Sole Expression of Laminin γ2 Chain in Invading Tumor Cells and Its Association with Stromal Fibrosis in Lung Adenocarcinomas

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Laminin-5 (LN-5), an important basement membrane (BM) protein consisting of laminin α3, β3 and γ2 chains, has been suggested to be involved in tumor cell invasion and tissue repair. In this study, the distribution of the LN-5 subunits in atypical adenomatous hyperplasia (AAH) and different types of adenocarcinomas of the lung was examined by immunohistochemical analysis. In AAH and non-sclerosing, well-differentiated adenocarcinomas, the LN γ2 chain was frequently detected along with the continuous BMs. These BMs were also positive for both LN α3 and β3 chains, suggesting that LN-5 had been deposited. In contrast, the cytoplasmic staining for the LN γ2 chain was frequently observed in tumor cells of sclerosing, well-differentiated adenocarcinomas, as well as of moderately and poorly differentiated adenocarcinomas, without any evidence of co-expression of the LN α3 and β3 chains. This staining pattern of the LN γ2 chain was prominent in carcinoma cells invading into interstitial stroma and was associated with the formation of a central scar in the tumor tissues. These results suggest that the LN γ2 chain monomer could be an important indicator of progression of lung adenocarcinoma.

Key words: Laminin-5 — Laminin γ2 chain — Lung adenocarcinoma — Tumor invasion — Immunohistochemistry

Laminin-5 (LN-5), which consists of laminin α3, β3, and γ2 chains, is a laminin (LN) isoform that is present in the basement membranes (BM)s of the skin and other epithelial tissues. It was originally found as an extracellular matrix protein secreted by cultured human keratinocytes and gastric carcinoma cells. This protein stabilizes the dermo-epidermal junction through binding to integrin α6β4, an important component of the hemidesmosome structures of basal epithelial cells. Mutation or deletion of the LN-5 genes (LAMA3, LAMB3, and LAMC2) is associated with epidermolysis bullosa, a lethal skin blistering disease. LN-5 strongly promotes adhesion, migration, and scattering of various types of cultured cells compared with other extracellular matrix proteins. The expression of LN-5 in tumor cells is stimulated by growth factors and a tumor promoter in vitro. Therefore, LN-5 has been supposed to play some roles in tumor invasion and metastasis.

Many past studies have shown that LN-5 or its subunits are expressed in various types of human cancers. Pyke et al. found, using in situ hybridization and immunohistochemistry, that the LN γ2 chain is highly expressed in tumor cells particularly at the invasion front and in budding tumor cells of colon carcinomas. Similar expression of the LN γ2 chain in invading tumor cells has been shown in pancreatic carcinomas, squamous cell carcinomas of the tongue and lung carcinomas. Although these studies suggested an important role of LN-5 in tumor invasion, they did not clarify whether or not these invading tumor cells produced the other LN-5 subunits and deposited LN-5. On the other hand, it has been reported that LN-5 is deposited on continuous BMs at the interface between granular tumor cells and stroma in gastric carcinomas, colorectal carcinomas and thyroid carcinomas. Furthermore, Sordat et al. reported that colorectal carcinoma cells budding from neoplastic tubules accumulate the LN β3 and γ2 heterodimer in the cytoplasm. The conflicts in these studies seem to have arisen from the differences in the analytical methods, as well as in the histological types of tumors. Our recent immunohistochemical study with gastric carcinomas demonstrated that there are two distinct patterns of LN γ2 chain expression: extracellular deposition as the LN-5 form and cytoplasmic accumulation. Well-differentiated carcinoma cells forming tubular structures often deposit LN-5 on the neoplastic BMs, whereas carcinoma cells invading the underlying stroma express only the γ2 chain and accumulate it intracellularly. The sole expression of the γ2 chain in invading tumor cells has not been reported in other kinds of cancers.

Lung cancer is one of the major causes of cancer death in Japan, the United States and other countries. Among...
various histological types of lung cancer, adenocarcinoma is the most frequent. It has been accepted that at least a subset of peripheral lung adenocarcinomas develop from atypical adenomatous hyperplasia (AAH) through in situ adenocarcinoma to invasive adenocarcinoma.24,25 During this progression, BM structures are gradually disrupted, while stromal fibrosis occurs and advances.26 It seems very likely that these dynamic changes in the BM structures and the stromal structures influence the behavior of tumor cells.27 Based on this assumption, we here examined the distribution of the important BM component LN-5 and its γ2 chain in different types of lung adenocarcinomas and atypical adenomatous hyperplasia tissues.

MATERIALS AND METHODS

Materials In the present study, 15 lesions of AAH from 13 patients (one or two lesions per patient) and 38 lesions of lung adenocarcinomas obtained from 30 patients (2 lesions each in 4 patients and 3 lesions each in 2 patients), who had undergone lobectomy or pneumonectomy for lung cancer at the Yokohama City University Hospital, Yokohama Municipal Citizen’s Hospital, Kanagawa Cancer Center Hospital, and Kanagawa Cardiovascular and Respiratory Center Hospital between 1991 and 1998, were analyzed for the expression of the LN γ2 chain. The classification of AAH and adenocarcinomas was performed according to the WHO classification28 with some modifications. In brief, the AAH lesions were divided into low-grade atypia AAH (n=4) and high-grade atypia AAH (n=11), depending upon the degree of nuclear atypia, structural atypia, and cell density.29 The adenocarcinoma lesions were classified into well-differentiated (n=23), moderately differentiated (n=7), and poorly differentiated adenocarcinomas (n=8). All of the 23 well-differentiated adenocarcinomas were of a bronchioloalveolar carcinoma (BAC) type, and these were further subclassified into 13 non-sclerosing BAC (pure form of BAC) and 10 sclerosing BAC (mixed subtype composed of BAC and other forms). There was no lesion of mucinous adenocarcinoma. In all of the 13 non-sclerosing BAC lesions, there was no evidence of tumor cell invasion. Among the 10 sclerosing BAC lesions, which showed a central area of marked stromal fibrosis, an invasive growth of tumor cells within the sclerotic area was identified in 6 and suspected in 2, while it was absent in 2 lesions.29

The resected lung lobes were fixed in 20% buffered formalin and embedded in paraffin. Sections (4-µm-thick) cut from the paraffin-embedded tissues were stained with hematoxylin-eosin for histological examination and used for immunohistochemistry of the LN γ2 chain as well.

For immunohistochemistry of the LN α3, β3, and γ2 chains with frozen sections, fresh tumor tissues were obtained at surgery from 9 additional age- and sex-matched patients, snap-frozen in O.C.T. compound (Embedding Medium, Sakura Finetechical Co., Tokyo), and stored at −80°C until use. These included 3 well-differentiated adenocarcinomas (one pure and two mixed form BAC), 3 moderately differentiated adenocarcinomas, and 3 poorly differentiated adenocarcinomas. Sections (4-µm-thick) were made with a cryostat and mounted on glass slides. Fresh normal skin tissue was also obtained from surgical material from an adult patient, prepared in the same manner, and used as the positive control. Antibodies The following monoclonal antibodies were used in this study: a monoclonal antibody to the LN α3 chain (P3H9) (Chemicon, Temecula, CA),29 a monoclonal antibody to the LN β3 chain (29E), and a monoclonal antibody to the LN γ2 chain (D4B5).30 The antibodies 29E and D4B5 were raised against human LN-5 and human recombinant LN γ2 chain, respectively, in our laboratory and used for immunohistochemical staining of formalin-fixed paraffin sections and/or paraformaldehyde-fixed frozen sections.

Immunohistochemistry For immunohistochemical staining, the paraffin sections were deparaffinized, rehydrated, immersed in 0.3% hydrogen peroxide-containing methanol for inactivation of intrinsic peroxidase and treated with Protease XXIV (Sigma, St. Louis, Mo.) for 20 min at room temperature. The frozen sections were also immersed in 0.3% hydrogen peroxide-containing methanol and treated with Protease XXIV for 5 min. Then the paraffin sections were incubated with the anti-γ2-chain antibody D4B5 at 4°C overnight, while frozen sections were incubated with each of the three antibodies (P3H9, 29E and D4B5) for 20–30 min at room temperature. The labeled antigens were detected with a HistoFine kit (Nichirei Pharmaceutical, Tokyo) and visualized by means of the 3,3-diaminobenzidine (DAB) reaction. Other experimental conditions were described previously.31 Statistical analysis Statistical analysis was performed using the χ² test. Differences were considered significant when P values were less than 0.05.

Analysis of LN γ2 chain monomer present in tumor tissue Ten-micrometer-thick sections from a frozen tumor tissue were combined and extracted with the sodium dodecylsulfate (SDS) sample buffer. The tumor extract was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 6% gels under non-reducing conditions, and the separated proteins were transferred onto nitrocellulose membranes. The LN γ2 chain was detected by the alkaline phosphatase method with the anti-γ2-chain antibody D4B5. As a control, pure LN γ2 chain monomer was run on the same gel. This protein was purified from serum-free conditioned medium of human gastric carcinoma cell line MKN45 treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) by affinity chromatography on a D4B5-conjugated Sepharose column.
RESULTS

Distribution of laminin γ2 chain  Expression of the LN γ2 chain was examined in 15 AAH lesions (4 low-grade and 11 high-grade) and 38 adenocarcinoma tissues (23 well-differentiated, 7 moderately differentiated and 8 poorly differentiated types) by immunohistochemical staining with the γ2-specific antibody D4B5. The well-differentiated adenocarcinoma tissues were further classified into the non-sclerosing type (pure form of BAC) and the

Table I. Summary of Immunohistochemical Analysis for Expression of LN γ2 Chain in Different Histological Types of Lung Lesions

| AAH (%) | Adenocarcinoma | Total cases |
|---------|---------------|-------------|
|         | Low-grade     | High-grade  | WD (%) | MD (%) | PD (%) |            |
| Basement membrane | 4/4 (100)     | 11/11 (100) | NSC BAC | 8/13 (62) | 2/7 (29) | 1/8 (13) | 29/53     |
| Cytoplasmic    | 1/4 (25)      | 5/11 (45)   | BAC     | 5/13 (38) | 9/10 (90) | 7/7 (100) | 8/8 (100) | 35/53     |

AAH, atypical adenomatous hyperplasia; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; BAC, bronchioloalveolar carcinoma; NSC, non-sclerosing; SC, sclerosing.

Fig. 1. Immunohistochemical staining of LN γ2 chain in normal lung (A), AAH (B) and well-differentiated adenocarcinomas (C, D). (A) Normal alveolar septa show continuous staining along the BMs for the LN γ2 chain. (B) Continuous BM staining for LN γ2 chain is seen in the stromal thickening lesion of the alveolar septa in an AAH lesion. (C) In a sclerosing, well-differentiated adenocarcinoma (mixed form BAC), the neoplastic BMs at the interface between tumor cell clusters and fibrous stroma are negative for the LN γ2 chain, but tumor cells show positive cytoplasmic staining for the LN γ2 chain. (D) Tumor cells infiltrating sclerotic space show positive cytoplasmic staining for the LN γ2 chain (arrows). This section also shows focal staining of different cell structures (arrowheads). These stained cells appear to be either degenerated tumor cells or unidentified stromal cells activated by tumor cells. Arrows, positive signal. Experimental conditions are described in the text. (Original magnification, A–C ×50, D×100)
sclerosing type (mixed form of BAC and others), the latter of which showed marked stromal fibrosis with scar formation. The results of immunohistochemical staining are summarized in Table I. All AAH lesions and the majority of non-sclerosing, pure form BAC showed positive BM staining for the LNγ2 chain, but most tumor cells show intense cytoplasmic staining. In a poorly differentiated adenocarcinoma, tumor cells having invaded the sclerotic space show diffuse cytoplasmic staining for the LNγ2 chain. Arrows, positive signal. Experimental conditions are described in the text. (Original magnification, ×50)

Table II. Degree and Frequency of Cytoplasmic Staining for LNγ2 Chain of Hyperplastic Cells or Tumor Cells in Different Types of Lung Lesions

| AAH (%) | WD (%) | Adenocarcinoma | MD (%) | PD (%) |
|---------|--------|----------------|--------|--------|
|         | Low-grade | High-grade | NSC BAC | SC BAC |       |       |
| Total cases | 4 | 11 | 13 | 10 | 7 | 8 |
| – | 3 (75) | 6 (55) | 8 (62) | 1 (10) | 0 | 0 |
| +/– | 1 (25) | 5 (45) | 5 (38) | 3 (30) | 0 | 0 |
| + | 0 | 0 | 0 | 6 (60) | 6 (86) | 7 (88) |
| ++ | 0 | 0 | 0 | 0 | 1 (14) | 1 (12) |

Immunoreactivities of tumor cells against anti-LNγ2 chain monoclonal antibody were evaluated as negative (−) when no positive cells were found, slightly positive (+/−) when positive cells accounted for less than 10% of the total numbers, positive (+) when positive cells were 10 to 50% of the total numbers, and diffuse (+++) when more than 50% tumor cells were positive.

AAH, atypical adenomatous hyperplasia; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; BAC, bronchioloalveolar carcinoma; NSC, non-sclerosing; SC, sclerosing.
Fig. 3. Expression of three LN-5 subunits in non-sclerosing (pure form BAC) carcinoma of the well-differentiated type. Adjacent frozen sections were immunostained for the LN α3 (A), β3 (B), and γ2 (C) chains. In a non-sclerosing carcinoma tissue, BMs surrounding glandular tumor cells are positive for the LN α3 (A), β3 (B) and γ2 (C) chains. Arrows, positive signal. Other experimental conditions are described in the text. (Original magnification, ×100)

Fig. 4. Expression of three LN-5 subunits in sclerosing (mixed form BAC) carcinoma of the well-differentiated type. Adjacent frozen sections were immunostained for the LN α3 (A), β3 (B), and γ2 (C) chains. In a sclerosing carcinoma tissue, tumor cells in the sclerotic space show positive cytoplasmic staining for the LN γ2 chain (C), but they are negative for the LN α3 (A) and β3 (B) chains. Arrows, positive signal. Other experimental conditions are described in the text. (Original magnification, ×100)
Cytoplasmic staining of the LN γ2 chain was also observed in all histological types of lung lesions. In contrast to the BM staining, the frequency and intensity of the cytoplasmic γ2 staining increased depending on the degree of the stromal invasion of tumor cells (Tables I and II). In AAH lesions, the cytoplasmic γ2 chain immunostaining was far weaker than in adenocarcinomas, and was sporadic. Among well-differentiated adenocarcinomas, the γ2 chain staining of tumor cells was stronger and more frequent in the sclerosing type than in the non-sclerosing type. In the sclerosing lesions, tumor cells bordering or having invaded the sclerotic area showed intracellular immunoreactivity for the LN γ2 chain (Fig. 1, C and D). All moderately and poorly differentiated adenocarcinoma lesions were positive for the γ2 chain (Tables I and II). In these adenocarcinomas, most of the tumor cells invading the sclerotic areas showed strong cytoplasmic staining for the γ2 chain (Fig. 2). The invasive tumors (sclerosing BAC and moderately and poorly differentiated adenocarcinomas) contained more γ2-chain-positive cells than the non-invasive tumors (AAH and non-sclerosing BAC) (P<0.001) (Table II).

**Distributions of LN α3, β3 and γ2 chains on frozen sections** To examine whether the LN γ2 chain observed in lung adenocarcinomas coexists with the LN α3 and β3 chains, frozen sections of human lung adenocarcinoma tissues were subjected to immunohistochemical analysis with the antibodies to the three LN-5 subunits. Tumor BMs in a non-sclerosing, pure form BAC were positive for all of the α3, β3 and γ2 chains (Fig. 3). Similar staining patterns were obtained in non-invasive areas of other types of adenocarcinoma, particularly those with a bronchioloalveolar pattern (data not shown). In contrast, tumor cells having invaded the sclerotic areas showed cytoplasmic staining for the γ2 chain, but neither the α3 nor β3 chain (Fig. 4). These distributions of the LN α3, β3 and γ2 chains in lung adenocarcinoma lesions suggest that in BMs of non-invasive adenocarcinomas the LN α3, β3 and γ2 chains are complexed and deposited as the LN-5 form, whereas in invading tumor cells the LN γ2 chain is solely expressed and accumulated in the cytoplasm.

**Analysis of LN γ2 chain monomer present in tumor tissue** To confirm the presence of the LN γ2 chain monomer, non-reducing western blotting analysis with the anti-LN-γ2-chain antibody was carried out with extracts from mixed form of BAC (Fig. 5). The control γ2 chain monomer showed a major band of 160 kDa and several proteolytic fragments with smaller molecular sizes. The tumor extract showed a major immunoreactive band of 130 kDa and at least two minor bands of 155 and 70 kDa. The 130 and 70 kDa bands co-migrated with the minor bands of the purified γ2 chain monomer, but the 155 kDa band had a slightly lower molecular size than the main component of the purified γ2 chain monomer (160 kDa) possibly due to difference in glycosylation or proteolysis.

**DISCUSSION**

In this study we examined the distribution of the LN γ2 chain in human lung AAH and various types of adenocarcinoma tissues. The results of our previous study on lung adenocarcinomas indicated that AAH and non-sclerosing BAC are intraepithelial non-invasive tumors, while sclerosing BAC and non-BAC tumors that form central scar tissues are invasive tumors. The LN γ2 chain was frequently detected in the epithelial or neoplastic BMs in AAH and non-sclerosing, pure form BAC, as well as in non-invasive areas of other types of tumors. In these BMs the LN α3 and β3 chains co-localized with the γ2 chain, probably forming the LN-5 complex. In contrast, the cytoplasmic accumulation of LN γ2 chain was observed in tumor cells invading into or surrounded by the sclerotic area, without any evidence of co-expression of the LN α3 and β3 chains.

Recently, Määttä et al.189 examined the expression of the LN γ2 chain in various types of lung carcinomas by immunohistochemistry and *in situ* hybridization. The expression of the LN γ2 chain was strongest in squamous cell carcinomas, followed by adenocarcinomas and large

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**Fig. 5. Western blotting analysis of LN γ2 chain monomer present in non-sclerosing BAC.** An extract from a frozen tissue of a well-differentiated adenocarcinoma (mixed type of BAC) (lane 1) and a control LN γ2 chain monomer which had been purified from conditioned medium of human gastric carcinoma cell line MKN45 (lane 2) were run on a 6% gel under non-reducing conditions, transferred onto a nitrocellulose membrane, and then probed with anti LN-γ2 chain (D4B5). Three bands (arrows) of 155, 130 and 70 kDa were detected in the carcinoma tissue. Other experimental conditions are described in the text.
cell carcinomas. The cytoplasmic staining of the γ2 chain was seen in carcinoma cells at the epithelial-stromal interface or in those infiltrating within desmoplastic fibrous stroma. They concluded that a LN γ2 chain-containing substrate, possibly LN-5, might be involved in the spread and growth of malignant tumors. Our results are consistent with their results regarding adenocarcinomas. However, the present study demonstrated that the γ2 expression in tumor cells is not accompanied with the expression of the α3 and β3 chains. The sole expression of the γ2 chain was confirmed by immunoblotting analysis of the tumor extract. The cytoplasmic staining of tumor cells for the LN γ2 chain seems due to the lack of synthesis of the α3 and β3 chains. We have previously shown that invading gastric carcinoma cells express and accumulate the LN γ2 chain monomer.20) Taken together, the cytoplasmic staining of the γ2 chain has been reported before in other types of carcinomas44–48) is most likely to reflect the expression of the γ2 chain monomer.

In peripheral lung adenocarcinomas the maintenance of intact BM structures is correlated with a better prognosis33–35) while the degree of stromal fibrosis is one of the important prognostic factors particularly at earlier stages of the tumors.26, 36) Kitamura et al.29) have shown that in peripheral lung adenocarcinomas the desmoplastic stromal fibrosis is closely associated with disruption of BM structures and a concomitant expression of the matrix metalloproteinase (MMP) gelatinase A and tissue inhibitor of metalloproteinases-2 (TIMP-2). Overexpression of MMP has been reported in many types of malignant tumors.37) In the present study, the cytoplasmic accumulation of LN γ2 chain in carcinoma cells was associated with the loss of neoplastic BMs and with the degree of stromal fibrosis. The BM structures surrounding or supporting tumor cell clusters can be disrupted by the loss of the ability to produce BM components by tumor cells, as well as by their proteolytic degradation. These changes are expected to allow tumor cells to invade into interstitial space. In fact, type IV and type VII collagens are often lost in poorly differentiated lung carcinomas.27) Our results suggest that the loss or decrease in the expression of the laminin α3 and β3 chains by tumor cells may contribute to the loss of BM structures in invasive carcinomas, since LN-5 is an important BM component in epithelial tissues.

The significance of the sole expression of the LN γ2 chain in invading carcinoma cells remains unknown. It has been reported that the limited cleavage of the LN γ2 chain by MMPs enhances the cell motility activity of LN-5.38, 39) This suggests that the LN γ2 chain plays an important role in regulating cell motility. We have reported that the LN γ2 chain monomer is secreted by gastric carcinoma cells23) and fibrosarcoma cells.40) Therefore, it seems likely that the LN γ2 chain monomer or its proteolytic fragment exerts some biological effects, though the direct stimulation of cell adhesion or motility by the γ2 chain monomer has not been proved. The mechanism of stromal fibrosis, or sclerosis, in tumor tissues has not been elucidated yet. Tumor cell-derived factors are expected to stimulate stromal cells to form fibrosis. It can be speculated that the LN γ2 chain monomer mediates some interaction between tumor cells and surrounding stromal cells. In this regard, it should be noted that in a considerable number of AAH lesions cytolytic staining of hyperplastic cells for the LN γ2 chain was detected. The cytoplasmic γ2 staining of hyperplastic cells might be an indicator for further progression to malignant carcinomas.

In conclusion, we found that lung adenocarcinoma cells infiltrating stromal tissues upregulated the LN γ2 chain monomer. The expression of the LN γ2 chain monomer was closely associated with the invasiveness of tumor cells and with the formation of stromal fibrosis in the tumor tissues. Therefore, the LN γ2 chain monomer could be an important indicator of progression of lung adenocarcinoma. However, the biological activity of the LN γ2 chain monomer remains to be clarified.

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