Deletion of Laminin-8 Results in Increased Tumor Noveascularization and Metastasis in Mice

Zhongjun Zhou,1,2 Masayuki Doi,2 Jianming Wang,2,4 Renhai Cao,3 Baohua Liu,1 Kui Ming Chan,1 Jarkko Kortesmaa,2 Lydia Sorokin,5 Yihai Cao,3 and Karl Tryggvason2

1Department of Biochemistry, Faculty of Medicine, University of Hong Kong, Hong Kong; 2Division of Matrix Biology, Department of Biochemistry and Biophysics, and Laboratory of Angiogenesis Research, Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden; 3Institute of Molecular Medicine, Department of Medicine, University of California at San Diego, La Jolla, California; and 4Lund University, Experimental Pathology, Lund, Sweden

Abstract

Laminin-8 (α4β1γ1) is one of the major laminin isoforms expressed in vascular endothelial basement membranes. Here we show that deletion of laminin-8 in mice affects angiogenesis under pathological conditions. Murine tumor models used in laminin α4-deficient mice results in hyperneovascularization and significant promotion of tumor growth and metastasis. The higher tumor growth rates in mutant mice correlate with decreased tumor cell apoptosis. Depletion of laminin α4 chain may alter the structure of vascular basement membranes, leading to increased angiogenesis. Our data suggest that the laminin-8 plays a critical role in the regulation of pathological angiogenesis.

Introduction

Angiogenesis is essential for tumor growth and metastasis. Like physiological angiogenesis, the process of tumor angiogenesis may be regulated by components in basement membranes, and break-down and reconstitution of the vascular basement membranes occurs simultaneously during angiogenesis. As proposed for healthy tissues, tumor neovascularization may involve angiogenesis, vasculogenesis, and intussusception. Tumor blood vessels can induce new blood vessel growth from the host, but remain fundamentally different from the host blood vessels. Morphologically, tumor vessels are irregular in shape, heterogeneous in cell composition and leaky. These features are considered as hallmarks of destruction of normal blood vessel integrity (1). The endothelial cells are disorganized and irregularly shaped, sometimes overlapping each other and with luminal projections, which lead to abluminal sprouts. It has been reported that blood vessels in tumors consist of mosaic cell types including tumor cells (2). Although mural cells have been found on tumor vessels, they are unusually loosely associated with endothelial cells (3). In addition, tumor vessels have abnormal basement membrane, which includes changes of matrix protein composition, assembly, and structure (4). The endothelial basement membrane may affect tumor growth and metastasis in two ways: First, the integrity of endothelial basement membrane of tumor blood vessels may function as a barrier for tumor cell invasion into the circulation system. In addition, structural components of basement membranes including collagens, laminins and proteoglycans may function as reservoirs for sequestration and release of tumor growth promoting factors such as fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor, platelet-derived growth factor, and transforming growth factor α. Laminins are complex multifunctional heterotrimeric proteins composed of α, β, and γ chains, held together via a coiled-coiled rod structure. To date, five α, four β, and three γ chains have been identified that give rise to at least 12 different laminin isoforms. The various laminins have wide, but often overlapping, tissue distributions and are differentially expressed during development. Laminins have been shown to play important roles in cell adhesion, migration, and differentiation (5). In vascular endothelial cell basement membranes, laminin-8 (α4β1γ1) and laminin-10 (α5β1γ1) are the two predominant isoforms (6, 7). The α4 chain-containing isoform, laminin-8, is found in the basement membranes of all blood vessels (6–8), whereas the α5 chain-containing isoform, laminin-10, occurs mainly in endothelial cell basement membranes of capillaries and some veins and venules (9, 10). The differential distribution of laminin-8 and laminin-10 in blood vessel basement membrane and recent studies of an in vitro inflammation model (8) suggest distinct functions for these two laminin isoforms. In the present study, we evaluate the effect of one of the vascular basement membrane components, laminin-8 on angiogenesis, tumor growth, and metastasis using laminin α4 chain null mice. We found accelerated tumor growth with a higher number of metastases in Lama4 null mice. The higher rate of tumor growth and metastasis is associated with increased neovascularization. Thus, laminin-8 plays a critical role in the regulation of tumor angiogenesis and tumor growth.

Materials and Methods

Animals and Breeding. The generation of Lama4 null mice has been described elsewhere (11). Heterozygous mutant mice were backcrossed to C57BL/6 mice (Charles River, Uppsala, Sweden) for seven generations. N7F1 mice were used for this study.

Cell Lines and Antibodies. Lewis lung carcinoma cells and the mouse melanoma cell line B16-F10 were obtained from Dr. Salomonri, University of Oulu, Finland. Monoclonal CD31 antibody was purchased from Pharmagen. The generation of laminin α1 and α2 polyclonal antibodies has been described previously (12, 13). The polyclonal antibody against laminin α3 chain was a gift from Daniel Aberdam, INSERM U385, Nice, France. Polyclonal laminin α5 chain antibodies (rabbit antiserum 8948) were kindly provided by Jeffrey Miner, Washington University, St. Louis, MO (14). Polyclonal antibody against the LG1–3 modules of laminin α4 chain was a gift from Rupert Timp, Max-Planck Institute for Biochemistry, Martinsried, Germany (15). Monoclonal antibodies against collagen IV, perlecain, and nidogen were from NeoMarkers. Horseradish peroxidase- and FITC-conjugated secondary antibodies were from DAKO.

Tumor Implantation. All of the animal experiments were performed with the permission of the Stockholm Animal Ethics Committee and were carried out in accordance with their guidelines. Mice 6–8 weeks of age were anesthetized, and 1 × 10⁶ Lewis lung carcinoma cells were inoculated into the dorsal s.c. tissue. Tumor size was measured with a caliper on alternate days from day 6 after tumor implantation. The growth of tumors was followed by measuring two dimensions of the tumor mass. Tumor volume was calculated as 0.5 × (width)² × length as described previously (16). At the end of the
experiment, the tumors were dissected and weighed. In separate experiments, aimed at evaluating tumor angiogenesis, tumors of similar size from mutant and control mice were excised. Tumor samples were snap-frozen in OCT embedding compound and were kept at −70°C until analyzed.

**In Vivo Metastasis Study.** Both i.m. injections and i.v. injections were used to evaluate metastasis. For i.m. injections, 1 × 10^6 B16-F10 cells were inoculated i.m. into the hind limb. For i.v. injections, 1 × 10^7 cells were injected into the tail vein. Mice were sacrificed and analyzed 3 weeks after the inoculation of tumor cells. Lung surface metastatic colonies were counted.

**Immunostaining.** Frozen sections (10 μm) were air-dried for 10 min and were fixed in 1% paraformaldehyde in PBS for 10 min. After washing and blocking, primary antibody was applied overnight at 4°C. For CD31 and laminin α chain double-staining, sections were first incubated with biotin-labeled antimouse CD31 at 1:100 dilution, and the signal was then amplified using the TSA Biotin System, before applying FITC-labeled secondary antibody. Polyclonal antibodies against laminin α4 or α5 chains were then applied and visualized by a tetramethylrhodamine isothiocyanate (TRITC)-secondary antibody. The signal was visualized by direct fluorescence microscopy when using fluorescent antibody, or light microscopy when using an horseradish peroxidase-labeled secondary antibody, followed by color development using diaminobenzidine.

**Quantification of Blood Vessel Density and Vessel Area.** Cryostat sections were immunostained with a CD31 antibody, without counterstain. Five images were randomly selected from each section and photographed with a charge-coupled device camera at a fixed magnification (×200). Images were analyzed with Scion Image software (Frederick, MD) according to the instructions. This program automatically measures the area of each blood vessel, including both the stained area and the interior lumen, and records the number of stained blood vessels in each image. Results from each tumor were presented as the average number of stained vessels per unit tumor area and the average vessel area per unit tumor area. Tumors from four mutant and four control mice were used in this study.

**Corneal Micropocket Assay.** The mouse micropocket assay was performed as described previously (11).

**BrdUrd Staining.** 5-Bromo-2′-deoxyuridine (BrdUrd; 100 μl of 10 mg/ml; Sigma) was injected i.p. 2 h before sacrificing the mice. BrdUrd staining (Zymed) was performed according to the manufacturer’s instruction and was documented with a digital camera. Five microscopic fields from each section were randomly chosen and photographed. All of the nuclei and BrdUrd-positive nuclei were counted using the Scion Image (Frederick, MD) software.

**TUNEL Assay.** Cryostat sections of tumor samples were fixed in 1% paraformaldehyde for 10 min at room temperature. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed with the Apoptag Peroxidase kit (DAKO). Sections were counterstained with methyl green (DAKO). Five microscopic fields from each section were randomly chosen and photographed. TUNEL-positive nuclei were counted with Scion Image software (Frederick, MD).

**Statistics.** All of the data were evaluated by the two-tailed Student t test.

**Results**

**Enhancement of Angiogenic Responses in Lama4/−/− Mice.** To study the effect of laminin α4 chain deletion on angiogenesis, we implanted angiogenic factors in the mouse corneas. Because of the avascular nature of the corneal tissue, corneal neovascularization allowed us to determine the angiogenic response, the structure of newly formed blood vessels and the vascular remodeling. Six days after implantation, FGF-2 induced a robust angiogenic response in the corneas of wild-type mice (Fig. 1A). The FGF-2-induced corneal vessels appeared as well-organized vascular networks that budded from the limbal vessel growing toward the implanted pellet. These vessels are separated from each other, and they nearly reached to FGF-2-containing pellets. By day 42, nearly all FGF-2-induced corneal vessels had regressed, with only a few thin vessels remaining (Fig. 1C). Similar to the earlier report (16), the same amount of FGF-2 (80 ng) induced an enhanced angiogenesis in Lama4/−/− mice 6 days after growth factor implantation (Fig. 1B). This newly formed vasculature consisted of multiple capillary branches growing in different directions. Unlike the well-organized vasculature in the wild-type mice, the unregulated growth of capillaries/microvessels in the corneal tissue of Lama4/−/− mice led to a “red” appearance, mainly because of the large number of microvessels and vessel dilation. Interestingly, these malformed corneal vascular networks underwent dramatic remodeling with increasing time. By day 42, the malformed vascular plexuses were reorganized into a well-defined vascular tree-like structure with the leading edge growing around the pellet, and the vessels appeared to have markedly larger diameters than in the wild-type mice (Fig. 1D). These results confirm that deletion of laminin α4 chain in mice results in an enhanced angiogenesis response, and demonstrate increased long-term stability of the experimentally induced corneal vessels.

**Accelerated Primary Tumor Growth and Tumor Neovascularization in Lama4/−/− Mice.** Previous studies (11) and the present result from corneal neovascularization in Lama4/−/− mice suggested that laminin-8 might also play a critical role in pathological angiogenesis. To test this hypothesis, we studied tumor growth and tumor neovascularization in Lama4/−/− mice. To study the role of the laminin α4 chain in tumor angiogenesis, tumor growth and metastasis, murine Lewis lung carcinoma cells were inoculated s.c. into Lama4/−/− mice or their littermate controls. Tumor nodules became visible at 6 days after implantation and thereafter. Three weeks after implantation, tumor volumes were significantly larger in Lama4/−/− mice (2.23 ± 0.24 cm^3, mean ± SE; n = 8) than in control mice (0.80 ± 0.24 cm^3, mean ± SE; n = 10; P < 0.01; Fig. 2, A–C). No difference was observed between wild-type and heterozygous mice. Similar results were obtained in two independent experiments. In addition, accelerated tumor growth in Lama4 null mice was also observed when B16-F10 melanoma cells were inoculated s.c. into Lama4/−/− mice or their littermate controls. Tumor nodules became visible at 6 days after implantation and thereafter. Three weeks after implantation, tumor volumes were significantly larger in Lama4/−/− mice (2.23 ± 0.24 cm^3, mean ± SE; n = 8) than in control mice (0.80 ± 0.24 cm^3, mean ± SE; n = 10; P > 0.05; Fig. 2, D and E). No difference was observed between wild-type and heterozygous mice. Similar results were obtained in two independent experiments. In addition, accelerated tumor growth in Lama4 null mice was also observed when B16-F10 melanoma cells were inoculated s.c. (data not shown). Another interesting finding was that the tumors growing on Lama4/−/− mice seemed more solid and less necrotic than those from control mice (Fig. 2A).

Tumor cell proliferation, measured by BrdUrd incorporation, was not significantly different between Lama4/−/− mice and control mice [5.8% ± 0.6% (mean ± SD) and 5.6% ± 0.2% (mean ± SD); P > 0.05] before the difference in tumor volumes became significant. However, the apoptotic index of tumors from mutant mice [0.39% ± 0.15% (mean ± SD)] was significantly lower than that of tumor cells from littermate controls [0.62% ± 0.15% (mean ± SD); P < 0.05; Fig. 2, D and E]. High blood vessel density was found in tumors from Lama4/−/− mice as compared with controls [Fig. 2, F and G; n = 4; density in Lama4/−/−, 82.53 ± 19.94 vessels/mm^2].
from littermate controls. E, littermate control mice (H11569/H11569/H11021) staining of tumors. Less TUNEL staining of tumor blood vessels in Lama4 from mutant mice were more vascularized than those from controls. F, percentage of TUNEL-positive cells in Lama4/mice and control mice (mean ± SE; *, *; P < 0.01). G, quantification of tumor blood vessel area in Lama4/mice and their littermate control mice (**, P < 0.01)

(mean ± SD); density in controls, 56.47 ± 6.24 vessels/mm² (mean ± SD); P < 0.05). Similarly, the total blood vessel area was also significantly larger in mutant mice [6.37% ± 1.02% (mean ± SD)] than in controls [3.61% ± 0.44% (mean ± SD); Fig. 1G; P < 0.01]. The tumor blood vessels from Lama4 null mutant mice were more dilated. These results indicate that tumors in Lama4 null mice exhibited increased vascularity.

Increase of Tumor Metastasis in Lama4−/− Mice. Previously, the malformation of blood vessels in tumors had been considered as a structural basis for tumor metastasis. To study whether the deletion of the laminin α4 chain could contribute to tumor metastasis, we first performed an i.m. inoculation of 1 × 10⁶ B16-F10 melanoma cells. Three weeks after the inoculation, the lung was dissected and examined for metastatic colonies. Four (33%) of 12 mutant mice developed lung metastases, whereas none developed in 21 littermate controls. We further performed i.v. inoculation of 1 × 10⁵ tumor cells into the tail vein. The presence of lung metastases was examined 3 weeks later. As shown in Fig. 3, the number of lung-metastatic colonies was considerably higher in Lama4 null mice, in comparison with their littermate controls (Fig. 3A and B). Moreover, the sizes of individual metastatic colonies were larger in mutant mice, compared with those in control mice (Fig. 3A). These results suggest that the deletion of the laminin α4 chain facilitates the lung metastasis of tumor cells.

Aberrant Expression of Laminin α5 Chain in Tumor Vessels. To understand the potential mechanisms contributing to the enhanced angiogenesis and tumor growth in Lama4−/− mice, we examined the expression of other laminin types in tumor vessels. In addition to laminin α4 chain, laminin α5 chain has also been found to be expressed in the vascular endothelial basement membranes. Laminin-10, an α5 chain-containing laminin, has been reported to support adhesion and migration of endothelial cells (17). Double-immunofluorescence staining with antibodies against CD31 and laminin α4 or α5 chains clearly showed the peri-endothelial distribution of both the α4 and the α5 chains (Fig. 4, B and C). As expected, laminin α4 expression was not detectable in tumors from Lama4 null mice (Fig. 4A), whereas this chain was prominent in the blood vessels of tumors from control mice (Fig. 4B). Laminin α5 chain expression was mainly seen in the smooth muscle cells that surrounded larger blood vessels in tumors grown in control mice, although a weak staining was also observed in a subset of the capillaries (Fig. 4D). In contrast, strongly positive immunoreactivity for the α5 chain was observed in most of the capillaries and larger tumor blood vessels of mutant mice, indicating that this chain, at least partially, compensated for the loss of laminin α4 in these tissues (Fig. 4C). Similar to our previous report in muscle (11), immunoreactivity for collagen IV, the major structural

Fig. 3. Tumor metastasis in Lama4−/− and control mice (wt, wild-type). A, representative lung metastasis in Lama4−/− mice and controls 3 weeks after injection of B16-F10 cells into the tail vein. B, numbers of lung surface metastatic colonies in Lama4−/− and control mice 3 weeks after injection of B16-F10 cells into the tail vein.

LAMININS AND TUMOR ANGIOGENESIS

Fig. 2. Tumor growth and angiogenesis in Lama4−/− and control mice. A, gross morphology of Lewis lung carcinoma (LLC) tumors dissected from a Lama4−/− mutant mouse and its littermate control 3 weeks after inoculation. Tumors grew faster and were less necrotic in Lama4−/− mice, compared with tumors in littermate controls. B, growth curve of LLC tumors from 8 Lama4−/− mice and 10 littermate controls. Significant size differences were observed from day 13 onwards (mean ± SE; P < 0.05). C, tumor weight at day 28 after inoculation (mean ± SE; **, P < 0.01). D, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) staining of tumors. Less TUNEL-positive cells are observed in tumors from Lama4−/− mice than from littermate controls. E, percentage of TUNEL-positive cells in Lama4−/− and control mice (mean ± SE; *, **; P < 0.05). F, CD31 staining of tumor blood vessels in Lama4−/− mice and controls. Tumors from mutant mice were more vascularized than those from controls. G, quantification of tumor blood vessel area in Lama4−/− mice and their littermate control mice (**, P < 0.01)
shown). No differences in perlecan immunostaining were observed.

Immunofluorescence (data not shown). Neither laminin $(\lambda_4\lambda_5)$ nor laminin $(\lambda_4\lambda_5)$ localization between wild-type and null mice was revealed by 5 antibodies (11) did not show laminin $\alpha_5$ localization in the blood vessels of newborn $\lambda_4\lambda_5$ null mice and no up-regulation of the laminin $\alpha_5$ mRNA in adult tissues using Northern blot analysis (11). This difference is likely because of the different ages of mice studied, because we found that the up-regulation of laminin $\alpha_5$ chain was more prominent in 6 day-old mutant mice than in the newborn mutant mice. The absence of an up-regulated laminin $\alpha_5$ mRNA in adult tissue of laminin $\alpha_4$ null mice is probably because blood vessels composed only a fraction of the organs. The up-regulated laminin $\alpha_5$ chain was observed only in vascular basement membranes, not in the basement membranes surrounding the muscle fibers.

In adult mutant mice, laminin $\alpha_5$ chain was readily detected in tumor vascular basement membranes (Fig. 4). The presence of $\alpha_5$ chain may, to some extent, contribute to the increased angiogenesis; an in vitro study using recombinant laminins showed that endothelial cells migrate faster on laminin-10 ($\alpha_5$ chain-containing laminin) than on laminin-8 ($\alpha_4$ chain-containing laminin; Ref. 17). The defective structure of the vascular basement membranes and/or the enhanced angiogenesis per se is more likely to play the major role in the accelerated tumor growth and metastasis. It is tempting to speculate that the increased number of blood vessels in the tumors in $\lambda_4\lambda_5$ null mice simply resulted in a better nutrient supply to the growing tumors. This possibility is supported by the reduction in necrosis observed in tumors of laminin $\alpha_4$ null mice. However, differences in basement membrane composition of vascular endothelium may also directly affect the endothelial cells or surrounding tumor cells, or may have an indirect effect by altering the concentration and/or availability of growth factors. Interestingly, when tumors growing in $\lambda_4\lambda_5$– and wild-type mice were compared, apoptosis was reduced in the tumors of the null mice whereas there was no difference in cell proliferation, which may reflect selective retention of survival factors by laminin $\alpha_5$. In conclusion, loss of laminin $\alpha_4$ chain results in earlier than normal deposition of laminin $\alpha_5$ chain in the endothelial basement membranes, increased angiogenesis, accelerated tumor growth, and metastasis.

**Acknowledgments**

We thank Ulla Mikkonen, Maria Laisi, and Dadi Niu for excellent technical support.

**References**


![Fig. 4. Expression of laminin $\alpha$ chains in tumor and brain. Double immunofluorescent staining of blood vessels using antibodies against CD31 together with either laminin $\lambda_4$ or $\lambda_5$ chain. A–D, expression of laminin $\alpha_4$ and $\alpha_5$ chains in Lewis lung carcinoma (LLC) tumors. In $\lambda_4\lambda_5$–/– mice, tumor blood vessels are negative for the $\alpha_4$ chain (A) and positive for the $\alpha_5$ chain (B) covering the endothelium. In control mice, tumor blood vessels stained positive for $\alpha_4$ (B), whereas a subset, mainly in the mature blood vessels, stained for the $\alpha_5$ chain (D). Expression of laminin $\alpha_5$ chain could also be detected in LLC tumor cells (C). E–H, Expressions of laminin $\alpha_4$ and $\alpha_5$ chains in the vascular basement membranes of brain from a 6-day-old mutant and its littermate control. Superimposed images of double staining of blood vessels stained with CD31 (green) and laminin $\alpha_4$ or $\alpha_5$ (red). Basement membranes of blood vessels in a mutant mouse are negative for the $\alpha_4$ chain (E) but positive for the $\alpha_5$ chain (G). In a control mouse, staining for $\alpha_5$ chain is much weaker compared with that in mutant mouse (H), but staining for $\alpha_4$ is positive (B).]


Deletion of Laminin-8 Results in Increased Tumor Neovascularization and Metastasis in Mice

Zhongjun Zhou, Masayuki Doi, Jianming Wang, et al.

Cancer Res 2004;64:4059-4063.