance while the other SCLC cell lines except NCI-H187 cells, attached but did not spread. Adhesion to laminin was associated with increased resistance to several cytotoxic drugs. Matrigel, an extract of basement membrane proteins, greatly accelerated tumor growth when coinjected with SCLC cells in athymic mice. A synthetic peptide from the B1 chain of laminin, cyclic-YIGSR (Tyr-Ile-Gly-Ser-Arg), inhibited laminin-induced SCLC cell adhesion and migration *in vitro* and reduced the size of the tumors they formed when coinjected with matrigel and YIGSR. These results suggest that the interaction of SCLC cells with laminin and possibly with other basement membrane proteins can enhance their tumorigenicity and drug resistance.**

Small cell lung cancer (SCLC) forms widespread metastasis, usually prior to detection (1). Despite the fact that this tumor is initially sensitive to chemotherapy, it reoccurs and becomes drug resistant with a survival of <2 years in >90% of the cases (1). Cell lines established from SCLC specimens are anchorage independent for growth and they are classified as either variant or classic based on morphological and biochemical features (2, 3). Cells of the classic but not of the variant type exhibit a range of neuroendocrine properties including high levels of L-dopa decarboxylase (3, 4), neuron-specific enolase (3, 4), polypeptide hormone secretion (4), and the presence of neurosecretory granules.

Metastatic tumor cells attach preferentially to laminin, the predominant glycoprotein in basement membranes (5, 6). Laminin stimulates tumor cell adhesion (7), collagenase IV production (8), cell motility (9, 10), and the formation of metastasis (6). We have previously reported the ability of various SCLC cell lines to attach to laminin (11). In this study, we present evidence that laminin alters the morphology and induces the motility of SCLC cell lines. Also, a reconstituted basement membrane (matrigel), of which laminin is the major component, increases the tumorigenicity of these cells in athymic mice. We report that SCLC cell lines are more resistant to various chemotherapeutic agents when attached to laminin. These observations suggest that the interaction of cells with laminin as well as with other basement membrane components may play a role in tumor growth and drug resistance.

MATERIALS AND METHODS

Extracellular Matrix Proteins, Matrigel, and Synthetic Peptides. Laminin, collagen IV, heparan sulfate proteoglycan, and matrigel were all isolated from the EHS tumor as described (12, 13) and were used as substrates *in vitro* in standard attachment assays as described (5, 6). Human fibronectin and bovine collagen I were purchased from Collaborative Research. Cyclic peptides were synthesized as described (14) with an automated synthesizer model 930A (Applied Biosystems), and their purity was ascertained by amino acid analyses and by high-performance liquid chromatography. The synthetic peptides were filtered to remove contaminating endotoxin (14).

Cell Culture. SCLC cell lines (NCI-N417, NCI-H187, NCI-146, NCI-H209, NCI-510) were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and Hepes buffer (25 mM) and were all purchased from GIBCO. NCI-H345 cells were grown in serum-free medium, HITES, as described (15).

Cell Motility Assay. Cell migration was tested by using modified Boyden chambers with a polyvinylpyrrolidone-free (pore size, 8 μm) polycarbonate filter (Neuroprobe, Cabin John, MD) placed over the lower well previously filled with RPMI 1640 medium (0.22 ml) containing 0.1% bovine serum albumin and increasing concentrations of laminin. Alternatively, laminin was dried directly on the lower surface of the filters. A suspension of SCLC cells (2×10^5 cells in 0.85 ml of RPMI 1640 medium/0.1% bovine serum albumin) was added to the upper well. After a 5-hr incubation at 37°C in 5% CO_2/95% air, the filters were removed, fixed, and stained with Diff-Quick stain (American Scientific Products, McGaw Park, IL). The area occupied by the cells that migrated to the lower surface of the filters was quantitated by image analysis (Optomax IV, Analytical Measuring Systems, Essex, U.K.).

***In Vivo* Tumor Studies.** Cultured SCLC cells were harvested by centrifugation (5 min at 1000 rpm), the cell clumps were disaggregated by gentle trituration, and an aliquot of the suspension was assessed for cell number and viability. Cells were resuspended in cold serum-free RPMI 1640 medium and mixed with either an equal volume of cold liquid matrigel (10 mg/ml), laminin (2 mg/ml), or collagen I (3 mg/ml, previously neutralized), and a final vol of 0.5 ml was immediately

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Abbreviation: SCLC, small cell lung cancer.

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injected subcutaneously into 4- to 8-week-old female NIH strain athymic nude mice. When tumors became apparent, calipers were used to determine their size.

Chemosensitivity Assay. The chemosensitivity of SCLC cell lines was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay as described (11, 16). The four drugs tested were doxorubicin, etoposide, cisplatin, and the alkylating agent nitrogen mustard. They are commonly used in the treatment of SCLC. Cells, harvested during logarithmic growth, were resuspended in the culture medium and seeded (1–5 × 10^4 cells per well) in 96-well plates either with or without a coating of laminin (2 μg per well). Serial dilutions of cytotoxic drugs over a 5 log range of concentrations were added to the appropriate wells 24 hr after seeding. Eight wells per plate were treated with each concentration of drug and each experiment was performed at least three times. On the 4th day, MTT was added to the medium and 4 hr later the absorbance of the fluid in the well at 540 nm was measured with an automated microplate reader (Bio-Tek Instruments, Winooski, VT). The ID_{50} is defined as the drug concentration causing a 50% reduction in absorbance of reduced MTT (11, 16) compared to the absorbance observed with cells not treated with drug. Statistical analysis was performed using the difference observed between the ID_{50} with laminin minus the ID_{50} without laminin. The null hypothesis that these differences have a mean value of zero for each of the combinations of cell lines and drugs was tested by the Wilcoxon signed rank test. A broader null hypothesis over the three adherent cell lines is that the probability of a difference being positive is equal to 50% for each drug and cell line; the sign test was used for this hypothesis. A modified Bonferroni procedure was applied to correct for the simultaneous tests of the four drugs (17). All *P* values are two sided.

RESULTS

Adhesion and Migration of SCLC Cells to Laminin. We tested the ability of six SCLC cell lines (NCI-N417, NCI-H345, NCI-H146, NCI-H187, NCI-H510, NCI-H209) to attach and spread on either laminin, fibronectin, collagens I and IV, and heparan sulfate proteoglycan (1–100 μg per 35-mm dish) and found that only laminin promoted SCLC cell attachment and spreading. The variant cell line NCI-N417, when attached to laminin, assumed an elongated and flattened shape, while cells of the classic SCLC cell line NCI-H345 adopted a neuron-like morphology with a round cell body from which processes projected (Fig. 1). These processes were 2–3 times the length of the cell body and appeared within 4 hr of exposure to laminin. Attachment of other SCLC lines including NCI-H146, NCI-H510, and NCI-H209 to laminin did not result in process formation or a flattened morphology. The NCI-H345 and NCI-N417 cells attached to laminin in a dose-dependent manner with the variant NCI-N417 line showing the highest affinity in repeated tests (Fig. 2). Still another cell line of the classic type (NCI-H187) did not attach to laminin (Fig. 2). The attachment and morphology of these SCLC cell lines on matrigel was similar to that on laminin, as previously reported (11).

Laminin caused a dose-dependent increase in the migration of NCI-N417 (Fig. 3), NCI-H345, and NCI-H146 but not of NCI-H187 cells. A similar migratory response was observed when the lower surface of the polycarbonate filters was coated with laminin (data not shown), suggesting that laminin is a haptotactic (immobilized) stimulant of cell motility as reported (9, 10).

Effect of Synthetic Peptides. One of the cell attachment sites in laminin includes the sequence YIGSR (Tyr-Ile-Gly-Ser-Arg) in the B1 chain (18). To evaluate the importance of this site in the interaction of SCLC cells with laminin, we tested the effect of a synthetic peptide, cyclic-YIGSR (14), on the adhesion of SCLC cells to laminin. Cyclic-YIGSR caused a dose-dependent inhibition of attachment of NCI-N417 cells to laminin, and at 50 μg of peptide per ml most of the cells remained as floating aggregates. Related peptides, cyclic-YIGSK (Tyr-Ile-Gly-Ser-Lys) (Fig. 2 *Inset*), and cyclic-YIGGR (Tyr-Ile-Gly-Gly-Arg) did not demonstrate inhibitory activity (data not shown). Furthermore, a peptide with the sequence GRGDS (Gly-Arg-Gly-Asp-Ser, an adhesion site on fibronectin) failed to inhibit the adhesion of NCI-N417, NCI-H345, and NCI-H146 cells to laminin. In addition, cyclic-YIGSR inhibited laminin-induced cell motility, while cyclic-YIGSK did not (Fig. 3 *Inset*).

Matrigel Can Enhance the Tumorigenicity of SCLC Cell Lines. To examine the effect of basement membrane proteins on tumor growth, four SCLC cell lines (NCI-N417, NCI-H345, NCI-H146, NCI-H187) were mixed with either liquid

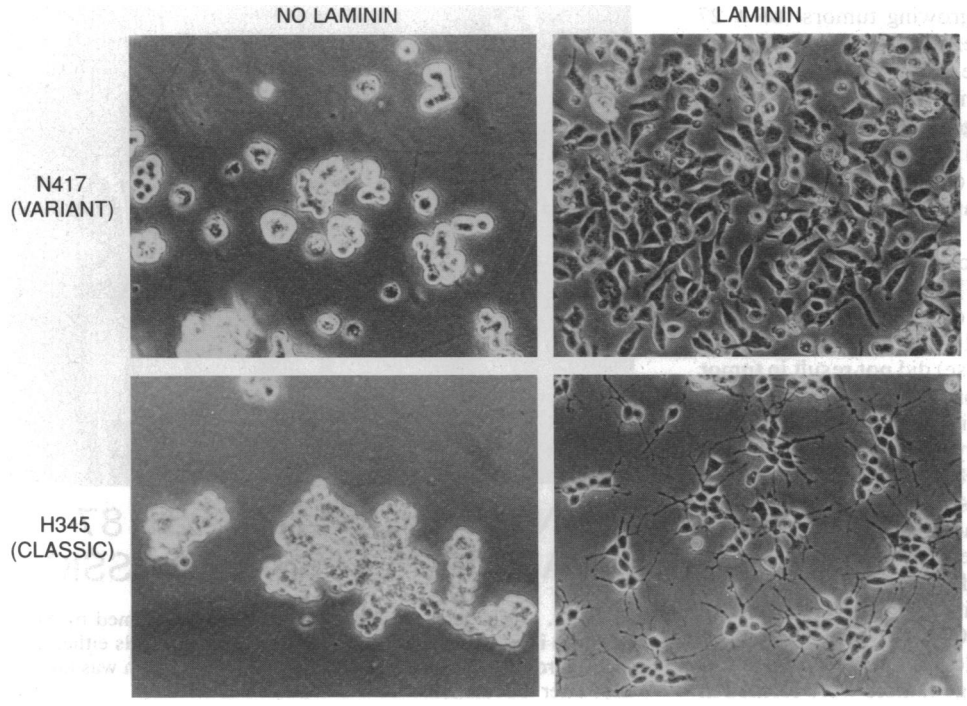

FIG. 1. Morphological appearance of NCI-N417 (variant) and NCI-H345 (classic) SCLC cells seeded on plastic tissue culture dishes with or without laminin coating. Tumor cells (2 × 10^5 cells per 35-mm dish) were seeded on plastic dishes either with or without 10 μg of laminin coating. The cells were photographed 8 hr after seeding. (×160.)

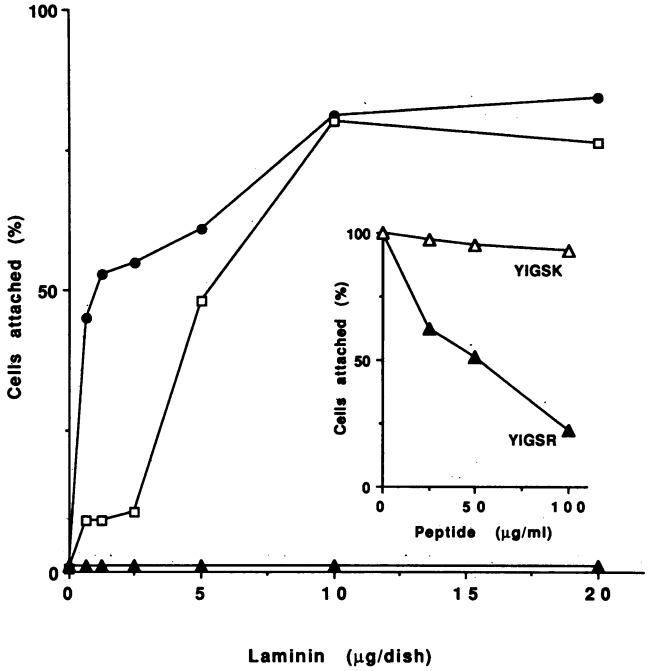

FIG. 2. Adhesion of SCLC cells to laminin. NCI-N417 (●), NCI-H345 (□), and NCI-H187 (▲) cells were seeded on dishes coated with increasing concentrations of laminin. After a 1-hr incubation, the firmly attached cells were counted electronically. (*Inset*) Effect of cyclic-YIGSR and cyclic-YIGSK on laminin-mediated adhesion of NCI-N417 cells. Tumor cells were preincubated (15 min) with peptides and then allowed to attach to laminin-coated dishes (5 μg per 35-mm dish) for 1 hr. Attached cells (100%) represent 60% of total added cells. Data represent the average of three separate experiments and each point represents triplicate samples that varied from one another by <15%.

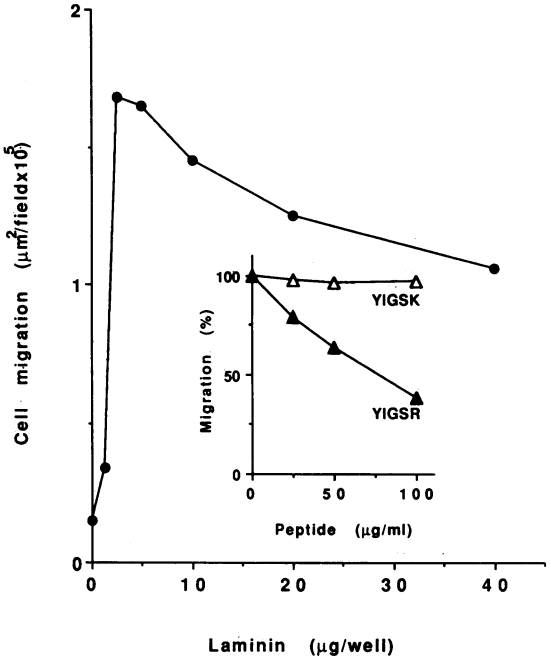

FIG. 3. Migration of NCI-N417 cells to laminin. Increasing concentrations of laminin in RPMI 1640 medium containing 0.1% bovine serum albumin were added to the lower well of a modified Boyden chamber. After a 5-hr incubation, the area occupied by the cells on the lower surface of the filter was determined by image analysis. (*Inset*) Migration assays were performed as described, except that the concentration of laminin in the lower well was 10 μg and various concentrations of either cyclic-YIGSR or cyclic-YIGSK were added to the upper wells. Data represent the average of three separate experiments and each point represents triplicate samples that varied from one another by <15%.

matrigel or medium and injected subcutaneously into athymic mice. All four of these SCLC cell lines produced tumors by 45 days in 80–100% of the mice when 5×10^5 cells were injected suspended in matrigel (Fig. 4). The time required for a tumor to reach a size of 1 cm (latency period) was 20.4 ± 2.8 days for NCI-N417 cells, and 29.4 ± 7.7 and 30.8 ± 3.6 days for NCI-H146 and NCI-H187 cells, respectively. NCI-H345 cells produced the slowest growing tumors (68 ± 27 days), as expected from the longer doubling time of these cells in culture (2, 3). In the case of NCI-N417 cells, the tumors formed in the presence of matrigel reached a size of ≈5 cm in diameter and an average weight of 33 g after 3 months. In the absence of matrigel and at concentrations of 5×10^5 cells per mouse, the three classic cell lines failed to produce tumors by 60 days, whereas the variant cells formed slow growing tumors in 20% of the mice. In contrast, tumor formation was observed by injecting as little as 25,000 NCI-N417 cells in matrigel (Fig. 5) and with 5×10^5 NCI-N417 cells suspended in matrigel diluted 1:10 (0.5 mg/ml) (data not shown). Injection of 5×10^5 NCI-N417 cells mixed with purified laminin (1 mg per mouse) did not result in tumor formation in 5 months. The injection of NCI-N417 cells (5×10^5 cells per mouse) suspended in collagen I (5 mg/ml) resulted in the formation of very slow growing tumors (latency period, 55 days) in 50–60% of the mice. The SCLC tumors which developed in the presence of matrigel showed the histopathological features characteristic of SCLC tumors and had little or no inflammatory reaction (data not shown). The tumors invaded locally but did not metastasize.

We also tested the tumorigenicity of NCI-N417 cells in matrigel mixed with either cyclic-YIGSR or cyclic-YIGGR. In the absence of matrigel, no visible tumors were apparent within a period of 45 days, while large tumors were formed in mice injected with cells in matrigel (Fig. 6). The tumors formed by cells inoculated with matrigel plus cyclic-YIGSR were considerably smaller. In contrast, the related peptide cyclic-YIGGR had no significant effect on tumor growth (Fig. 6). When NCI-H187 cells, which do not attach to laminin, were

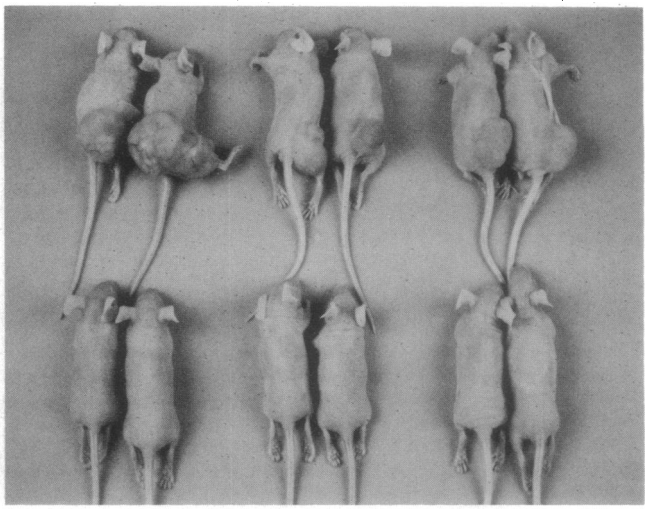

FIG. 4. Representative subcutaneous tumors formed by SCLC cell lines in athymic mice inoculated with 5×10^5 cells either alone (bottom row) or in matrigel (top row). The photograph was taken 45 days after inoculation.

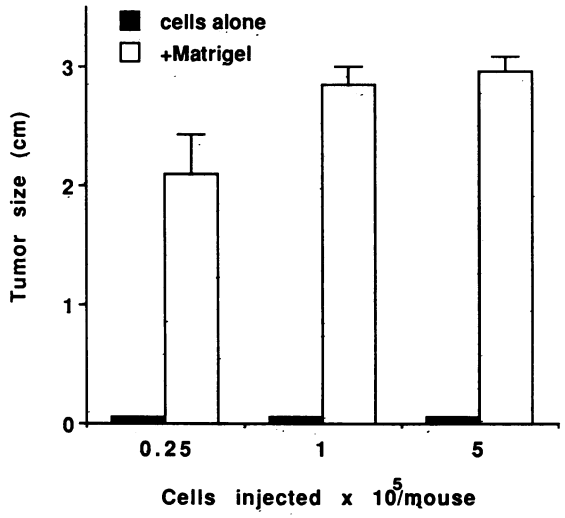

FIG. 5. Effect of matrigel on NCI-N417 tumor growth as a function of the number of cells inoculated. Various amounts of NCI-N417 cells (25×10^3, 1×10^5, 5×10^5) were mixed either with matrigel or with medium alone and were injected subcutaneously into athymic mice. Tumor size was periodically measured with a caliper and represents the mean size in a group of five mice. Values represent the sizes of the tumors 45 days after inoculation. Similar results were obtained in three independent experiments.

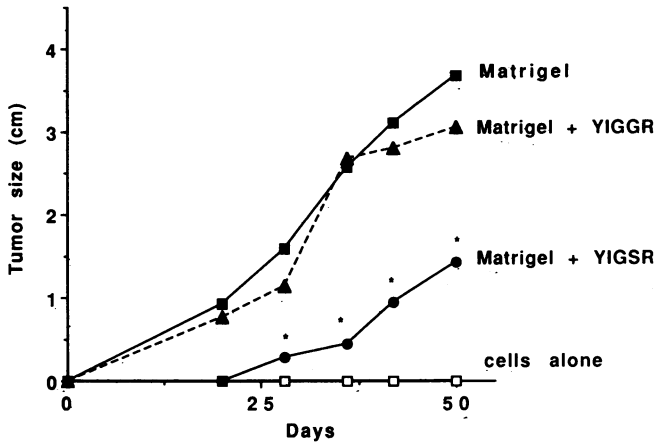

FIG. 6. Effect of cyclic-YIGSR and cyclic-YIGGR on NCI-N417 tumor growth in the presence of matrigel. NCI-N417 cells (5×10^5 cells per mouse) were mixed with 1 mg of either cyclic-YIGSR or cyclic-YIGGR and after 5 min were mixed with an equal volume of liquid matrigel (10 mg/ml). The cells were then injected subcutaneously into athymic mice (five mice per group). Tumor size was determined as described in Fig. 5. Statistical analysis was performed by *t* test. *, The level of statistical significance relative to cells injected with or without matrigel is $p < 0.05$. This experiment was repeated three times with similar results.

injected in matrigel plus cyclic-YIGSR, the peptide did not alter tumor growth (data not shown). Furthermore, collagen I failed to stimulate the proliferation of NCI-H187 cells in athymic mice, while matrigel at the same protein concentration stimulated tumor formation in four of five mice injected with 5×10^5 cells per mouse after 1 month of observation. Taken together, these results suggest that both laminin and other matrigel-specific components may be involved in the stimulatory effect of matrigel on SCLC growth *in vivo*.

Chemosensitivity Studies. We examined the drug sensitivity of SCLC cell lines (NCI-N417, NCI-H345, NCI-H146, NCI-H187) grown on either laminin or plastic. The presence of laminin significantly reduced the cytotoxicity of the three adherent cell lines (NCI-417, NCI-H345, NCI-H146) to various degrees (Table 1) but did not alter the sensitivity of the nonadherent line NCI-H187 (data not shown). Since a reduced proliferation of SCLC cells when grown in serum-free medium on high concentrations of laminin (30 μg/ml) was reported (11), we measured the proliferation of SCLC cells under the conditions used for the chemosensitivity assays (16) (serum-containing medium, different cell density) and did not find an altered growth rate whether the cells were grown in the presence or absence of laminin by using either the MTT assay or [^{3}H]thymidine incorporation into DNA to measure cell proliferation. These studies suggest that adhesion of SCLC cells to laminin is associated with increased resistance to cytotoxic drugs.

DISCUSSION

In this study, we show that the interaction of a number of SCLC cell lines with laminin and with a reconstituted basement membrane matrix greatly influences their behavior. Laminin, but not other extracellular matrix proteins, promotes SCLC cell adhesion and, in the case of NCI-N417 and NCI-H345 cells, induces a significant change in morphology, with NCI-H345 cells developing neurite-like processes. Previously, a similar differentiation to a neuron-like morphology was shown to occur in another SCLC cell line (PC-6) exposed to dibutyryl-cAMP in culture (19). Laminin had no effect on the level of expression of various neuroendocrine properties including L-dopa decarboxylase, gastrin-releasing peptide, chromogranin A, Leu-7, neuron-specific enolase, and synaptophysin in both NCI-N417 (variant) and NCI-H345 (classic) cell lines but did enhance the expression of neurofilaments in the variant cell line (unpublished observation). The differential response of various SCLC cell lines to laminin

Table 1. Chemosensitivity of SCLC cells on laminin

		Median ID_{50} value, μM			
Cell line	Laminin	Etoposide	Cisplatin	Doxorubicin	Nitrogen mustard
NCI-H146	+	5.3	19	19.4	10
	−	2.6	15.8	0.47	4.3
NCI-H345	+	20	83	16.2	24.7
	−	16.5	29.8	5.4	14.9
NCI-N417	+	71.6	25.6	2.8	29.3
	−	29.3	24.3	3.2	19.5
All lines	+	22	26	11	20
	−	16	18	1	14
	P value	0.0034	0.0010	0.0018	0.0129

The chemosensitivity of SCLC cells was tested as described. Statistical analysis was performed by the Wilcoxon test. The mean of the standard deviation of each experiment was ≈10%. These values represent the mean of three or more independent experiments.

may be related to the state of differentiation of each particular tumor cell line. Laminin induces the migration of SCLC cells, probably by haptotaxis (20), and this could play a role during metastasis formation as suggested for other types of tumor cells (9, 10, 20).

Human lung cancer cells do not grow readily in athymic mice, even when a large number of cells ($>1 \times 10^7$ cells per mouse) (21–23) and/or immunosuppressive agents (22) are used to obtain tumors. Our data demonstrate that low inocula of SCLC cells from both variant and classic cell lines readily form large tumors when coinjected with matrigel in athymic mice. Both the incidence and growth rate of the tumors were increased, while the latency period was reduced. Such tumors retained the histopathological characteristics of SCLC but were not metastatic. Injection of SCLC cells with purified laminin did not produce a similar increase in tumorigenicity, probably because isolated laminin is both soluble and readily degraded, while matrigel is insoluble and may persist for extended periods. However, a collagen I gel was found unable to induce the formation of rapidly growing tumors by SCLC cells, suggesting that some specific matrigel constituents may be important. We have found that coinjection of NCI-N417 cells with matrigel and cyclic-YIGSR, a synthetic peptide that inhibits the adhesion and migration of SCLC cells to laminin, resulted in a slower rate of tumor growth. Thus, laminin may be partly responsible for the effect of matrigel on SCLC cell proliferation *in vivo*. However, matrigel, but not collagen I, stimulated tumor formation by NCI-H187 cells, which do not attach to laminin *in vitro*, and their tumorigenicity was not affected by the presence of cyclic-YIGSR. These data suggest that various mechanisms may be involved, including providing a protected environment for the tumor cells. It has been previously shown that a fibrin coagulate can protect tumor cells from cytotoxic cells (24). It is possible that the encasement of tumor cells with matrigel may help the SCLC cells to escape from natural killer (NK) cells in the athymic mice. We have found that NCI-N417 cells formed rapidly growing tumors in NK cell-deficient beige nude xid (*bg/nu/xid*) mice only when coinjected with matrigel (data not shown). Furthermore, SCLC cells coinjected with matrigel in immunocompetent mice did not form tumors. These studies suggest that specific cell–matrix interactions may play a role in tumor cell proliferation *in vivo*. The use of matrigel for growing certain human tumors in animal models may be useful for many studies in cancer research.

An interesting finding of this study was the increased chemoresistance of SCLC cells attached to laminin. Resistance was not specific for a particular drug, although doxorubicin sensitivity was the most affected. The enhanced drug resistance was not due to an effect on cell growth since the proliferation of SCLC cells was not altered by their adhesion to laminin. In a previous study, Luk and Baylin (25) reported that adhesion of two SCLC cell lines to fibronectin induced a decreased sensitivity to an inhibitor of polyamine biosynthesis. The mechanisms involved in the acquisition of drug resistance by SCLC cells are still unclear. Overexpression of the multidrug resistance gene MDR1 is uncommon in SCLC cell lines when measured in cells in suspension, suggesting that this is not a dominant mechanism of drug resistance in lung cancer (26). We have found no difference in the level of expression of MDR1 mRNA when the SCLC cell lines are attached to laminin (unpublished observation). The increased resistance of SCLC cells on laminin suggests that SCLC cells might exist in a more drug-resistant state when attached to basement membranes *in vivo*.

Note. Since submission of this paper, we have studied the growth of other tumor cells, including early passage human renal carcinoma cell lines, a line of human epidermoid carcinoma cells (A253), as well as B16 murine melanoma cells. We found enhanced tumorigenicity when they were coinjected with matrigel in mice. Still other cell lines, including some human ovarian carcinoma and other human renal carcinoma cell lines, coinjected with matrigel did not result in enhanced tumorigenicity.

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