Expression of Laminin-5 Enhances Tumorigenicity of Human Fibrosarcoma Cells in Nude Mice

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Laminin-5 (LN5), which consists of laminin α3, β3 and γ2 chains, is a laminin isoform produced by various kinds of normal epithelial cells and tumor cells. Strong activity of LN5 in adhesion, migration and scattering of cells in vitro and its frequent detection in human tumor tissues have suggested a possible role of LN5 in the malignant growth of tumor cells. To examine whether LN5 affects the malignant potential of tumor cells, we prepared human fibrosarcoma HT1080 cell lines producing LN5 by transfecting a cDNA of laminin α3 chain into the parent cell line, which constitutively expressed the laminin β3 and γ2 chains. The exogenous α3 chain associated with the endogenous β3 and γ2 chains to secrete the LN5 heterotrimer that has strong cell-scattering and cell adhesion activities. The HT1080 transfectants expressing LN5 efficiently adhered to culture dishes in a serum-free condition as compared with control HT1080 cells, which secreted the monomers and heterodimer of the β3 and γ2 chains. When injected into nude mice subcutaneously, the HT1080 transfectants expressing LN5 grew faster and formed much larger tumors than the control cells. This suggests that LN5 promotes tumor growth in vivo.

Key words: Laminin-5 — Cell adhesion — Cell migration — Tumor growth — HT1080 cells

The basement membrane proteins laminins are a family of extracellular matrix (ECM) components that consist of laminin α, β and γ chains linked by disulfide bonds.1) Five α chains, three β chains and three γ chains have been identified so far. These three chains form at least 15 laminin isoforms (laminin-1 to laminin-15) with different chain combinations. Lamins play essential roles in construction and maintenance of various epithelial and non-epithelial tissues,1) and hence mutation or deletion of laminin genes causes severe degeneration and dysfunction of tissues.2, 3) On the other hand, various types of cancer cells also produce distinct laminin isoforms.4, 5) However, the pathological meaning of tumor-derived laminins remains unknown. Laminin-1, which was originally identified in mouse Engelbreth-Holm-Swarm tumor, has been suggested to be associated with growth, metastasis and invasion of cancer cells.6) The reconstituted basement membrane proteins of melanoma cells in vitro,7) and the synthetic peptide YIGSR (Tyr-Ile-Gly-Ser-Arg), a sequence of the laminin β1 chain, suppresses the Matrigel-induced tumor growth in athymic mice.7) On the other hand, it has been shown that synthetic peptides of the laminin α1 chain, which bind to integrin α6β1, accelerate invasion of melanoma cells in vitro.8)

Laminin-5 (LN5), which consists of laminin α3, β3 and γ2 chains, was originally found as an ECM protein produced by keratinocytes9, 10) and a cell-scattering factor (ladsin) secreted by human gastric carcinoma cells.11) LN5 is a structurally and functionally unique laminin isoform. It has the truncated amino-terminal structures of the α3, β3 and γ2 chains.1) It potently promotes cell adhesion and migration,11–13) and these activities are mediated mainly by the cell surface receptor integrin α3β1.12) However, interaction of LN5 with integrin α6β4 in the hemidesmosomal structure is essential for the stable adhesion of basal keratinocytes to the underlying basement membrane and connective tissue.14, 15) On the other hand, LN5 is frequently produced by squamous carcinoma and gastric adenocarcinoma cells, and expression of its subunits is induced by growth factors and a tumor promoter5, 11) In vitro, LN5 or its subunit is up-regulated in invading carcinoma cells16–18) and at the site of wound healing of the skin.19) These facts, as well as the strong stimulation of cell migration by LN5 in vitro, suggest possible involvement of LN5 in tumor growth and invasion. To clarify the role of LN5 in tumor growth and invasion, we introduced the laminin α3 chain cDNA into HT1080 human fibrocarcinoma cells constitutively expressing the laminin β3 and γ2 chains and examined the effect of LN5 production on the behavior of the cancer cells in vitro and in vivo.
MATERIALS AND METHODS

Construction of expression vectors Human laminin α3 chain cDNA has been cloned from the human gastric carcinoma cell line STKM-1. The full-length cDNA (−1 nt to 5279 nt) was constructed in a plasmid vector pGEM3Zf(+) (Promega, Madison, WI). The five prime portion of the cDNA was replaced by a cDNA fragment generated by PCR to add a XbaI site for subcloning and to alter the Kozak consensus sequence using an unique SacII site. PCR primers used are 5′-CATCTAGACCACCAGGGGATGGCCTG-3′ and 5′-CTGTGTTTCCTGTTGACC-3′, where the underline indicates the Kozak sequence. The full-length cDNA was ligated into a XbaI site of mammalian expression plasmid vector pEF-BOS-CITE-NEO30.

Cell culture and transfection Human fibrosarcoma cell line HT1080 and human squamous adenocarcinoma cell line C-4I were obtained from the Japanese Cell Resources Bank and maintained in DME/F12 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum, streptomycin sulfate, and penicillin. Buffalo rat liver epithelial cell line BRL was described previously. The laminin α3 chain cDNA was transfected by the calcium phosphate method. Transfectants were cloned by the limiting dilution method and maintained in the medium containing 500 μg/ml Genetin (GIBCO BRL).

Preparation of conditioned medium (CM) HT1080 transfectants were grown to confluency in 90-mm culture dishes (Becton Dickinson, Bedford, MA) with DME/F12 medium containing 10% fetal calf serum. The confluent cultures were washed twice with and incubated in serum-free DME/F12 medium. The serum-free CM was collected after a 2-day incubation, clarified by centrifugation, and subjected to protein precipitation with 80%-saturated ammonium sulfate. The precipitated proteins were dissolved in a 1/40 volume of 20 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer.

Antibodies, immunoprecipitation and immunoblotting Antisera against human laminin α3 chain was prepared by immunizing a rabbit with a recombinant glutathione-S-transferase (GST) fusion protein of domain IIIa (amino acid residues 109 to 200) and reacted with an appropriate antibody at 4°C overnight. Immune complexes were precipitated with anti-mouse-IgG-conjugated Sepharose 4B (ICN Pharmaceuticals, Inc., Aurora, OH), washed three times with STEN buffer, and boiled in Laemmli’s SDS-PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol. SDS-PAGE was carried out on 6% polyacrylamide slab gels (85 mm in width, 1 mm in thickness, and 70 mm in length). The proteins separated on the slab gels were electrophoretically transferred onto nitrocellulose membranes and probed with the antibodies against the laminin α3 chain, β3 chain (kalinin B1), and γ2 chain (D4B5). The immunoreactive proteins were detected by the alkaline phosphatase method.

Preparation of extracellular matrix (ECM) of HT1080 cells Transfectants of HT1080 cells with the laminin α3 chain cDNA (5×10⁴ cells) were seeded into each well of
96-well culture plates in serum-containing medium and cultured for 4–5 days, and then cells were detached from the plates by incubating with 10 mM EDTA in phosphate-buffered saline (PBS). Complete detachment of cells was confirmed under a microscope. The plates on which the matrix had been deposited were washed twice with PBS, and used for attachment assay, as described previously. In some experiments, the matrix was prepared in 90-mm culture dishes, dissolved in the SDS-PAGE sample buffer and subjected to immunoblotting.

**Assay of tumorigenicity**  HT1080 transfectants with the laminin α3 chain cDNA (1×10^7 cells) were suspended into 0.2 ml of DME/F12 containing 10% fetal calf serum and subcutaneously injected into 6-week-old male nude mice. Tumor volume was measured one month after the injection by the method reported by Akamatsu et al.

**RESULTS**

**Expression of LN5 subunits in HT1080 cells**  Human fibrosarcoma cell line HT1080 was chosen as a recipient for exogenous laminin α3 chain cDNA transfection, because this cell line is known to express only the β3 and γ2 chains of LN5. Although the treatments of HT1080 cells with 12-O-tetradecanoylphorbol-13-acetate (100 ng/ml) and with epidermal growth factor (30 ng/ml) increased the transcriptional levels of the β3 and γ2 chains, the transcript of the α3 chain was never detected (data not shown).

The parent HT1080 cells were transfected with the laminin α3 chain cDNA or the control vector without the cDNA, and the neomycin-resistant transfectants were cloned. Secretion of the laminin α3, β3, and γ2 chains from three control transfectants (V1, V2, and V3) and three cDNA transfectants (T1, T2, and T3) was examined by immunoblotting of their CMs (Fig. 1). Similar levels of the β3 (140 kDa) and γ2 (150 kDa and 105 kDa) chains were detected in all clones. On the other hand, the 190-kDa precursor form and the proteolytically processed 160-kDa form of the α3 chain were specifically detected from the CMs of T1, T2 and T3 (Fig. 1). In addition, another processed form (130 kDa) of the α3 chain was detected from the CM of one clone (T1).

Secretion of the LN5 heterotrimer from the HT1080 transfectants was verified by immunoprecipitation of the α3β3γ2 heterotrimer with the anti-laminin-γ2 antibody. As shown in Fig. 2, the γ2 chain antibody precipitated both laminin α3 and β3 chains from the CMs of the cDNA transfectants T1, T2 and T3, indicating that the exogenous α3 chain formed the heterotrimer with the endogenous β3 and γ2 chains. The anti-laminin-γ2 antibody also precipitated the β3 chain from the CMs of control transfectants, indicating that the majority of the β3 chain was associated with the γ2 chain. When the CMs of control transfectants were directly subjected to immunoblotting with anti-β3 and -γ2 antibodies under non-reducing conditions, the monomers and the heterodimer of the β3 and γ2 chains were detected (data not shown).

Deposition of the LN5 subunits on the matrix was also examined with control (V1) and cDNA (T1) transfectants. As shown in Fig. 3, all of the three LN5 chains α3, β3 and γ2 were detected in the matrix of the cDNA transfectant

![Fig. 2. Formation of LN5 heterotrimer. Concentrated CMs of the control transfectants (V1, V2, and V3) and the laminin α3 cDNA transfectants (T1, T2, and T3) were subjected to immunoprecipitation with the anti-laminin γ2 chain monoclonal antibody D4B5 as described in the text. Immunoprecipitates (left panel) and supernatants (right panel) were separated with 6% SDS polyacrylamide gel under reducing conditions and subjected to immunoblotting with the anti-laminin α3 chain antibody (top panels, α3), the anti-laminin β3 chain antibody (center panels, β3), or the anti-laminin γ2 chain antibody (bottom panels, γ2). Arrowheads indicate LN5 subunits.](image-url)
T1, suggesting that LN5 had been assembled into the matrix. The laminin β3 and γ2 chains, but not the α3 chain, were deposited by the control transfectant V1. There was a tendency that the expression of the α3 chain increased the amounts of the β3 and γ2 chains deposited onto the culture plates. Similar results were obtained with the other transfectants (data not shown).

Activity of LN5 produced by HT1080 cells LN5 promotes rapid cell adhesion and cell scattering.11, 12) We next examined whether or not the LN5 produced by HT1080 cells is functional. First, cell adhesion activity of matrices deposited by the HT1080 transfectants was tested with human squamous adenocarcinoma cell line C-4I. This cell line adhered to purified LN5, but hardly to fibronectin (data not shown), which was secreted at a high level by HT1080 cells. C-4I cells weakly attached to the matrices of the control transfectants (Fig. 4). In contrast, the matrices of T1, T2 and T3 supported rapid attachment and spreading of C-4I cells, although that of T3 showed a relatively low activity. The attachment of C-4I cells to the matrix of T1, as well as to purified LN5, was effectively inhibited by pretreatment of the cells with function-blocking antibodies to α3 and β1 integrins but neither anti-α6- nor anti-β4-integrin antibody (Fig. 5).

Next, we examined the cell-scattering activity of the CMs of the HT1080 transfectants. As expected, the CMs of the three cDNA transfectants showed potent cell-scattering activity towards Buffalo rat liver cell line BRL,
whereas those of the three control transfectants showed no activity (data not shown). The relative cell-scattering activity of the CMs correlated with the amount of the laminin α3 chain. These results indicated that the α3 chain cDNA transfectants of HT1080 secreted and deposited recombinant LN5 indistinguishable from the natural LN5.

**Attachment and migration of transfectants** The cDNA transfectants of HT1080 cells were compared with the control transfectants with respect to the abilities of adhesion to substrates, migration and growth. When cells were plated onto plastic plates in serum-containing medium, both the control and cDNA transfectants attached to the substrates at a similar rate (Fig. 6A). In serum-free medium, the control transfectants hardly attached to the culture plates, whereas the cDNA transfectants T1 and T2 attached to and spread on the substrates at almost the same rates as they did in the serum-containing medium (Fig. 6B). T3 most poorly attached to the substrate among the three cDNA transfectants, though its capacity to attach was much higher than that of the control transfectants (V1, V2 and V3). The cell attachment of the cDNA transfectants was blocked by the anti-α3-integrin antibody, suggesting that the self-produced LN5 was responsible for efficient cell attachment (data not shown).

When the ability of cell migration was tested in Boyden chambers, there was no significant difference between the control and cDNA transfectants (data not shown). The cell growth rates of the control and cDNA transfectants in 10%-serum-containing medium were also comparable (data not shown).

**Growth of transfectants in vivo** Growth of the control and cDNA transfectants of HT1080 cells in vivo was compared by injecting them into the subcutaneous region of the back of nude mice (Fig. 7, A and B). One control transfectant V1 produced no tumor, and the other two controls grew poorly, producing small tumors. In contrast, all the three cDNA transfectants secreting LN5 rapidly grew to form big tumors. The frequency of tumor formation was also higher in the cDNA transfectants than in the controls (Fig. 7A). However, invasive growth was not evident in either tumor of the control or cDNA transfectant of HT1080 cells (data not shown). Histological examination did not show any significant difference between the two types of tumors. When cells were recovered from the tumors of the cDNA transfectants, they secreted LN5 at essentially the same level as the cells before transplantation. These results indicated that LN5 supported tumor growth in vivo.

**DISCUSSION**

In this study, we established stable transfectants of HT1080 cells with an exogenous laminin α3 cDNA, which continuously secreted recombinant LN5. We could not detect any significant difference in cell motility or growth rate between the control and cDNA transfectants in culture. However, the LN5-producing transfectants more rapidly attached to and spread on plastic plates in serum-free medium than the control transfectants. More importantly, the expression of the laminin α3 cDNA conferred on HT1080 cells a potent capacity to grow in vivo. The LN5-producing transfectants grew faster and produced bigger tumors in the subcutaneous region of nude mice than the controls.

Interaction between ECM proteins and integrins on the cell surface induces complex intracellular signal transduction that regulates cellular growth, adhesion, migration, differentiation and apoptosis. For example, it has long been known that loss of cell attachment to substrates leads to apoptosis (or anoikis) in many cell types. The inte-
grin-mediated signals are essential for survival and growth of many types of normal adhesive cells. Since different ECM proteins bind different integrins and have different effects on the same cells, the induced signals seem to differ depending on the kind of integrins or ECM proteins. However, the difference in ECM-specific integrin signalings have not been clarified yet. It is also expected that integrin-specific signals differently regulate tumor growth in vivo. Tumor cells or transformed cells often show decreased expression of integrin α5β1 and decreased fibronectin assembly.27) Overexpression of fibronectin or integrin α5β1 cDNA suppresses transformed phenotype in vitro or tumorigenicity in vivo in some cell lines.23, 28) Therefore, fibronectin seems to have a suppressive effect on tumor growth. LN5 is a much better substrate than fibronectin, vitronectin and laminin-1 for adhesion and migration of many types of cultured cells.12, 13) As shown in this study, HT1080 cells and C-4I adenocarcinoma cells preferentially adhered to LN5 via the cell surface integrin α3β1. This is true in many other types of cells (Kikkawa et al., unpublished data). In vivo, LN5 is an important component of basement membranes in many types of epithelial tissues. For example, in the skin LN5 plays an essential role for the stable anchorage of basal keratinocytes to the basement membrane and underlying connective tissue.9, 10, 15) However, the basal keratinocytes undergo active cell division, keeping contact with the basement membrane. This implies that LN5 supports both growth and stable adhesion of keratinocytes. In addition, it has been reported that LN5-deficient keratinocytes exhibit reduced survival compared to normal keratinocytes.15) These facts suggest that LN5 is a suitable substrate for the proliferation and survival of tumor cells in vivo. In this study, all of the LN5-producing clones T1, T2 and T3 showed much higher tumor growth in vivo than the control clones. However, the cell adhesion activity produced by the three LN5-producing clones did not correlate with their capacity to grow in vivo. For example, T3 showed the lowest cell adhesion activity but the highest tumor growth. We speculate that a low level of LN5 production is sufficient for the survival and/or growth of the tumor cells, and some other factors produced by these cells may influence the tumor growth.

In earlier studies, we found that LN5 (ladsin), which was isolated as a cell scattering factor from a human gastric carcinoma cell line, strongly stimulates cell adhesion and migration in vitro.11, 12) This suggested the possible involvement of LN5 in tumor invasion and metastasis. In fact, Pyke et al.16, 17) showed in their immunohistological studies with an anti-γ2 chain antibody that LN5 is overexpressed at the invasion front of human cancer tissues. Since their findings, overexpression of LN5 has been reported in many types of human carcinomas. Histological distribution of LN5 or its γ2 subunit in human cancer tissues can be classified into two typical cases.18) In one case, tumor cells forming glandular structures deposit LN5 con-

![Graph](image-url)  
**Fig. 7.** Growth of control and laminin α3 cDNA transfectants of HT1080 cells on the back of nude mice. (A) Each of the control transfectants (V1, V2, and V3) and the laminin α3 cDNA transfectants (T1, T2, and T3) (1×10⁶ cells) was subcutaneously injected into the back of nude mice. After 1 month, the tumor volume was measured. Each point represents the mean±SE (bar) for the tumor-bearing mice in each group. The numbers of the total mice used (Total) and the mice bearing tumors (Tumor) are shown under the abscissa. (B) Photographs of one mouse each from the control transfectants V2 (upper) and the laminin α3 cDNA transfectants T3 (lower) are shown.
tinuously along the underlying neoplastic basement membranes. Integrins α6β4 and/or α3β1, the major receptors of LN5, are also upregulated in various tumors, suggesting that these integrins mediate tumor cell adhesion to the neoplastic basement membranes. In the other case, the laminin γ2 chain is found in the cytoplasm of invading tumor cells. This type of overexpression of the laminin γ2 chain is associated with a poor prognosis for patients with pancreatic carcinoma, squamous cell carcinomas and lung adenocarcinomas. However, these studies have not clarified whether or not the invading tumor cells produce the LN5 heterotrimer. We recently demonstrated that invading tumor cells overexpress the laminin γ2 monomer without expressing the laminin α3 and β3 chains in gastric carcinomas and lung adenocarcinomas. In vitro human gastric carcinoma cells secrete the γ2 monomer into culture medium. Therefore, we speculate that the secreted γ2 monomer may play some role in tumor invasion, while the LN5 deposited on surrounding ECM may contribute to tumor growth rather than tumor invasion.

Since HT1080 is a human fibrosarcoma cell line, its transfectants with the laminin α3 cDNA may not be a suitable model for human carcinomas. However, like many carcinoma cells, HT1080 cells efficiently adhere to LN5.

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