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Difference in Laminin Expression Between High and Low Metastatic Cell Clones Derived from Murine Lewis Lung Carcinoma

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ABSTRACT. **We investigated the expression of laminin in two cell lines with different metastatic potentials established from murine Lewis lung carcinoma. Immunostaining of the cells with anti-laminin antibody and Northern blot analysis of laminin mRNA demonstrated that the high metastatic clone expressed less laminin than the low metastatic one. In contrast, expressions of 67 kDa-laminin receptor were at similar levels between these two lines. These findings show the possibility that endogenous laminin may contribute to the difference in metastatic properties in the murine Lewis lung carcinoma cell lines examined.**

Laminin (LN), a high-molecular-weight glycoprotein (Mr = 850,000–1,000,000), is a major component of basement membrane and possesses binding sites in the molecule to cell surface receptors, type IV collagen and heparin (1). Laminin has been shown to play important roles in various cellular activities such as adhesion, spreading, migration, differentiation, neurite outgrowth and tumor cell metastasis. In a metastatic process, attachment of malignant cells to LN of basement membranes is thought to initiate a cascade of invasion and metastasis (3). In some cell lines, e.g., murine BL6 from B16 melanoma cell and PM2 from PMT fibrosarcoma cell, it has been shown that LN promotes attachment of metastatic tumor cell to type IV collagen (14). However, in murine macrophage-derived M5706 cell (2), murine glioma cell (4) and human squamous carcinoma cell (16), LN inhibits the attachment of the cells to type IV collagen.

In an experiment of chemotaxis using a modified Boyden chamber, exogenous soluble LN stimulates the directional migration of B16 F1 and B16 F10 in a dose-dependent manner (7). However, the function of soluble LN which may be autocrined by the cell has not been fully elucidated yet.

Recently two cell lines with different metastatic potentials have been established from murine Lewis lung carcinoma (3LL) cells (8). LM60-D6 is a cell line with high metastatic potential and P29 is one with low metastatic potential. In this study, using these cell lines we examined the levels of expression of endogenous LN together with 67 kDa-laminin receptor (67 kDa-LNR) (17) and fibronectin (FN) by immunostaining and Northern blot analysis. As a result, it was shown that the expression level of LN was markedly decreased in the highly metastatic cell line as compared with that in the low metastatic one. In contrast to LN, no significant difference was found in the levels of 67 kDa-LNR.

MATERIALS AND METHODS

Cells. Two cell lines LM60-D6 and P29 were established from mouse 3LL as described previously (8). The cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and maintained at 37°C in 5% CO_2. Murine teratocarcinoma cell line F9 was used as the positive control in some experiments, since F9 differentiates into endodermlike form and synthesizes a large amount of LN (13). Hereafter in this paper, differentiating F9 (dF9) means the F9 cells cultured with both 10^{-7} M retinoic acid and 10^{-3} M dibutyryl cyclic AMP for 3 days.

Experimental metastasis. Tumor cells (1×10^5) suspended in 0.5 ml phosphate-buffered saline were injected into the tail vein of C57BL/6 mice. After 21 days, the mice were sacrificed and the number of visible metastases on the surface of the lung was counted.

Anti-LN antiserum and anti-67 kDa-LNR antiserum. Rabbit polyclonal anti-LN antiserum was purchased from Advance (Tokyo, Japan). Rabbit polyclonal anti-67 kDa-LNR antibodies were raised by repeated intravenous injection of the bovine serum albumin (BSA)- con-

jugated synthetic peptide (RGTISREHPWEEVMPD) which corresponds to a part of the 67 kDa-LNR molecule (18). The antiserum was passed through a column of BSA-coupled Sepharose 4B to remove anti-BSA antibodies.

Immunostaining of the cells. The tumor cells (1×10^5) were seeded onto LAB-TEK chamber (Nunc, Roskilde, Denmark) and cultured for 24 hours. Induction of F9 to make dF9 was carried out in LAB-TEK chamber for 3 days. After washing and fixing with cold acetone, cells were incubated with 1 : 2500 dilution of anti-LN, or 1 : 3 dilution of anti-67 kDa-LNR antiserum diluted in PBS containing 0.3% Triton X-100 at 4°C overnight. After washing, the cells were incubated with a 1 : 80 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin antiserum (Cappel, Malvern, PA) at 4°C overnight. Staining was carried out with diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and hydrogen peroxide. After washing in water, the cells were counterstained with hematoxylin.

cDNA probes. Mouse LN cDNAs, A-E3 for A chain (10), p24 for B1 chain (9) and P7 for B2 chain (11), were generously provided by Dr. M. Sasaki (National Institute of Dental Research, Bethesda, MD). Human FN cDNA pFH154 (5) was obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). Human cDNA for 67 kDa-LNR (17) and human cDNA for β-actin were cloned from the cDNA library derived from human lung fibroblast cell line IMR90 (Clonetech, Palo Alto, CA) as reported previ-

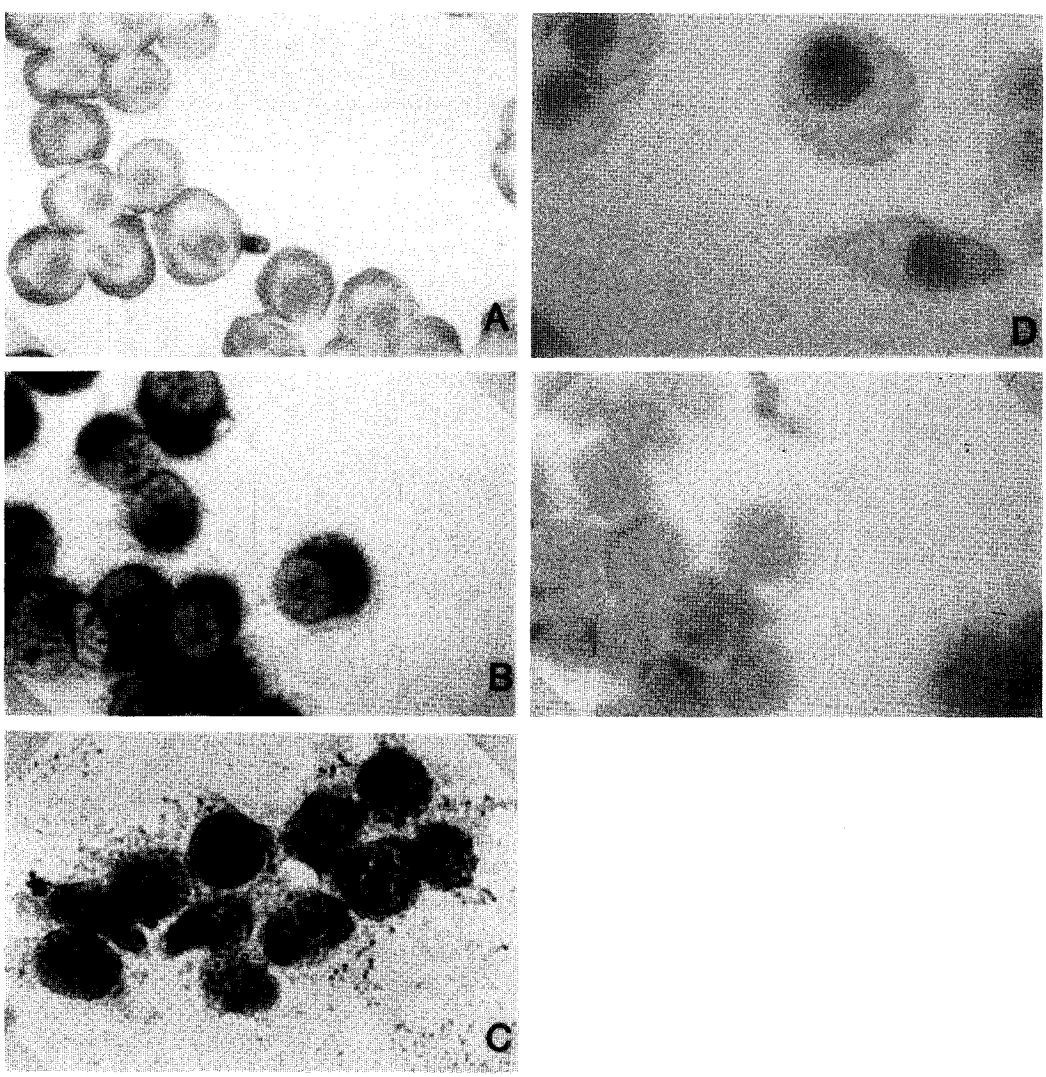

Fig. 1. Immunostaining of 3LL and dF9. Cells grown on LAB-TEK chamber were fixed in 70% ethanol and acetone and then stained at 4°C with anti-LN antiserum (A, B and C), or with anti-67 kDa-LNR antiserum (D and E) followed by treatment with peroxidase-conjugated goat anti-rabbit immunoglobulin antiserum. A and D; LM60-D6, B and E; P29, C; dF9.

ously (12). These cDNAs were labeled by nick translation using ^{32}P-deoxynucleotides and used as probes.

Preparation of total cellular RNA. LM60-D6, P29 and dF9 (1×10^7 cells) were lysed in 2 ml of 4 M guanidine isothiocyanate, 200 mM sodium acetate (pH 6.0) and 120 mM β-mercaptoethanol (GIT buffer). In 5 ml centrifuge tubes, guanidine-lysed sample was overlaid on 1.7 ml of 5.7 M cesium chloride, and 25 mM sodium acetate (pH 6.0). After centrifugation at 35,000 rpm at 20°C for 21 hours with a SW 55 rotor (Hitachi, Tokyo, Japan), pelleted RNAs were purified by ethanol precipitation.

Northern blot hybridization. Total RNA (15 μg) obtained from each cell line was electrophoresed in 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the RNA was transferred to a nitrocellulose filter (Schleicher & Schuell, BA85, Keene, NH). The filters were baked at 80°C under vacuum for 2 hours and prehybridized in 50% formamide, 5 × SSC (750 mM NaCl, 75 mM trisodium citrate, adjusted to pH 7.0 with 1 M HCl), 45 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.1% sodium dodecyl sulfate, 10 × Denhardt's solution (0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll 400) and 0.25 mg/ml salmon sperm DNA at 45°C for at least 3 hours. Hybridization was carried out at 45°C overnight with ^{32}P-labeled cDNA probes (1.0×10^6 cpm/ml) in the prehybridization buffer. The filters were then washed in 1 × SSC buffer containing 0.05% disodium pyrophosphate at room temperature several times, then in the same buffer at 45°C several times and exposed to X-ray film at −80°C for 2 days. The amount of mRNA was estimated by scanning of the densities on X-ray film with a densitometer (Fujiriken, Tokyo, Japan).

RESULTS

Metastatic incidence of tumor cells. Two clones isolated from mouse 3LL were examined for their lung-colonizing abilities after intravenous injection of the cells (1×10^5 cells) into syngeneic C57BL/6 mice. As shown in Table I, a striking difference was found in their metastatic potentials, i.e., LM60-D6 formed more than 100 metastatic foci while P29 essentially formed no metastasis.

Immunostaining with anti-LN and anti-67 kDa-LNR antibodies. Two clones of 3LL and dF9 were examined for the expression of LN and LNR by immunostaining of the cells. With anti-LN antiserum, highly metastatic clone LM60-D6 (Fig. 1A) was stained only weakly, whereas low metastatic clone P29 (Fig. 1B) was stained as strongly as dF9 (Fig. 1C). On the other hand, there was no difference in staining between LM60-D6 and P29 stained with anti-67 kDa-LNR antiserum (Fig. 1D and Fig. 1E, respectively). The control experiments with pre-immune rabbit serum showed no staining of these cells at any appreciable level (not shown).

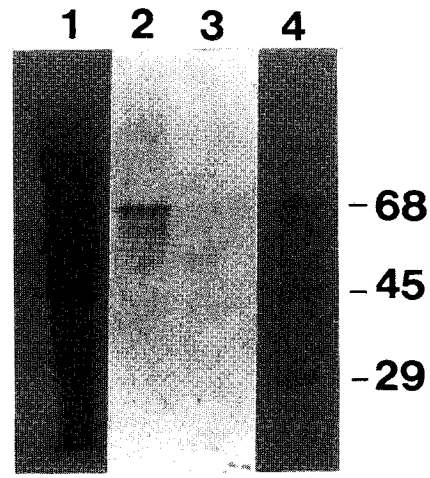

Fig. 2. The specificity of anti-LNR antiserum. The electrophoresed 4M urea extract of Lewis lung carcinoma cells was blotted onto nitrocellulose membrane. The membrane was stained with Amido Black (lanes 1 and 4) or by using anti-LNR antiserum (lanes 2 and 3). Lanes 1 and 2, cell extract; lanes 3 and 4, standard protein mixture including bovine serum albumin (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Note that the antiserum reacted with the limited protein bands including that with 67 kDa of the extract (lane 2) and not with those of the standard mixture including bovine serum albumin (lane 3). Molecular weights of standard proteins are marked in kDa.

The specificity of the anti-LNR antiserum was demonstrated by the Western blot analysis (Fig. 2). The antiserum reacted with 67 kDa-LNR of parental Lewis lung carcinoma cells together with some lower molecular weight proteins which could be explained by proteolytic degradation as described previously (18).

Expression of the genes of LN, 67 kDa-LNR and FN

Total cellular RNA samples of the cells from three different culture dishes in the same condition were analyzed by Northern hybridization. The filters were hybridized with six different probes. As shown in Fig. 3, there was no significant difference in mRNA size of all LN chains and 67 kDa-LNR between LM60-D6 and P29.

Densitometry of the autoradiograms revealed that the amounts of both LN B1 and LN B2 mRNAs were significantly lower in LM60-D6 than in P29 (Table II) ($p < 0.001$ by two sample t-test). The amounts of 67 kDa-LNR mRNA were almost comparable between LM60-D6 and P29. This finding is consistent with the result of immunostaining of the cells. Neither LM60-D6 nor P29 expressed FN gene as long as examined by the Northern blot analysis. The immunostaining of the cells with anti-FN antiserum showed absence of the protein around the cells and in the cytoplasm, corresponding to the re-

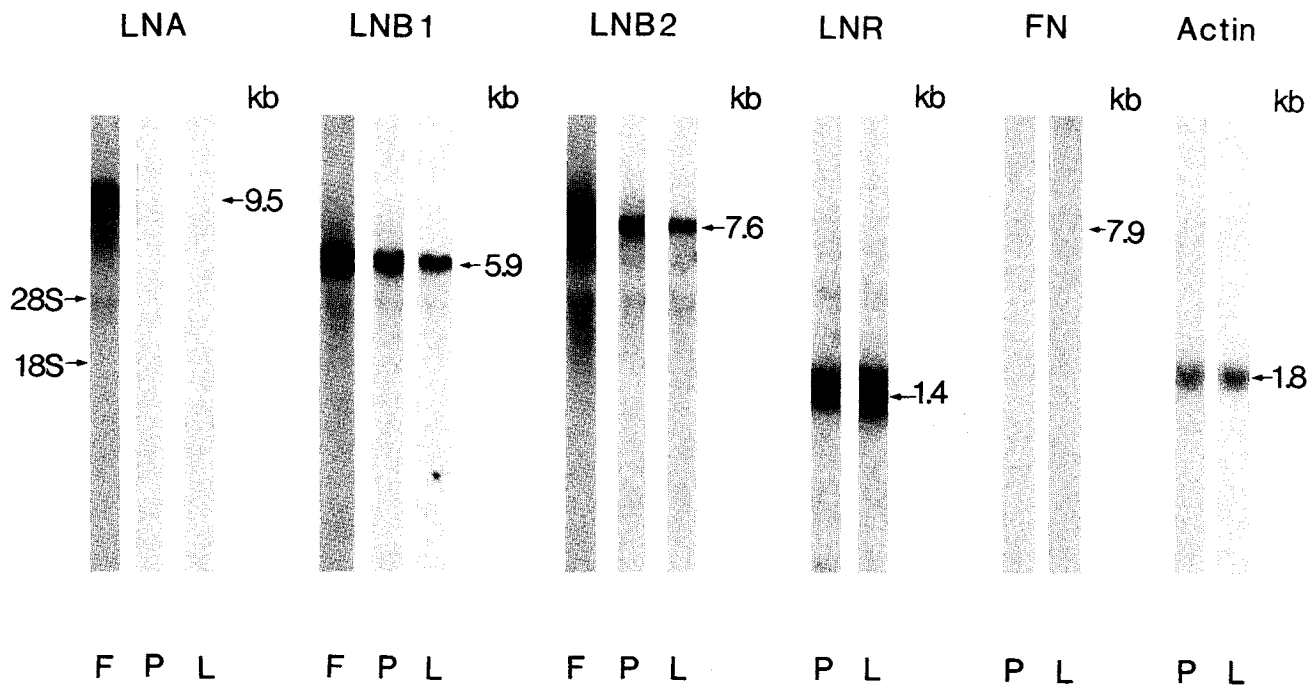

Fig. 3. Northern blot analyses of mRNA for three chains of laminin, 67 kDa-laminin receptor, fibronectin and β-actin. Total RNA samples (15 μg/lane) from 3LL or dF9 were electrophoresed in 1% formaldehyde-denaturing agarose gel and was transferred to a nitrocellulose filter. Each cDNA insert was labeled with ^{32}P by nick translation and was used for hybridization of the nitrocellulose filters. Lanes F, P. and L indicate expressions of mRNA of dF9, P29 and LM60-D6, respectively. LNA; laminin A chain, LNB1; laminin B1 chain, LNB2; laminin B2 chain, LNR; 67 kDa-laminin receptor, FN; fibronectin, Actin; β-actin.

sult of Northern blot analysis (data not shown).

DISCUSSION

In the present study, we used two cell clones derived from murine 3LL cells with different metastatic potentials. The difference in activities of pulmonary metastasis after intravenous injection of these cells (Table I) was consistent with the previous data obtained with intramuscular and subcutaneous inoculation (8). Immunostaining of the cells with anti-LN demonstrated that high metastatic LM60-D6 was stained less intensely than low metastatic P29 (Fig. 1), suggesting that the high metastatic 3LL cells synthesized less LN than the low metastatic ones (P29) did. This finding was compatible with the difference in the LN mRNA level (Fig. 3).

Table I. METASTATIC POTENTIALS OF CLONED LEWIS LUNG CARCINOMA CELLS*.

Cell line	Visible lung metastatic foci	
	Mean number	Range
P29	0.4	0–1
LM60-D6	171.9	138–222

* The cells (1×10^5/mouse) were injected iv. Values are means for seven mice.

The difference, however, in the immunostaining appeared to be greater than that in the mRNA level, which may be explained by the difference in the efficiency of the secretion of LN synthesized.

In contrast to LN, there was no significant difference in the level of expression of 67 kDa-LNR between the two cell clones shown by immunostraining and Northern blot analysis (Fig. 1 and Fig. 3).

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Table II. LEVELS OF LAMININ A, B1 AND B2 CHAIN mRNAs*.

	Laminin A		Laminin B1		Laminin B2	
	P29	LM60-D6	P29	LM60-D6	P29	LM60-D6
EXP. 1**	0	0***	100	64	100	53
EXP. 2	0	0	100	54	100	62
EXP. 3	0	0	100	65	100	43
p value			<0.001		<0.001	

* Equal amounts (15 μg) of total RNA, isolated from cultured cells by CsCl density gradient centrifugation, were electrophoresed, transferred to nitrocellulose filters and hybridized with cDNAs for murine laminin A, B1 or B2 chain. The mRNA levels were estimated by scanning of the autoradiograms with a densitometer.

** Experiments were independently performed three times.

*** The mRNA level of LM60-D6 was culculated in comparison with that of P29 assigned as 100 in each hybridization experiment.

LNR primarily contributes to high metastatic potential of some malignant cells (12, 15). The present results, however, appear to exemplify that the level of LNR mRNA alone is not necessarily correlated to the metastatic potentials. The relationship between endogenous LN and metastatic potential of the cancer cell is not fully elucidated. The present results suggest that LM60-D6 may possess more free (unoccupied by endogenous LN) LNR than P29 and consequently LM60-D6 may be able to bind to LN in basement membranes better than P29, resulting in the higher metastatic potential. This possibility appears to be pertinent to the previous data which indicate that self-synthesized LN is partially responsible for the competitive inhibition of cell attachment with exogenous LN (2, 4, 6, 16).

As shown in Figure 3, neither LM60-D6 nor P29 expresses the gene of the LN A chain. This suggests that the A chain of LN is not directly associated with metastatic or adhesive activity of 3LL cells. It is also interesting that this un-orthodox LN synthesized by 3LL is unlikely to be secreted into culture medium compared with the usual LN by dF9 (unpublished results). Further studies are needed to clarify the functional significance of aberrant LN which is devoid of the A chain.

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