Overexpression of Laminin γ2 Chain Monomer in Invading Gastric Carcinoma Cells

Naohiko Koshikawa, Kayano Moriyama, Hiroyuki Takamura, Hiroto Mizushima, Yoji Nagashima, Shunsuke Yanoma, and Kaoru Miyazaki

Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, Yokohama 244-0813 [N. K., K. M., H. T., H. M., K. M.]; Department of Pathology, Yokohama City University School of Medicine, Yokohama 236-0004 [Y. N.]; and Clinical Research Institute, Kanagawa Cancer Center, Yokohama 241-8015 [S. Y.], Japan

ABSTRACT

Laminin (LN)-5, a heterotrimer of α3, β3, and γ2 chains, has been suggested to be involved in tumor cell invasion. The present immunohistochemical study investigated the distribution of the LN γ2 chain in 48 different human gastric adenocarcinomas. The immunohistochemical analysis showed two distinct patterns of LN γ2 chain expression: (a) extracellular deposition; and (b) cytoplasmic accumulation. The extracellular deposition of the LN γ2 chain was typically observed at neoplastic basement membranes of well-differentiated adenocarcinomas. The immunoreactivity was continuous along tumor basement membranes in these tumors but was irregular and diffuse in poorly differentiated carcinomas. These tumor cells coexpressed the LN α3 and β3 chains, suggesting that the LN γ2 chain was deposited as the LN-5 complex. In contrast, tumor cells at the invading fronts showed strong cytoplasmic staining for the LN γ2 chain without any detectable signal for the LN α3 or β3 chain in both well- and poorly differentiated carcinomas. On the other hand, in vitro analysis by two-dimensional SDS-PAGE demonstrated that human gastric carcinoma cells secrete a high level of LN γ2 chain monomer in addition to the LN-5 complex into culture medium. These results indicate that the LN γ2 chain can be secreted as a single subunit and might be involved in tumor cell invasion.

INTRODUCTION

LN-5,1 which consists of LN α3, β3, and γ2 chains, is a LN isoform that is present in the basement membranes of the skin and various epithelial tissues. This protein was originally found in two different sources. Three groups reported this protein as an extracellular matrix protein secreted by cultured human keratinocytes referred to as kalinin, epiligrin, and nicein (1–3). In the skin, LN-5 stabilizes epidermal/dermal junction through binding with integrin αvβ5, which forms the hemidesmosome structure (1, 4). Mutation or deletion in the LN-5 genes (LAMA3, LAMB3, and LAMC2) is associated with epidermolysis bullosa, a lethal skin blistering disease (5–7). On the other hand, we identified this protein as a large cell-scattering protein (ladsin) secreted by human gastric carcinoma cells (8). LN-5 promotes the adhesion, migration, and scattering of various types of cultured cells much more strongly than other known extracellular matrix proteins (8–10). These activities are mediated mainly by integrin αvβ3 (2, 9, 11, 12). In culture, LN-5 is produced by human gastric carcinoma cell lines and squamous cell carcinoma lines as well as by normal keratinocytes (8, 13). Expression of LN-5 is regulated by growth factors and a tumor promoter in vitro (13, 14). These properties of LN-5 suggest its possible roles in tumor invasion and metastasis.

There are some reports showing the expression of LN-5 or its subunits in human cancer tissues. Pyke et al. (15, 16) analyzed the expression of the LN γ2 chain by in situ hybridization and immunohistochemistry and showed that LN-5 is highly expressed in invading colon carcinoma cells. LN-5 is also up-regulated at the site of wound healing of the skin (17). In most cases of pancreatic adenocarcinomas, tumor cells show cytoplasmic immunoreactivity for the LN γ2 chain, but the degree of γ2 expression is inversely correlated with the invasive and metastatic potential of the tumor (18). In prostate carcinomas, the LN γ2 chain is not detected, although its mRNA is expressed (19). Decreased expression of LN-5 has been reported in transformed keratinocytes (17) and breast carcinoma cells (20). In gastric carcinomas, LN-5 is continuously deposited at the interface between tumor cells and stroma (21). A recent study has shown discontinuous deposition of LN-5 in colon carcinomas and enhanced intracellular accumulation of the LN β3γ2 heterodimer in the dissociating tumor cells (22). There are apparent discrepancies among these studies that might arise from the differences in the tumor types and the antibodies used. Thus, the role of LN-5 in tumor malignancy remains unclear.

We recently prepared a monoclonal antibody capable of detecting the LN γ2 chain with high sensitivity in both immunoblotting and immunohistochemistry (23). Using this antibody, we examined the distribution of the LN γ2 chain in human gastric carcinoma tissues from 48 patients. Immunohistochemical analyses with antibodies against the LN β3 and α3 chains were also carried out for some tumor tissues to show the coexistence of these LN-5 subunits.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibody D4B5 was prepared using the human recombinant LN γ2 chain (amino acid residues 382–608) as the antigen (23). Rabbit polyclonal antibody was raised against the human recombinant LN α3 chain (residues 109–331). Mouse monoclonal antibody against the LN β3 chain (clone 29E) was prepared using purified LN-5 as the antigen. These antibodies could be used for immunohistochemical staining of both formalin-fixed paraffin sections and paraformaldehyde-fixed frozen sections. Mouse monoclonal antibody against the LN β3 chain (kalinin B1) was purchased from Transduction Laboratories (Lexington, KY) and used for immunoblotting.

Tumor Specimens and Sample Preparation. Small tumor specimens were obtained from surgically resected gastric cancer tissues and used with the permission and informed consent of patients or their families. They were immediately fixed in 10% formalin and embedded in paraffin. Four-μm-thick paraffin sections were mounted on aminoacetyl silane-coated glass slides and used for immunohistochemical analysis of the LN γ2, α3, and β3 chains. For immunohistochemical staining, the paraffin sections were dewaxed, rehydrated, and immersed in 0.5% hydrogen peroxide-containing methanol for inactivation of intrinsic peroxidase. All sections were treated with Protease XXIV (Sigma, St. Louis, MO) for 15 min at room temperature. The sections were then incubated with each antibody at 37°C for 1 h. The labeled antigen was detected by a HistoFine kit (Nichirei Pharmaceutical, Tokyo, Japan) and visualized by the 3,3’-diaminobenzidine reaction. Other experimental conditions were as described previously (24).

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2 To whom requests for reprints should be addressed, at Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 642-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan. Phone: 81-45-820-1905; Fax: 81-45-820-1901; E-mail: miyazaki@yokohama-cu.ac.jp.

3 The abbreviation used is: LN, laminin.
Analysis of LN-5 and LN γ2 Monomer Secreted from Cultured Cancer Cells. A confluent culture of human gastric carcinoma cell line MKN-45 was incubated in serum-free medium for 2 days in the presence of 100 ng/ml of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate to stimulate LN-5 production, and the resulting conditioned medium was collected and concentrated as reported previously (8). The concentrated conditioned medium was subjected to the two-dimensional SDS-PAGE essentially by the method of LeMosy et al. (25). The one-dimensional SDS-PAGE was carried out on a 4% (v/w) polyacrylamide disc gel (2 mm in diameter and 70 mm in length) under nonreducing conditions. After electrophoresis, the gel was incubated in SDS sample buffer containing 5% (v/v) 2-mercaptoethanol at room temperature for 15 min, placed on a 6% (v/w) polyacrylamide slab gel (85 mm wide, 1 mm thick, and 70 mm long), and run for the two-dimensional SDS-PAGE under reducing conditions. The proteins separated on the slab gel were electrophoretically transferred onto a polyvinylidene difluoride membrane and probed sequentially with rabbit polyclonal antibody against the LN α3 chain, mouse monoclonal antibody against the LN β3 chain (kalinin B1), and mouse monoclonal antibody against the LN γ2 chain (D4B5). The immunoreactive proteins were detected by the chemiluminescence method using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom).

RESULTS

Distribution of the LN γ2 Chain. Human gastric adenocarcinoma tissues from 48 patients were classified as 24 differentiated carcinomas (or tubular adenocarcinomas), 19 poorly differentiated carcinomas, and 5 other carcinomas (2 signet ring carcinomas, 2 papillary adenocarcinomas, and 1 mucinous adenocarcinoma), based on the formation of neoplastic glandular structures or neoplastic tubules. When these tumor specimens were subjected to immunohistochemical analysis with the anti-γ2 monoclonal antibody D4B5, the immunostaining patterns were described in the text.

<table>
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<th>Histology</th>
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Table 1: Summary of immunohistochemical analysis of human gastric carcinomas for the LN γ2 chain

The gastric adenocarcinoma tissues from 48 patients were histologically classified into three groups and four subgroups: well (Well) and moderately (Moderately) differentiated tumors, solid (Solid) and non-solid (Non-solid) types of poorly differentiated tumors, and others (Others). “Others” consisted of two signet ring carcinomas, two papillary adenocarcinomas, and one mucinous adenocarcinoma. Positive staining for the LN γ2 chain was divided into extracellular staining and cytoplasmic staining, and the former was subdivided into the staining of tumor basement membranes (BM) and diffuse staining (Diffuse) around tumor cells. Tumor specimens showing focal staining for the LN γ2 chain were included in positive cases. Experimental conditions are described in the text.

Fig. 1. Extracellular deposition of the LN γ2 chain in gastric carcinomas. In two highly differentiated carcinomas (A and B), basement membranes surrounding neoplastic glandular structures are intensely and continuously stained for the LN γ2 chain. In a poorly differentiated carcinoma (C), the immunoreactivity is diffuse around tumor cells. In a signet ring carcinoma (D), the immunoreactivity is localized around a single tumor cell or several tumor cells, showing basement membrane-like structures. Arrowheads, a positive signal for the LN γ2 chain. Experimental conditions are described in the text. Magnification: ×200 for A and C; ×400 for B and D.
distinct types, extracellular staining and cytoplasmic staining. The results of the immunohistochemical analysis are summarized in Table 1. Most typically in differentiated carcinomas, the basement membranes surrounding neoplastic glandular structures showed intense and continuous immunoreactivity for the $\gamma_2$ chain of LN-5 (Fig. 1, A and B). This type of staining was essentially identical to that observed in adjacent normal gastric epithelia. In some cases, almost all parts of the neoplastic basement membranes were positive for the $\gamma_2$ chain (Fig. 1B), whereas in other cases, only parts of the basement membranes, especially surface areas, were positive for the antigen (data not shown). The basement membrane staining of the LN $\gamma_2$ chain was less frequent in poorly differentiated carcinomas than in differentiated carcinomas (Table 1). In another extracellular staining pattern, the immunoreactivity for the LN $\gamma_2$ chain was irregular and fibrous around tumor cells scattered in the stroma (Fig. 1C). This staining pattern was focally observed in many cases of the solid type of poorly differentiated carcinomas (Table 1). In signet ring carcinomas, the immunoreactivity showed small basement membrane-like structures surrounding a single tumor cell or several tumor cells (Fig. 1D).

The most characteristic staining pattern for the LN $\gamma_2$ chain was the cytoplasmic staining of tumor cells. This staining pattern was observed in about half of both differentiated and poorly differentiated carcinomas (Table 1). The most typical case in a highly differentiated carcinoma is shown in Fig. 2, A and B. In this case, cells in the tumor nests showed little immunoreactivity for the LN $\gamma_2$ chain, whereas only cells budding or dissociating from the tumor nests showed intense cytoplasmic staining. In another differentiated carcinoma, the immunoreactivity was seen preferentially in tumor cells invading deep into the stroma (Fig. 2C). On the other hand, the cytoplasmic immunoreactivity for the LN $\gamma_2$ chain in poorly differentiated carcinomas differed considerably from that seen in differentiated carcinomas. The majority of tumor cells diffusing into the stroma showed a positive signal for the LN $\gamma_2$ chain in poorly differentiated carcinomas (Fig. 2D). In some of these cases, diffuse extracellular staining for the LN $\gamma_2$ chain was also evident (data not shown).

**Distribution of the LN $\alpha_3$ and $\alpha_3$ Chains.** To examine whether the LN $\gamma_2$ chain produced by gastric carcinoma cells exists as the LN-5 complex, some tumor tissues were subjected to immunohistochemical staining with antibodies against the LN $\alpha_3$ and $\beta_3$ chains. Both the rabbit polyclonal antibody against LN $\alpha_3$ chain and the mouse monoclonal antibody against LN $\beta_3$ chain (clone 29E) reacted with the respective intracellular antigens but not the antigens deposited in basement membranes as the LN-5 complex in formalin-fixed paraffin sections. When tumor tissues that showed positive basement membrane staining for LN $\gamma_2$ chain were analyzed with the anti-$\alpha_3$ and anti-$\beta_3$ antibodies, tumor cells showed positive signals with both antibodies (Fig. 3, A–C). The immunoreactivity for the LN $\alpha_3$ chain was stronger in inner ductal cells.
than in surface glandular cells, whereas the immunoreactivities for the LN β3 and γ2 chains showed an inverse gradient (data not shown).

When invading gastric carcinoma cells showing an intracellular positive signal for the LN γ2 chain were analyzed with the anti-α3 and anti-β3 antibodies, no immunoreactivity with either anti-α3 or anti-β3 antibody was detected in the tumor cells (Fig. 3, D–F). These distributions of LN α3, β3, and γ2 chains in gastric adenocarcinoma

Fig. 3. Expression of three LN-5 subunits in two highly differentiated gastric carcinomas. Close paraffin sections from two highly differentiated carcinomas were subjected to immunohistochemical staining for the LN α3 (A and D), β3 (B and E), and γ2 (C and F) chains as described in the text. Tumor cells with positive basement membrane staining for the LN γ2 chain (C) show positive cytoplasmic staining for the LN α3 (A) and β3 (B) chains. Invading tumor cells showing cytoplasmic staining for the LN γ2 chain (F) are negative for cytoplasmic staining with the anti-α3 (D) or anti-β3 (E) antibody. Arrowsheads, positive signal for each LN chain. Experimental conditions are described in the text. Magnification: ×400 for A–C, ×200 for C–E.
coexpressed with the LN chain, in the cytoplasm. LN-5 forms of described in the text. Arrowheads (or budding) tumor cells express only the LN chain, and invading or budding tumor cells, which did not express the LN chain, secrete the LN chain as a monomer (or a single subunit), the three subunits of LN-5 were secreted by human gastric carcinoma cell line MKN-45 were secreted as a monomer (or a single subunit), the three subunits of LN-5, the LN-5 secreted by human gastric carcinoma cell line MKN-45 were secreted in the one-dimensional SDS-PAGE (nonreducing SDS-PAGE and reducing conditions were used for the one-dimensional SDS-PAGE and subsequent immunoblotting with antibodies against the α3 (A), β3 (B), and γ2 (C) chains, as described in the text. Arrowheads indicate the respective subunits derived from two major LN-5 forms of M, 460,000 and M, 400,000 (the former is composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 155,000 γ2 chain, and the latter is composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 155,000 γ2 chain). An arrow indicates the single M, 155,000 γ2 chain (C). Experimental conditions are described in the text.

Fig. 4. Analysis of three subunits of LN-5 secreted from a human gastric carcinoma cell line by two-dimensional SDS-PAGE. Serum-free conditioned medium from human gastric carcinoma cell line MKN-45 was concentrated and analyzed by two-dimensional SDS-PAGE (nonreducing SDS-PAGE and reducing conditions were used for the one-dimensional SDS-PAGE and subsequent immunoblotting with antibodies against the α3 (A), β3 (B), and γ2 (C) chains, as described in the text. Arrowheads indicate the respective subunits derived from two major LN-5 forms of M, 460,000 and M, 400,000 (the former is composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 155,000 γ2 chain, and the latter is composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 155,000 γ2 chain). An arrow indicates the single M, 155,000 γ2 chain (C). Experimental conditions are described in the text.

tissues suggest that in glandular tumor cells, the LN γ2 chain is coexpressed with the LN α3 and β3 chains and deposited as the LN-5 form in the underlying basement membranes, whereas invading or budding tumor cells express only the LN γ2 chain and accumulate it in the cytoplasm.

Secretion of the LN γ2 Monomer by Human Gastric Carcinoma Cells in Culture. To examine whether the LN γ2 chain can be secreted as a monomer (or a single subunit), the three subunits of LN-5 secreted by human gastric carcinoma cell line MKN-45 were analyzed by two-dimensional SDS-PAGE (nonreducing SDS-PAGE in the first dimension and reducing SDS-PAGE in the second dimension) and subsequent immunoblotting with antibodies against the α3, β3, and γ2 chains. MKN-45 cells were incubated in serum-free medium for 2 days in the presence of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate to stimulate LN-5 production, and the resulting conditioned medium was concentrated and subjected to the two-dimensional SDS-PAGE. During the 2 days of serum-free culture, few dead cells were observed under microscopic observation. As shown in Fig. 4, the three immunoblots indicated the presence of two major LN-5 forms of approximately M, 460,000 and M, 400,000 in which the former was composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 155,000 γ2 chain, and the latter was composed of M, 160,000 α3 chain, M, 140,000 β3 chain, and M, 105,000 γ2 chain. In addition, LN-5 forms that migrated more slowly than the M, 460,000 form in the one-dimensional SDS-PAGE were weakly detected. Furthermore, the immunoblot with the anti-γ2 antibody showed the most intense spot, which migrated to the position corresponding to M, 155,000 in both the one- and two-dimensional SDS-PAGE. Neither anti-α3 nor anti-β3 antibody showed any spot in the position of M, 140,000–160,000 in the one-dimensional SDS-PAGE. These results demonstrated that the gastric carcinoma cells secrete the LN γ2 chain as a single subunit.

DISCUSSION

The present study demonstrated that the localization of the LN γ2 chain in human gastric carcinomas is divided into two distinct types: (a) extracellular deposition; and (b) cytoplasmic accumulation. Differentiated carcinoma cells forming neoplastic glandular structures expressed all of the LN α3, β3, and γ2 chains and deposited the γ2 chain (possibly as the LN-5 form) in underlying basement membranes. In contrast, the cytoplasmic accumulation of the LN γ2 chain was typically observed in invading or budding tumor cells, which did not express the LN α3 or β3 chain at a detectable level.

Some previous studies have shown preferential expression of the LN γ2 chain in invading carcinoma cells (15, 16, 18). However, most of these studies did not clarify whether LN α3 and β3 chains were coexpressed with the LN γ2 chain in those tumor cells. As an exception, Sordat et al. (22) reported that the heterodimer of the LN γ2 and LN β3 chains is accumulated in the cytoplasm of dissociating (or budding) tumor cells from neoplastic tubules of colon carcinomas. The LN β3 and γ2 chains are found only in LN-5 among 11 LN species identified thus far. It has been reported that gene expression of the three subunits of LN-5 (α3, β3, and γ2) is regulated differently in cancer cell lines and in normal and malignant tissues (13, 19, 26). In the present study, both normal gastric epithelial cells and tumor cells surrounded by basement membranes containing LN-5 showed no cytoplasmic immunoreactivity for the LN γ2 chain. This suggests that in cells expressing all three subunits of LN-5, the γ2 chain is secreted immediately as the complex of LN-5.

The marked cytoplasmic accumulation of the LN γ2 chain in tumor cells that express neither the α3 nor β3 chain suggests that the γ2 chain may not be secreted as the monomer. Indeed, it is known that in LN-1, the α1 chain can be secreted as a single subunit, whereas the β1 and γ1 chains cannot (27). When β1 and γ1 chains are overexpressed separately or together, they remain intracellularly as the disulfide-linked dimer of β1γ1 or β1β1. Similarly, it has been reported that in LN-5, the β3 and γ2 chains first form heterodimer β3γ2 and then bind to the α3 chain to produce and secrete LN-5 (28). No previous studies have shown the secretion of the LN γ2 chain monomer. In this study, however, we showed that a high level of the LN γ2 chain was secreted from cultured human gastric carcinoma cells as the monomer, in addition to the heterotrimer with the α3 and β3 chains. We have also obtained data that tumor cell lines expressing the β3 and γ2 chains but not the α3 chain secrete the γ2 chain as the monomer or the
heterodimer with the β3 chain. Thus, the behavior of the LN γ2 chain seems different from that of the LN γ1 chain. It seems very likely that in the absence of the α3 and β3 chains, the LN γ2 chain is accumulated intracellularly in gastric carcinoma, but a part of the overexpressed γ2 chain is secreted.

What is the meaning of the sole expression of the LN γ2 chain at the invasion fronts of tumors? There are several possibilities for positive roles of the LN γ2 chain in tumor invasion. First, secreted LN γ2 monomer or its proteolytic fragments might have some biological activities that promote tumor cell invasion. The LN γ2 chain receives some proteolytic processing (8, 29). It has been reported that the limited proteolysis of the LN γ2 chain by gelatinase A (MMP-2) increases the cell motility activity of LN-5, indicating the important role of the γ2 chain (30). It seems possible that the γ2 chain acts directly on cells. Second, the LN γ2 monomer may modulate the extracellular deposition and biological activity of LN-5 or other LN chains. It is known that the NH2-terminal region of the γ2 chain contains sites to bind with fibulin-2 (31) and type VII collagen (32). Therefore, the secretion of the γ2 chain monomer may alter the extracellular location and interaction of LN-5 with target cells. Third, the cytoplasmic accumulation of the LN γ2 chain might disturb the formation of other LN species such as LN-10 and LN-11. Multiple LN subunits are found in basement membranes surrounding differentiated gastric carcinoma cells and normal gastric epithelia (21, 26). As shown here and in other studies (21, 26), tumor cells forming tumor cell nests from the tumor cell nests.

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