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Amino-terminal fragments of laminin γ2 chain retract vascular endothelial cells and increase vascular permeability

Hiroki Sato,[1,2] Jun Oyanagi,[1,2] Eriko Komiya,[1,2] Takashi Ogawa,[2] Shouichi Higashi[1,2] and Kaoru Miyazaki[1,2]

[1]Department of Genome Science, Graduate School of Integrated Science and Nanobioscience, Yokohama City University, Yokohama; [2]Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan

Key words

Laminin γ2 chain, laminin-332, tumor invasion, vascular endothelial cells, vascular permeability

Correspondence

Kaoru Miyazaki, Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan.
Tel: +81-45-820-1905; Fax +81-45-820-1901;
E-mail: miyazaki@yokohama-cu.ac.jp

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Laminin γ2 (Lmγ2) chain, a subunit of laminin-332, is a typical molecular marker of invading cancer cells, and its expression correlates with poor prognosis of cancer patients. It was previously found that forced expression of Lmγ2 in cancer cells promotes their invasive growth in nude mice. However, the mechanism of the tumor-promoting activity of Lmγ2 remains unknown. Here we investigated the interaction between Lmγ2 and vascular endothelial cells. When treated with an N-terminal proteolytic fragment of γ2 (γ2pf), HUVECs became markedly retracted or shrunken. The overexpression of Lmγ2 or treatment with γ2pf stimulated T-24 bladder carcinoma cells to invade into the HUVEC monolayer and enhanced their transendothelial migration *in vitro*. Moreover, γ2pf increased endothelial permeability *in vitro* and *in vivo*. As the possible mechanisms, γ2pf activated ERK and p38 MAPK but inactivated Akt in HUVECs. Such effects of γ2pf led to prominent actin stress fiber formation in HUVECs, which was blocked by a ROCK inhibitor. In addition, γ2pf induced delocalization of VE-cadherin and β-catenin from the intercellular junction. As possible receptors, γ2pf interacted with heparan sulfate proteoglycans on the surface of HUVECs. Moreover, we localized the active site of γ2pf to the N-terminal epidermal growth factor-like repeat. These data suggest that the interaction between γ2pf and heparan sulfate proteoglycans induces cytoskeletal changes of endothelial cells, leading to the loss of endothelial barrier function and the enhanced transendothelial migration of cancer cells. These activities of Lmγ2 seem to support the aberrant growth of cancer cells.

Epithelial tissues are separated from connective tissues by thin membrane structures called basement membranes. Loss of this structure is closely associated with cancer progression. Laminins, large glycoproteins composed of α, β, and γ chains, are essential components of various basement membranes.[1] Seventeen laminin isoforms with different combinations of three chains (α1-α5, β1-β3, and γ1-γ3), including a recently identified vascular-type laminin (laminin-3B11), have been identified so far.[2,3] Among the laminin families, laminin-332 (Lm332) consisting of α3, β3, and γ2 chains, previously known as laminin-5, is a major type of laminin in epithelial tissues.[4–6] In the skin, Lm332 is an essential component of hemidesmosomes and stably anchors basal keratinocytes to the basement membrane.[5,7] We initially identified Lm332 as a cancer cell-derived scatter factor that strongly stimulates cell adhesion and migration.[8] Indeed, unlike other types of laminins, a soluble form of Lm332 stimulates cell migration.[9] The cell adhesion and motility activities of Lm332 are regulated by the proteolytic cleavage of the short arm of laminin γ2 (Lmγ2) chain.[10,11] Metalloproteinases such as BMP-1 and MT1-MMP have been reported to cleave Lmγ2 at its N-terminal short arm.[12,13] Recent studies have shown that the N-terminal region of Lmγ2 is essential for the assembly of Lm332 into extracellular matrix.[14] Laminin-332 matrix assembled by cultured cells stably anchors cells, whereas a soluble form of Lm332 highly promotes cell migration.[15]

Because of the unique properties of Lm332, much attention has been focused on its possible roles in cancer malignancy.[16–19] Laminin-332 is found in basement membranes of many types of differentiated carcinomas. A number of immunohistochemical studies have shown that Lm332 or its subunits are overexpressed in invasive human carcinomas.[20] In particular, Lmγ2 is overexpressed at invasion fronts of many types of human cancers such as colorectum,[21,22] pancreas,[23] stomach,[24] lung,[25] and esophagus.[26] Such characteristic expression of the Lmγ2 chain is associated with poor prognosis and metastasis.[23,26,27] Thus, Lmγ2 has been regarded as a typical molecular marker of invasive carcinomas. Our past studies have shown that Lmγ2 is expressed mainly as a monomer form, but not as the Lm332 heterotrimer, by invading carcinomas cells.[24,25] In human cell culture, excess Lmγ2 is secreted as the monomer or the β3/γ2 heterodimer into culture medium,[15,28,29] and its short arm fragment is released into circulation of patients with head and neck squamous cell carcinomas.[30] Expression of Lmγ2 is regulated together with

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MT1-MMP by Wint-β-catenin signaling[22] and stimulated by epidermal growth factor (EGF), tumor necrosis factor-α, and transforming growth factor-β in association with the epithelial–mesenchymal transition of human carcinoma cells.[28,31] The characteristic expression and localization of Lmγ2 suggest that Lmγ2 monomer, apart from Lm332, has some special roles in tumor invasion. We recently found that forced overexpression of the Lmγ2 monomer or its short arm (γ2SA) in T-24 human bladder carcinoma cells promotes their invasive growth in nude mice.[28] However, the mechanism by which Lmγ2 promotes tumor invasion remains totally unknown.

It has well been established that stromal tissues surrounding cancer cells, such as fibroblasts, inflammatory cells, and vascular endothelial cells, are deeply involved in tumor progression.[32] In particular, tumor angiogenesis plays critical roles in tumor growth, invasion, and metastasis.[33–35] In this study, we investigated the possibility that Lmγ2 might have some activity towards vascular endothelial cells. Our results show that Lmγ2 induces retraction of vascular endothelial cells and enhances vascular permeability *in vitro* and *in vivo*.

Materials and Methods

Antibodies. Mouse mAbs used were anti-β-catenin and anti-VE-cadherin antibodies from BD Transduction Laboratories (Lexington, KY, USA), anti-His-tag antibody from MBL (Nagoya, Japan), anti-α-tubulin antibody from Millipore Merck (Temecula, CA, USA), anti-Δ-heparan-sulfate antibody (anti-heparan sulfate proteoglycan [HSPG] antibody) from Seikagaku Kogyo (Tokyo, Japan), and anti-β-actin antibody from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies used were anti-phospho-ERK antibody from Sigma Aldrich, anti-pan-ERK, anti-phospho-Akt (Ser^{473}), anti-pan-Akt, and anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibodies from Cell Signaling Technology (Beverly, MA, USA), and anti-pan-p38 MAPK antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The FITC-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody were purchased from Vector Laboratories (Burlingame, CA, USA).

Cell culture. The human bladder carcinoma cell line T-24 (EJ-1 strain) and its transfectants (Mock-T24 and γ2SA-T24) were used in our previous study.[28] These cells were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Nichirei Biosciences, Tokyo, Japan) and antibiotics. Human umbilical vein endothelial cells were purchased from Kurabo (Osaka Japan) and maintained on type I collagen (40 μg/mL) (Nitta Gelatin, Osaka, Japan) coated plates in MCDB131 medium (Sigma-Aldrich) supplemented with 10 ng/mL EGF (Wako, Osaka Japan), 5 ng/mL basic fibroblast growth factor (Wako), 50 μg/mL heparin (Wako), 10% FCS, and antibiotics.

Preparation of recombinant proteins. Recombinant proteins of Lmγ2 and its fragments, γ2 proteolytic fragment (γ2pf) and γ2 domain V (γ2dV), all of which contained the His-tag sequence in their C-termini, were prepared as previously reported.[36] Expression vectors for three deletion mutants of γ2dV with His-tag, named NE1/2, NE1/3 and NE2/3, were constructed with the pSecTag2B/Zeo vector (Invitrogen). The expression vectors were transfected into human embryonic kidney cell line HEK293 using the X-tremeGENE 9 DNA transfection reagent (Roche, Basel, Switzerland). Each protein was purified from the conditioned medium of the stable transfectants using cOmplete His-Tag Purification Resin (Roche). The detailed methods for the construction of the γ2-expression vectors and the protein purification are described in Document S1.

***In vitro* and *in vivo* endothelial permeability assays.** The *in vitro* permeability assay was carried out using the two-chamber methods reported by Chen *et al.*[37] Briefly, a HUVEC monolayer that had been prepared on type I collagen-coated culture insert (0.4-μm pore size; BD Bioscience) was treated with test proteins in the growth medium for 18 h and then with FITC-dextran (40 kDa; Sigma-Aldrich) for 3 h. The amount of FITC-dextran diffused into the lower chamber was measured by excitation at 485 nm and emission at 590 nm. Miles vascular permeability assay was carried out using the BALB/c strain of mice (5–6 weeks old) essentially by the method of Zhang *et al.*[38] All mice were handled according to the recommendations of the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Care Committee of Kihara Institute for Biological Research (Yokohama, Japan). Evans blue dye (200 μL of a 0.5% solution in 0.9% NaCl) was injected i.v. into each mouse. Ten minutes later, 50 μL of test samples were injected intradermally into the back skin. Phosphate-buffered saline, as the vehicle control, and test samples were injected into separate points of the same mouse. Thirty minutes later, the animals were killed, and the skin area that covered the entire injection site was removed by punch biopsies. Evans blue dye diffused from blood vessels was extracted from the resected skin tissues and measured for absorbance at 595 nm.

Assays of T-24 cell invasion into endothelial monolayer and transendothelial migration. For the invasion assay, HUVECs were inoculated at 2×10^5 cells per well of 8-well Lab-Tek chamber slides (Nunc, Naperville, IL, USA) pre-coated with type I collagen (40 μg/mL) and incubated for 3 days. T-24 cells that had been labeled with Cell Tracker Green or Orange (Lonza, Walkersville, MD, USA) were incubated on the HUVEC monolayer for 18 h. Fluorescence images were obtained with BZ-8000 fluorescence microscope (Keyence, Osaka, Japan). For the transendothelial migration assay, fluorescence-labeled T-24 cells were incubated with test samples on the HUVEC monolayer prepared in culture inserts (8-μm pore size) for 18 h. Fluorescence images were obtained for the cells that had migrated onto the lower surface of membrane filters.

Immunofluorescent staining of cytoskeletal and membrane-bound proteins. The HUVECs were inoculated at 5×10^4 cells per well of a Lab-Tek 8-well chamber slide precoated with fibronectin (1 μg/mL) and grown to reach suitable density. After treatment with test samples, the cells were fixed in acetone/methanol (1:1, v/v) for 15 min on ice, blocked with 3% BSA in PBS for 1 h and incubated with primary antibody against α-tubulin, VE-cadherin, or β-catenin diluted with the blocking buffer. The cells were then stained with FITC-labeled secondary antibody, rhodamine phalloidin, and DAPI for 1 h. The fluorescence images were obtained using a confocal microscope (FV1000; Olympus, Tokyo, Japan).

Statistical analysis. Statistical significance was evaluated with an unpaired Student's *t*-test. A P-value < 0.05 was considered significant.

Results

Induction of morphological changes of endothelial cells by Lmγ2 chain. The Lmγ2 chain is cleaved at a specific site of domain III in the short arm (γ2SA) by some proteinases, releasing the N-terminal fragment, named γ2pf (Fig. 1a). It

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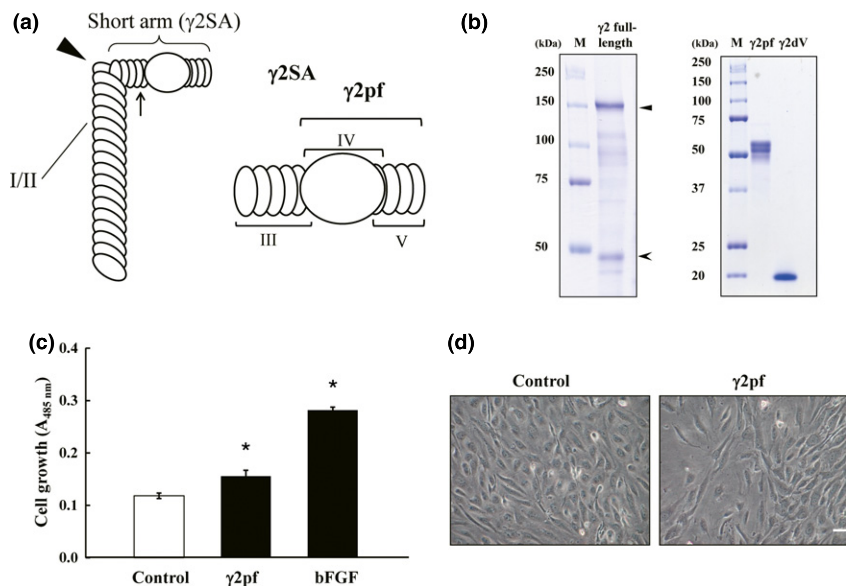

Fig. 1. Domain structure of laminin γ2 (Lmγ2) and its biological activities toward endothelial cells. (a) Domain structure of Lmγ2 chain. Left, full-length; right, short arm (γ2SA). Laminin γ2 consists of domains I/II, III, IV (globular domain), and V. Arrow, proteolytic cleavage site. Domain V (γ2dV) consists of three N-terminal epidermal growth factor-like repeats (NE1–3). (b) SDS-PAGE profiles of purified γ2 full-length (left panel) and γ2pf and γ2dV (right panel) as detected by Coomassie Brilliant Blue staining. Approximately 2 μg protein was run in each lane, as reported previously.[28] A 50-kDa protein (arrowhead) in γ2 full-length seems to be γ2pf. M, molecular weight markers and their size. (c) Effect of γ2pf on proliferation of vascular endothelial cells. Cells (HUVECs) were incubated with PBS as vehicle control (Control) or with 2 μg/mL γ2pf or 10 ng /mL basic fibroblast growth factor (bFGF) in MCDB131 medium supplemented with 1% FCS and 10 ng/mL epidermal growth factor on collagen-coated 96-well plates for 3 days. The relative number of grown cells was determined by MTT formazan assay with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Each point represents the mean ± SD (bar) of triplicate wells. $^{*}P < 0.05$. (d) Morphological change of HUVECs by γ2pf treatment. A confluent culture of HUVECs in serum-free basal medium was treated with PBS (Control) or 2 μg/mL γ2pf for 2 h. Phase-contrast micrographs were taken at an original magnification of ×200. Scale bar = 20 μm.

was previously found that γ2SA has tumor-promoting activity *in vivo*.[28] To examine the biological activities of the γ2 chain toward vascular endothelial cells, we prepared the full-length γ2 and its various N-terminal fragments, including γ2pf, γ2dV, and γ2SA (Fig. 1b). Among them, γ2pf and γ2dV were mainly used in this study because of their high purity and relative importance.[36] When the growth effect of γ2pf on endothelial cells (HUVECs) was examined, it showed a statistically significant but faint growth-stimulatory effect (Fig. 1c). In the two-chamber assay, γ2pf scarcely stimulated the migration of HUVECs (Fig. S1a). However, when γ2pf was added to the monolayer culture of HUVECs, these cells became markedly retracted or shrunken compared with untreated cells (Fig. 1d). Such a morphological change was not observed when the epithelial cell line MDCK was treated with γ2pf (data not shown). We also confirmed that γ2SA induced similar morphological changes in endothelial cells (Fig. S1b,c). However, γ2SA showed little growth effect on endothelial cells (data not shown).

We next examined the effects of Lmγ2 on the interaction between cancer cells and endothelial cells, using the human bladder carcinoma cell line T-24 overexpressing γ2SA (γ2SA-T24) and its control cell line transfected with the empty vector (Mock-T24) (Fig. 2a). These cell lines were labeled with fluorescence (Cell Tracer Orange) and then placed on the monolayer culture of HUVECs. Under a fluorescent microscope, Mock-T24 cells spread poorly on the HUVEC monolayer, whereas γ2SA-T24 cells were well spread and extended many cell protrusions (Fig. 2b, upper panels, c). When Mock-T24 cells were treated with purified γ2pf protein, they showed a similar spreading morphology to that of γ2SA-T24 cells (Fig. 2b, upper right panel, c). The addition of γ2pf dose-dependently promoted the protrusion formation of Mock-T24 cells (Fig. 2d). Fluorescence labeling of cancer cells and filamentous actin staining of whole culture with rhodamine phalloidin revealed that both γ2SA-T24 cells and the γ2pf-treated Mock-T24 cells had invaded into the HUVEC monolayer and spread on the plastic surface (Fig. 2b, lower panels).

Induction of transendothelial migration of cancer cells by Lmγ2 chain. The results shown above suggested that γ2pf might induce migration of cancer cells through the endothelial cell sheet. This was tested by the Transwell chamber assay. Fluorescence-labeled Mock-T24 cells were placed on the HUVEC monolayer in the upper chamber and incubated in the presence or absence of purified γ2pf. The number of cells that migrated through the endothelial monolayer to the lower chamber increased 2.5 times in the presence of γ2pf (Fig. 3a,b). This clearly indicated that γ2pf stimulated the cancer cell migration through the endothelial monolayer sheet.

Enhancement of vascular permeability by Lmγ2 chain. The γ2pf-induced retraction of endothelial cells seemed to lead to their loss of barrier integrity. To confirm this possibility, we examined the activity of γ2pf on endothelial permeability *in vitro* (Fig. 4a). When the monolayers of HUVECs on the culture inserts were treated with purified γ2pf, the endothelial cell permeability, as measured by the diffusion of FITC-dextran through the HUVEC sheet, significantly increased compared to the untreated control cultures. In addition, enhanced permeability was observed with the full-length γ2 chain and its N-terminal domain V (γ2dV) (Fig. 4b, see also Fig. 1a). The order of the permeability activity was γ2dV > γ2pf > full-length γ2. Furthermore, we examined the effect of Lmγ2 on vascular permeability *in vivo* by Miles permeability assay with mice (Fig. 5). The intradermal

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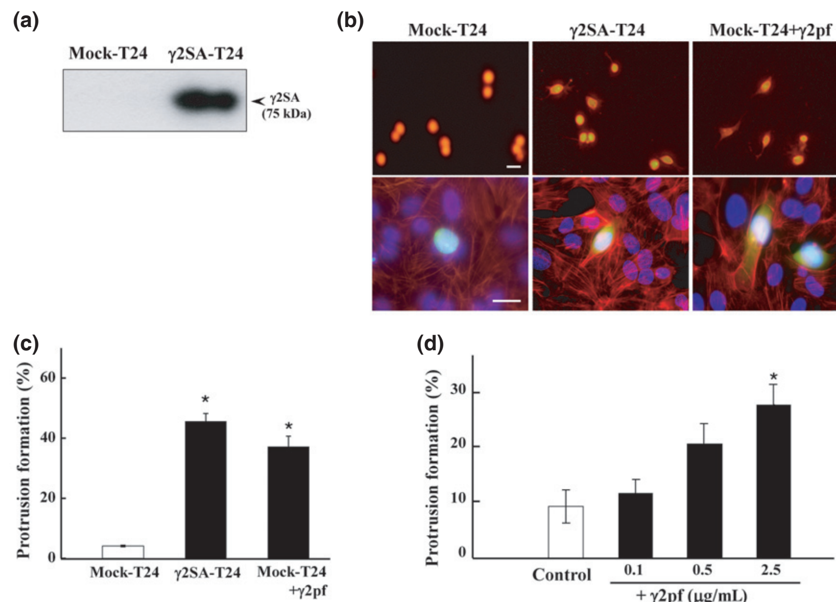

Fig. 2. Effects of laminin γ2 expression or γ2 proteolytic fragment (γ2pf) protein on morphology of T-24 carcinoma cells on a HUVEC monolayer. (a) Comparison of expression of γ2 short arm (γ2SA) between Mock-T24 and γ2SA-T24 cells. Amounts of γ2SA secreted into serum-free conditioned media were analyzed by immunoblotting with the anti-γ2 antibody D4B5, as reported previously.[28] (b) Upper panels, fluorescence-labeled (orange) Mock-T24 or γ2SA-T24 cells were incubated overnight on a HUVEC monolayer without (left and center panels) or with 2 μg/mL γ2pf (right panel) in a 24-well plate. Lower panels, fluorescence-labeled (green) Mock-T24 or γ2SA-T24 cells were treated as above on Lab-Tek chamber slides. The resultant cultures were fixed and stained for F-actin with rhodamine phalloidin (red) and for nuclei with DAPI (blue). Scale bars = 20 μm. The γ2SA-T24 cells (center) and γ2pf-treated Mock-T24 cells (right), but not control Mock-T24 cells (left), invaded into the HUVEC monolayer. (c) Quantification of cancer cells with protrusions. In the experiment shown in (a) (upper panels), the percentage of cells with protrusions was determined by scoring T-24 cells (150–200 cells in total) present in the center field of each well. Each point represents the mean ± SD (bar) of triplicate wells. $^*P < 0.05$. (d) Protrusion formation of Mock-T24 cells by increasing concentrations of γ2pf.

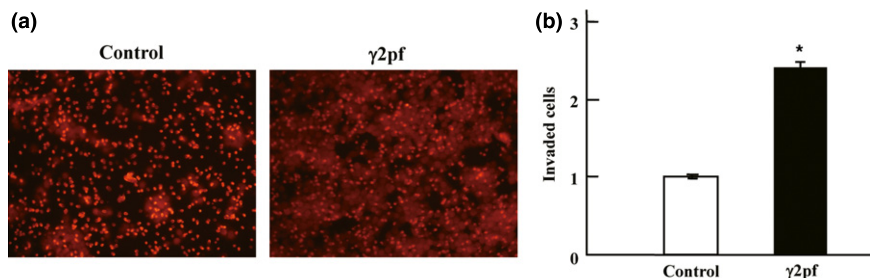

Fig. 3. Effect of laminin γ2 proteolytic fragment (γ2pf) on transendothelial migration of T-24 carcinoma cells. (a) Fluorescence-labeled Mock-T24 cells were incubated on a HUVEC monolayer with PBS (Control) or with γ2pf (2 μg/mL) in Transwell chambers. After incubation for 18 h, the cells that had migrated onto the lower surface of membrane filters were fixed and photographed under a fluorescence microscope. Small homogeneous spots are pores of the membrane filters. (b) Quantitative analysis of migrated cells. The areas of cells on the lower surface of membrane filters were measured by Image-J. Each point represents the mean ± SD (bar) of triplicate chambers. $^*P < 0.05$. Essentially the same results were reproduced in an additional experiment.

injection of γ2pf increased the leakage of Evans blue dye two-fold compared to the PBS injection as control (Fig. 5a). Purified γ2dV also increased vascular permeability two-fold (Fig. 5b), but domain III of Lmγ2 did not show any significant activity (Figs 5c, S2, see also Fig. 1a). These results suggest that the N-terminal fragments of Lmγ2 chain function as vascular permeability-promoting factors in pathological conditions.

Rearrangement of cytoskeleton by γ2pf in HUVECs. To elucidate the mechanism by which the laminin γ2 chain disrupts the barrier function of vascular endothelial cells, we next examined effect of γ2pf on the localization of cytoskeletal proteins and adherence junction proteins (Fig. 6). In a sparse culture of HUVECs, double fluorescence staining of F-actin and microtubules showed that γ2pf strongly induced actin stress fibers in the cytoplasm, while it reduced or disassembled microtubule structures (Fig. 6a). As expected, the γ2pf-induced actin stress fiber formation was effectively blocked by the treatment with the ROCK inhibitor Y-27632 (Fig. 6b).

The induction of stress fiber formation by γ2pf was also found in confluent culture of HUVECs (Fig. 6c). Although VE-cadherin and β-catenin were localized linearly along with cell–cell borders in the untreated cells, this localization was disrupted or became unclear on treatment with γ2pf (Fig. 6c,d).

We also investigated effects of Lmγ2 on major signal transduction for cytoskeletal regulation. The results showed that γ2pf weakly activated ERK and p38 MAPK but inactivated Akt in HUVECs (Fig. S3).

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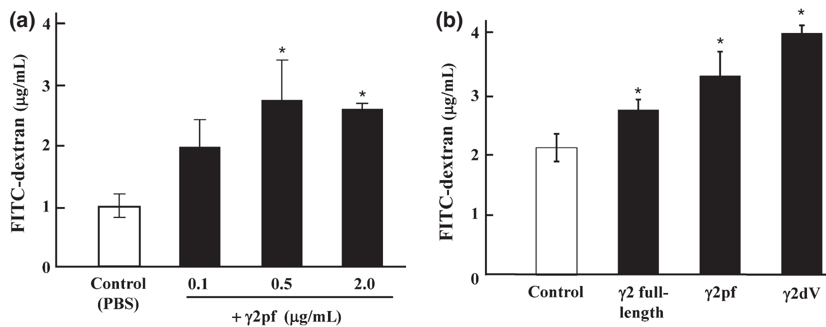

Fig. 4. Effect of laminin γ2 (Lmγ2) chain on endothelial permeability *in vitro*. (a) Dose-dependent effect of γ2 proteolytic fragment (γ2pf) on the permeability of a HUVEC monolayer using FITC-dextran. Each point represents the mean ± SD (bar) of triplicate chambers. $^*P < 0.05$. (b) Effects of three kinds of Lmγ2 proteins on the permeability of a HUVEC monolayer. Cells were stimulated with PBS (Control), full-length γ2 chain (1.5 μg/mL), γ2pf (0.5 μg/mL), or γ2 N-terminal domain V (γ2dV; 0.2 μg/mL) for 18 h, and the permeability was determined as above.

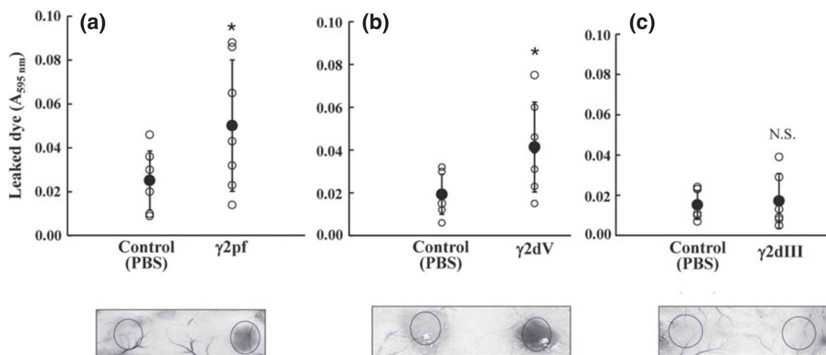

Fig. 5. Vascular permeability-inducing activity of laminin γ2 chain *in vivo*. Miles assay was carried out by injecting 5 μg each of γ2 proteolytic fragment (γ2pf) (a), γ2 N-terminal domain V (γ2dV) (b), or domain III (γ2dIII) (c) into the back skin of BALB/c mice. Each mouse was injected with PBS (Control) and a test sample on the left and right sides of the skin, respectively. Evans blue dye leaked from blood vessels was extracted and quantified. Lower panels show representative examples from the respective experiments. Similar results were reproduced in two additional experiments. Closed circles indicate the mean ± SD (n = 6); $^*P < 0.05$. N.S., not significant.

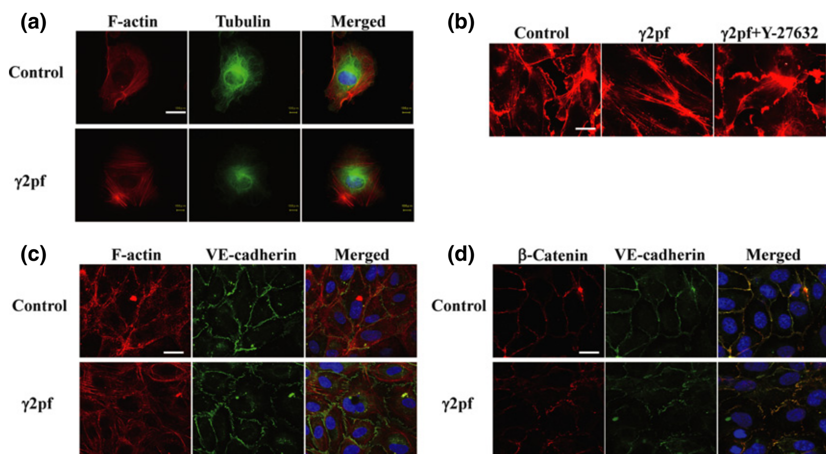

Fig. 6. Effect of γ2 proteolytic fragment (γ2pf) on localization of cytoskeletal and intercellular junctional proteins. (a) F-actin and tubulin. Cells (HUVECs) in sparse culture were incubated for 2 days with PBS (Control) or γ2pf (2 μg/mL) in a factor-free and 1% FCS-supplemented medium. Green, tubulin; red, F-actin. (b) Effect of ROCK inhibitor (Y-27632) on γ2pf-induced actin stress fiber formation. Cells were pretreated with 10 μM Y-27632 for 30 min then treated with γ2pf as described above. (c, d) Effect of γ2pf on VE-cadherin (c, green) and β-catenin (d, red). Confluent cultures of HUVECs were treated with PBS (Control) or γ2pf. Other experimental conditions were the same as above. Scale bars = 20 μm.

Interaction of Lmγ2 chain with membrane receptors on HUVECs. It has previously been suggested that the γ2 chain interacts with syndecan-1 and other anionic molecules on the cell surface.[36,39] In the present analysis by cell ELISA, γ2pf bound to the cell surface of HUVECs in a dose-dependent manner and this binding was blocked efficiently by the presence of heparin and completely by 1 M NaCl (Fig. S4). We also attempted to identify the membrane receptors of the γ2 chain on HUVECs. Experiments with a γ2pf-conjugated column showed that it bound to HSPGs with a molecular mass range from 100 to 150 kDa as core proteins (Fig. S4a). However, syndecan-1 was detected in neither the membrane fractions nor the column fractions (data not shown). Furthermore, pull-down assay with anti-His-tag antibody showed that γ2pf interacted with membrane proteins with 50–75 kDa in addition to HSPGs (Fig. S4b).

As shown in Figure 4(b), the most N-terminal domain of Lmγ2, domain V (γ2dV) had the highest activity to stimulate the endothelial cell permeability. It is composed of three N-terminal EGF-like-repeats, that is, disulfide bond-linked loop structures (NE1, 2, and 3). To localize the active site in domain V, we prepared three combinations of two repeats, NE1/2, NE1/3, and NE2/3 (Fig. 7a). In the pull-down assay with heparin-Sepharose, NE1/2 most strongly bound to heparin-Sepharose (Fig. 7b). NE1/3 weakly bound to heparin but not NE2/3 at all. Moreover, NE1/2 most evidently induced the delocalization of VE-cadherin from the cell–cell borders, but NE2/3 did not (Fig. 7c). Consistent with these results, NE1/2 increased vascular permeability *in vivo* more evidently than γ2dV (Fig. 7d). Neither NE1/3 nor NE2/3 showed significant activity. These data suggest that the first EGF-like repeat NE1 plays a critical role in the biological activities and heparin-binding activity of the Lmγ2 chain.

Discussion

Dysfunctions of the vascular system in cancer tissues are strongly involved in cancer progression. For example,

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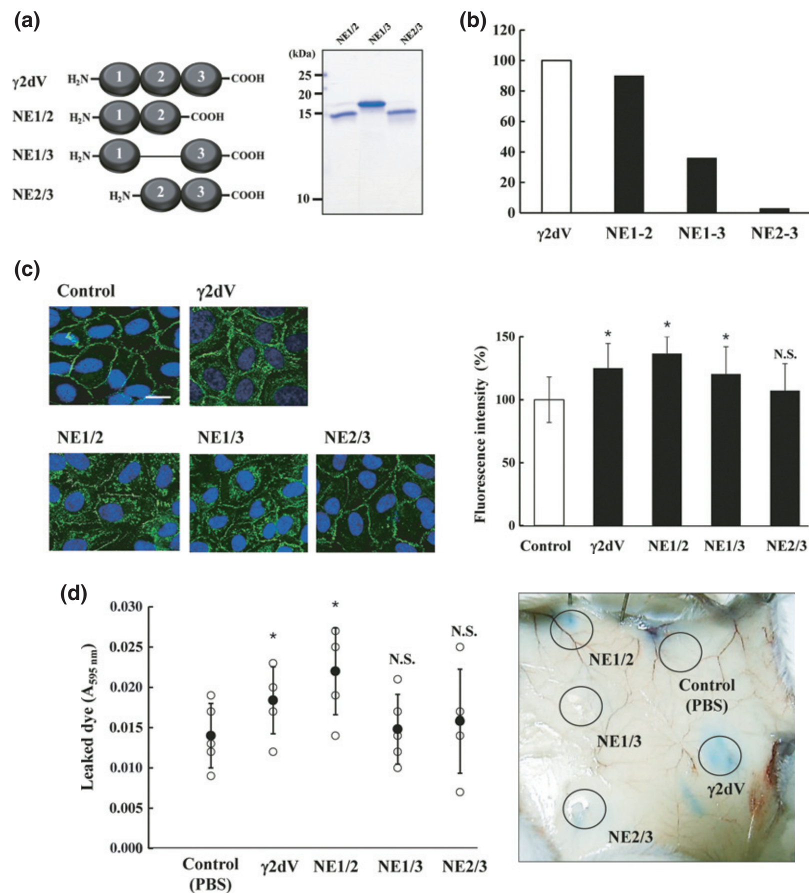

Fig. 7. Identification of the active site of laminin γ2 chain using deletion mutants of domain V. (a) Three recombinant proteins, NE1/2, NE1/3, and NE2/3, were prepared by deleting each of three epidermal growth factor-like repeats in domain V. Right panel, SDS-PAGE profiles of purified NE1/2, NE1/3, and NE2/3 proteins; 1 μg protein was run in each lane. (b) Heparin-binding activity of three recombinant proteins. NE1/2, NE1/3, or NE2/3 (1 μg) was incubated with 100 μL heparin-Sepharose beads for 120 min, and bound proteins were analyzed by immunoblotting with anti-His-tag antibody. Relative intensity of the immunopositive bands was measured by ImageJ. (c) Effects of γ2 N-terminal domain V (NE) proteins on VE-cadherin localization. Confluent cultures of HUVECs were treated with PBS (Control) or 1 μg each recombinant protein. Through morphological changes of HUVECs, VE-cadherin at intercellular junctions moved to the cytoplasm. Right panel shows the average intensity of green fluorescence per cell in the cytoplasmic area, which was analyzed by ImageJ. Each value was obtained from 50 cells in 15 different images. $^{*}P < 0.05$. N.S., not significant. Scale bars = 20 μm. (d) *In vivo* vascular permeability. Miles assay was carried out. Each mouse was injected with PBS (Control) and 1 μg each of γ2dV, NE1/2, NE1/3, and NE2/3 at separate points on the back skin ($n = 5$). One representative example of mouse skin is shown in the right panel.

enhanced angiogenesis supports tumor growth and metastasis.[34,35] Abnormal structures and loss of the barrier function of vasculature impair normal blood circulation. This causes hypoxia in cancer tissue and induces hypoxia-inducible factor-1α, increasing the invasive potential of cancer cells.[40] The present study showed for the first time that the tumor invasion marker Lmγ2 had profound activities toward vascular endothelial cells. Laminin γ2 induced cytoskeletal changes and retraction of endothelial cells. These activities enhanced vascular permeability *in vitro* and *in vivo* and transendothelial migration of cancer cells through the endothelial cell sheet. Although we do not currently have direct evidence, our results suggest the hypothesis that Lmγ2 produced by invading cancer cells acts on surrounding blood vessels and accelerates the abnormal vascular structure and functions as well as cancer progression. Recently we reported that expression of Lmγ2 monomer in T-24 bladder carcinoma cells enhances their invasive growth and accumulation of ascites fluid when the cells are i.p. transplanted into nude mice.[28] These previous results support the above hypothesis. The stimulation of transendothelial migration of cancer cells by Lmγ2 also suggests the possibility that it may enhance intravasation or extravasation of cancer cells, leading to the enhanced metastasis. Although this possibility was preliminarily tested, we failed to obtain enough evidence (data not shown). Laminin γ2 scarcely stimulated the proliferation or migration of vascular endothelial cells. However, the disruption of the intercellular junction of endothelial cells is an important initial step of tumor angiogenesis. Therefore, it is also possible that Lmγ2 may be involved in tumor angiogenesis. These possible functions of Lmγ2 in cancer vasculature and cancer progression remain to be clarified in further studies.

In the Lm332 molecule, the short arm of Lmγ2 has important effects on Lm332 activity. The loss of γ2pf from Lm332 decreases cell adhesion activity and increases cell motility activity,[11] and the cell adhesion effect of γ2pf is mediated by the interaction of domain V with syndecan-1 on the cell surface.[36] Moreover, domain IV of Lmγ2 is critical for the matrix assembly of Lm332.[14] One research group showed that domain III of Lmγ2, which is not contained in γ2pf, is important for the cell motility activity of Lm332, and this active site is released by MMP2 and MT1-MMP.[13,41] However, mammalian tolloid metalloproteinase (or BMP-1) has been shown to be a major γ2pf-releasing enzyme.[12] In addition, matrilysin (MMP7)[42] and neutrophil elastase[43] have been reported to cleave Lmγ2. The present study showed that γ2pf could bind to some HSPGs and lower molecular weight proteins in the membrane fraction of HUVECs. Synecan-1 was undetectable even in the membrane fraction. The interaction between γ2pf with HSPGs seems to be responsible for the biological activities of γ2pf because heparin inhibited the interaction. Moreover, we found that NE1 of domain V in Lmγ2 plays an essential role in Lmγ2 activities. Interestingly, the activities toward HUVECs were highest in γ2dV, then γ2pf, and the full length Lmγ2, in this order. Although γ2dV and γ2pf similarly increased vascular permeability *in vivo,* the NE1/2 fragment showed higher activity than γ2dV. It is supposed that smaller fragments containing the active site NE1 may exert higher permeability activity than larger fragments. Laminin γ2pf or similar fragments are produced from Lm332,

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the β3γ2 dimer, and the γ2 monomer.[15,28,29] Unidentified fragments of Lmγ2 short arm have been detected in sera from patients with pancreatic cancers[30] and in squamous cell carcinomas.[44,45] Laminin γ2 is expressed by not only invading cancer cells but also cancer-associated fibroblasts.[46] Our preliminary analysis has shown that γ2pf and smaller fragments containing domain V are indeed produced in invasive human cancer tissues (unpublished data, 2013). These results imply that domain V fragments are released by some proteinases in human cancer tissues. It seems very likely that the γ2dV-containing fragments produced by invasive cancer cells and activated fibroblasts act on surrounding blood vessels.

The present study also showed that γ2pf and γ2dV strongly induced actin stress fiber formation, while diminishing microtubule structures. Moreover, γ2pf and γ2dV activated ERK and p38 MAP kinases, and Rho kinase was involved in the γ2-mediated cytoskeletal changes of endothelial cells. These activities of Lmγ2 and its N-terminal fragments seem to cause the retraction of endothelial cells and loosen the cell–cell junction, mediated by VE-cadherin. These activities of Lmγ2 are similar to those of vascular endothelial growth factor.[47,48] It is well known that the cell surface HSPG syndecans cooperate with integrins to induce actin cytoskeletal changes and regulate cell adhesion.[49,50] It is very likely that the interaction of γ2pf or γ2dV with unidentified cell surface HSPGs induces similar cytoskeletal changes in vascular endothelial cells. In conclusion, the present study strongly suggests that Lmγ2 or its N-terminal fragments produced in human cancer tissues are involved in aberrant vascular functions in cancer tissues.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Preparation of laminin γ2 (Lmγ2) recombinant proteins.

Fig. S1. Effect of γ2 proteolytic fragment (γ2pf) on migration of vascular endothelial cells and effect of γ2 short arm (γ2SA) on their morphology.

Fig. S2. Vascular permeability-inducing activity of γ2 fragments *in vivo*.

Fig. S3. Analysis of signal transduction induced by γ2 proteolytic fragment (γ2pf) in HUVECs.

Fig. S4. Analysis of membrane receptors for laminin γ2 chain on endothelial cells.

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