Develop. Growth Differ. (2008) **50**, 97–107 doi: 10.1111/j.1440-169x.2007.00979.x

Cryptic fragment α4 LG4-5 derived from laminin α4 chain inhibits de novo adipogenesis by modulating the effect of fibroblast growth factor-2

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Cleavage of the extracellular matrix (ECM) by proteolysis unmasks cryptic sites and generates novel fragments with biological activities functionally distinct from those of the intact ECM molecule. The laminin G-like (LG)4-5 fragment has been shown to be excised from the laminin α4 chain in various tissues. However, the functional role of this fragment has remained unknown to date. To investigate this, we prepared α4 LG1-3 and α4 LG4-5 fragments by elastase digestion of recombinant α4 LG1-5, and examined their effects on de novo adipogenesis in mice at the site of injection of basement membrane extract (Matrigel) and fibroblast growth factor (FGF)-2. Although the addition of whole α4 LG1-5 suppressed adipogenesis to some extent, the α4 LG4-5 fragment could strongly suppress adipogenesis at a concentration of less than 20 nM. Addition of the α4 LG4 module, which contains a heparin-binding region, had a suppressive effect, but this was lost in mutants with reduced heparin-binding activity. In addition, antibodies against the extracellular domain of syndecan-2 and -4, which are known receptors for the α4 LG4 module, suppressed adipogenesis. Thus, these results suggest that the cryptic α4 LG4-5 fragment derived from the laminin α4 chain inhibits de novo adipogenesis by modulating the effect of FGF-2 through syndecans.

Key words: extracellular matrix, fibroblast growth factor-2, heparin, Matrigel, syndecan.

Introduction

The extracellular matrix (ECM) is a 3D structure consisting of various molecules, such as collagens, laminins, fibronectin, and proteoglycans (PGs), and is important not only as a structural scaffold, but also as an instructive environment for tissues and cells. The ECM transmits signals from the extracellular environment to cells and *vice versa* to control cell migration, proliferation, differentiation and survival (Boudreau & Bissell 1998; Lukashev & Werb 1998; Ruoslahti 1999). Cells use several receptors to interact with the ECM, including integrins and cell surface PGs (Larsen *et al*. 2006). During tissue remodeling, the composition of the ECM and its cellular receptors are altered in a number of ways. Furthermore, tissue remodeling may result in alterations in existing ECM proteins. ECM components are known to contain cryptic domains, which are exposed by proteolysis or conformational changes, and which elicit biological responses that are not observed with the intact molecule (Davis *et al*. 2000; Schenk & Quaranta 2003; Bix & Iozzo 2005). To date, many cryptic fragments have been found in various ECM molecules. For example, the intact non-collagenous (NC1) domain of collagen XVIII does not inhibit angiogenesis, but endostatin, a globular 20 kDa fragment derived from the C-terminal NC1 domain of collagen XVIII, inhibits tumor growth and angiogenesis by inducing apoptosis in endothelial cells (O'Reilly *et al*. 1997). Tumstatin, a 28 kDa fragment derived from the NC1 domain of collagen IV, can inhibit tumor growth by blocking protein synthesis via αVβ3 integrin in endothelial cells (Maeshima *et al*. 2002).

Laminins consist of three subunits, α, β, and γ chains. Currently, five α, three β, and three γ chains

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Received 10 October 2007; revised 12 November 2007; accepted 13 November 2007.

have been identified, and 16 heterotrimers of different combinations of each subunit have been described (Aumailley *et al*. 2005). All five α chains contain a large C-terminal globular domain (called the G domain) consisting of five laminin G-like (LG) modules (LG1 to LG5). These modules of 180–200 amino acid residues show up to 45% amino acid sequence identity. Altogether, 400 LG modules have been proposed in diverse families of extracellular and transmembrane proteins, such as perlecan, agrin and neurexin I (Timpl *et al*. 2000; Rudenko *et al*. 2001). The LG modules of laminin α chain have been shown to function as binding sites for various cell surface receptors, including integrins, dystroglycan, and syndecans (Ruoslahti 1999; Utani *et al*. 2001; Ido *et al*. 2004; Narita *et al*. 2004; Yamashita *et al*. 2004; Kikkawa *et al*. 2007).

Among laminin isoforms, laminin-411 ($\alpha4\beta1\gamma1$) was first identified in endothelial cells (Aratani & Kitagawa 1988; Tokida *et al*. 1990) and 3T3-L1 preadipocytes (Niimi *et al*. 1997). Expression of laminin-411 was enhanced during adipose conversion in 3T3-L1 cells (Niimi *et al*. 1997). It has recently been shown that the laminin α4 chain is widely expressed in various mesodermal tissues, including skeletal muscle, smooth muscle, heart and endothelium (Petajaniemi *et al*. 2002). Recent interest in the LG module of laminin α4 chain has focused on its processing and role *in vivo*. We previously showed that the α4 LG4-5 fragment seemed to be released from intact α4 LG1-5 by unidentified protease(s) during purification from the conditioned medium of cultured cells (Yamaguchi *et al*. 2000). We also showed that the G domain of the laminin α4 chain has a higher affinity for heparin than any other laminin α chain, and that the α4 LG4 module has the ability to bind to cells through syndecans (Yamashita *et al*. 2004). Other groups have also indicated that the α4 LG4-5 fragment is proteolytically cleaved *in vivo,* but is not retained within the basement membranes of capillary wall, adipose and neural tissues (Talts *et al*. 2000). Matsuura *et al*. (2004) showed that the epitope recognized by an antibody against the α4 LG4-5 region was located in the basement membrane zone of capillary vessels and in an area adjacent to fibroblast-like cells. Despite increasing information about the *in vivo* processing of the LG domain, the significance of this processing and the role of the α4 LG4-5 fragment *in vivo* still remain unclear.

In the present study, we report that the α4 LG4-5 fragment derived from the laminin α4 chain inhibits de novo adipogenesis. When added to a mixture of Matrigel and fibroblast growth factor (FGF)-2 for subcutaneous injection into nude mice, the α4 LG4-5 fragment efficiently blocked de novo adipogenesis at a concentration of less than 20 nM. This inhibitory effect of the α4 LG4-5 fragment was stronger than those of the α4 LG1-5 and α4 LG1-3 fragments. The α4 LG4 module, which is the heparin-binding region within α4 LG4-5, also suppressed adipogenesis, but α4 LG4 mutants without heparin-binding activity did not. In addition, an antibody against the extracellular domain of syndecan, which is a cell surface receptor of the α4 LG4 module, also blocked adipogenesis. Therefore, we conclude that the cryptic α4 LG4-5 fragment generated by elastase digestion modulates the effect of FGF-2 mainly via its heparin-binding region, and leads to the suppression of de novo adipogenesis.

Materials and methods

Reagents

Porcine pancreas elastase (ES438; Elastin Products Company, Owensville, MO, USA), FGF-2 (R & D Systems, Minneapolis, MN, USA), heparin (Novo Nordisk, Bagsværd, Denmark), and antithrombin III (Wako, Osaka, Japan) were obtained from commercial sources. The monoclonal antibodies 8G3, recognizing the cytoplasmic domain of human syndecan-4, and 10H4, recognizing the extracellular domain of human syndecan-2, were generous gifts from Dr Guido David (University of Leuven, Belgium) (Lories *et al*. 1989). The monoclonal antibody 5G9, recognizing the extracellular domain of human syndecan-4, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of recombinant laminin fragments

Recombinant mouse α4 LG1-5 was overexpressed in Chinese hamster ovary (CHO) cells and purified by heparin-column chromatography as described previously (Yamaguchi *et al*. 2000). α4 LG1-3 and α4 LG4-5 fragments were obtained by digestion of α4 LG1-5 with porcine pancreas elastase. Recombinant α4 LG1-5 (330 μg/mL) was incubated in 10 mM Tris-HCl (pH 7.4) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (buffer A) with elastase at 2.7 μg/mL for 2 h at 37°C. Digest products were applied to a 1 mL Hi-Trap heparin column (GE Healthcare, Piscataway, NJ, USA). After washing the column with 5 mL of buffer A, bound protein was eluted with a 50 mL linear gradient of NaCl from 0 to 1 M in buffer A, and 1-mL fractions were collected. The purity and identity of α4 LG1-3 and α4 LG4-5 fragments were confirmed based on their affinity to the heparin column, N-terminal sequencing, and sizes

estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Glutathione s-transferase (GST)-fusion protein of the mouse laminin α4 chain LG4 module (residues 1453–1636) (Liu & Mayne 1996) and its site-directed mutants were prepared as described previously (Yamashita *et al*. 2004).

Preparation of Matrigel

Matrigel was prepared from Engelbreth-Holm-Swarm (EHS) sarcoma as described (Yamashita *et al*. 2004). Briefly, the EHS sarcoma was excised from mice, and homogenized in a buffer containing 3.4 M NaCl, 50 mM Tris-HCl (pH 7.4), 4 mM EDTA and 2 mM N-ethylmaleimide. After centrifugation, the precipitate was extracted for 12 h at 4°C with an equal volume of 2 M urea solution containing 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl, and centrifuged at 10 000 *g* for 30 min. The extract was dialyzed against 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl. Dialysate with a protein concentration of 18–20 mg/mL was adjusted to 10 mg/mL for injection.

De novo adipogenesis by injection of Matrigel plus FGF-2

All animal procedures were conducted under the approval of the Nagoya University Institutional Animal Care and Use Committee. After anesthetization with diethyl ether, Balb/c nude mice (5.5–6.5 weeks old) were injected with 100 μL of Matrigel with or without 1 μg/mL of FGF-2 and the additives specified in each experiment, subcutaneously, over the chest or at the boundary between chest and abdomen. Each mouse had four or five injections of Matrigel with additives. At 2 weeks after injections, mice were killed, and the transplants were excised for histological analysis. Specimens were fixed for 12 h in 10% buffered formaldehyde, dehydrated, and then embedded in paraffin wax. Sections of 4 μm were stained with hematoxylin-eosin, and examined. Specimens were observed using an Axioskop photomicroscope (Carl Zeiss, Oberkochen, Germany).

Specimens with active adipogenesis showed positive Sudan IV staining as described previously (Kawaguchi *et al*. 1998). For the quantification of adipogenesis, assays for biochemical markers, such as accumulated triglycerides and glycerol 3-phosphate dehydrogenase activity, were used as alternatives. However, to determine the specific amount of adipogenic activity precisely, careful isolation of Matrigel plugs freed from surrounding tissues would have been critical. Instead, we carried out the following semiquantification of adipogenesis by estimating the width of the white area occupied by adipocytes in the Matrigel plug sections, from which accumulated triglycerides were removed during the fixation and dehydration of specimens. We found that this was a valid method for evaluating de novo adipogenesis since the space occupied by a Matrigel plug could be specified at the microscopic level and the histological analysis is well correlated with the results from above assays for biochemical markers (Kawaguchi *et al*. 1999). A similar method was reported to evaluate *in vivo* formation of adipose tissue in rat (Hiraoka *et al*. 2006).

Semiquantification of de novo adipogenesis

For semiquantification of adipogenesis, eight to 10 Matrigel plugs were prepared for every combination of additives. Sections of paraffin-embedded specimens from the centers of Matrigel plugs were selected, and color images of the sections were captured using a Leica DC300 digital camera (Wetzlar, Germany) in order to cover the whole area of each Matrigel plug. The images were analyzed using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, CA, USA). In brief, color images were first converted to gray scale, the brightness and contrast were then adjusted, the signal of each pixel was determined to be either white or black, and the proportion of white pixels within the whole image of a Matrigel plug was used as an index of adipogenesis. For this analysis, many images covering one Matrigel plug were analyzed and the data were combined. Indices from four or five Matrigel plugs with the same combination of additives were statistically evaluated. The age of mice, ranging from 5.5 to 6.5 weeks old at the time of injection, Matrigel preparations with potential contamination from various growth factors, the staining efficiency of the sections, adjustment of the brightness and contrast of the microscopic images, and other variables, were possible arbitrary factors different among experiments. To make the results comparable, positive (Matrigel plus FGF-2) and negative (Matrigel alone) injections were included in all experiments. All experiments were independently repeated at least twice. The *P*-values were obtained using the Student's *t*-test.

Results

Preparation of elastase-generated fragments from the LG1-5 region of the laminin α4 chain

To analyze the function of the α4 LG4-5 fragment *in vivo*, we first prepared α4 LG1-3 and α4 LG4-5

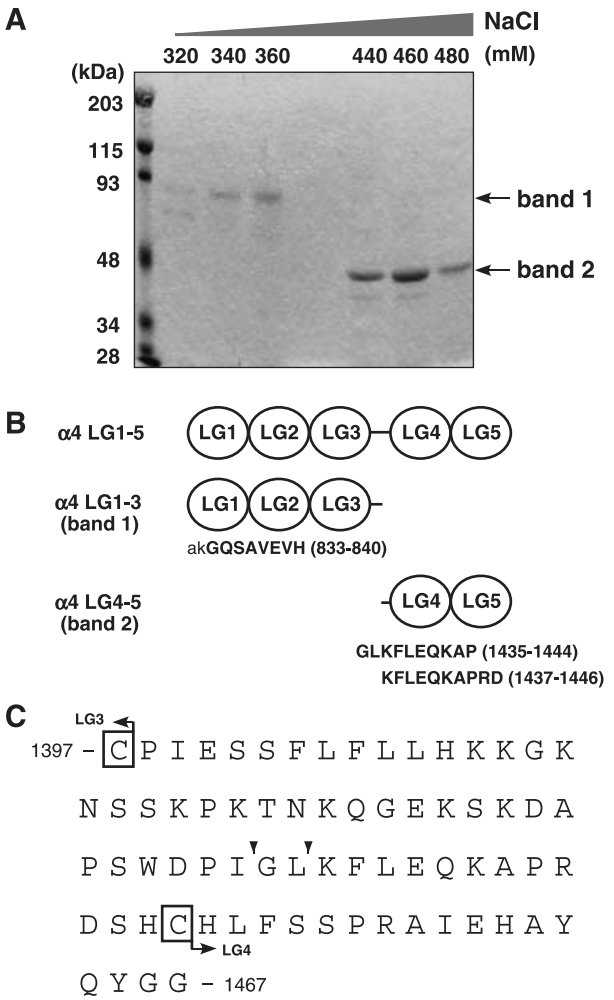

Fig. 1. Purification of elastase-digested fragments from recombinant laminin α4 laminin G (LG1-5) domain. (A) Recombinant mouse laminin α4 LG1-5 was digested with elastase and the digested fragments were purified by heparin column chromatography. Fractions eluted at the NaCl concentration indicated at the top were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the gel was stained with Coomassie Brilliant Blue. Marker size is shown at the left. (B) Schematic modular structures of α4 LG domains and the N-terminal amino acid sequences of the elastase-digested fragments (bands 1 and 2). (C) Amino acid sequence of the linker region between LG3 and LG4 of mouse laminin α4 chain. The linker region starts after cysteine 1397 and ends at cysteine 1450. The end of α4 LG3 and the beginning of α4 LG4 are shown by the bent arrow. Estimated elastase cleavage sites in the linker region are indicated by the arrowhead.

fragments by elastase digestion of the recombinant laminin α4 LG1-5 protein (Fig. 1A). The α4 LG1-3 fragment (band 1) was obtained as a 75 kDa polypeptide eluted from a heparin-affinity column at a NaCl concentration of 360 mM, whereas the α4 LG4-5 fragment (band 2) was obtained as a 42 kDa polypeptide at 460 mM NaCl, which is the same concentration as that for elution of the α4 LG1-5 protein (Yamaguchi *et al.* 2000). N-terminal amino acid sequencing of band 1 revealed a sequence of akGQSAVEVH corresponding to a start at residue 833, which is localized in the LG1 module. The two N-terminal residues in this fraction, 'ak', showed that the signal sequence of the erythropoietin receptor, equipped in the expression vector for secretion of recombinant products, remained without *in vivo* cleavage. Sequencing of band 2 gave two sequences, GLKFLEQKAP corresponding to a start at residue 1435, and KFLEQKAPRD corresponding to a start at residue 1437, suggesting that band 2 comprised a mixture of fragments cleaved at two sites close to each other within a hinge region between LG3 and LG4 modules (Fig. 1B,C). These results also suggested that elastase is one of the candidate proteases for the specific cleavage of the laminin α4 G domain *in vivo*.

The laminin α4 LG4-5 fragment suppresses de novo adipogenesis

We have previously shown that subcutaneous injection of Matrigel in combination with FGF-2 induced migration of endogenous stem cells into the Matrigel space, and that these proliferate and differentiate to generate adipose tissue de novo (Yamaguchi *et al.* 2000). In this process, invading endothelial cells first formed a microcapillary network within 3 days; preadipocytes migrated to the vicinity of microcapillaries and then accumulated intracellular lipid droplets within a week (Toriyama *et al.* 2002). These processes were strictly dependent on the addition of FGF-2 to the Matrigel.

Using this model system of de novo adipogenesis, we explored a potential function of the α4 LG4-5 fragment in the modulation of growth factor signaling. When the α4 LG4-5 fragment was added at a concentration of 800 nM, the Matrigel space observed in the section (panel α4 LG4-5 in Fig. 2A) remained quiescent in contrast to abundant differentiation of adipocytes in the positive control section (panel one in Fig. 2A), induced by injection of only Matrigel plus FGF-2. We quantified such suppression of de novo adipogenesis by image analysis of photographs of Matrigel space sections, to calculate the percentage of the total Matrigel area consisting of white area occupied by adipocytes (see Materials and methods). When this semiquantification was carried out over four Matrigel plugs formed by independent injection, the average percentage area that was white was as low as that in the negative control, with injection of Matrigel alone (Fig. 2B). When the concentration of

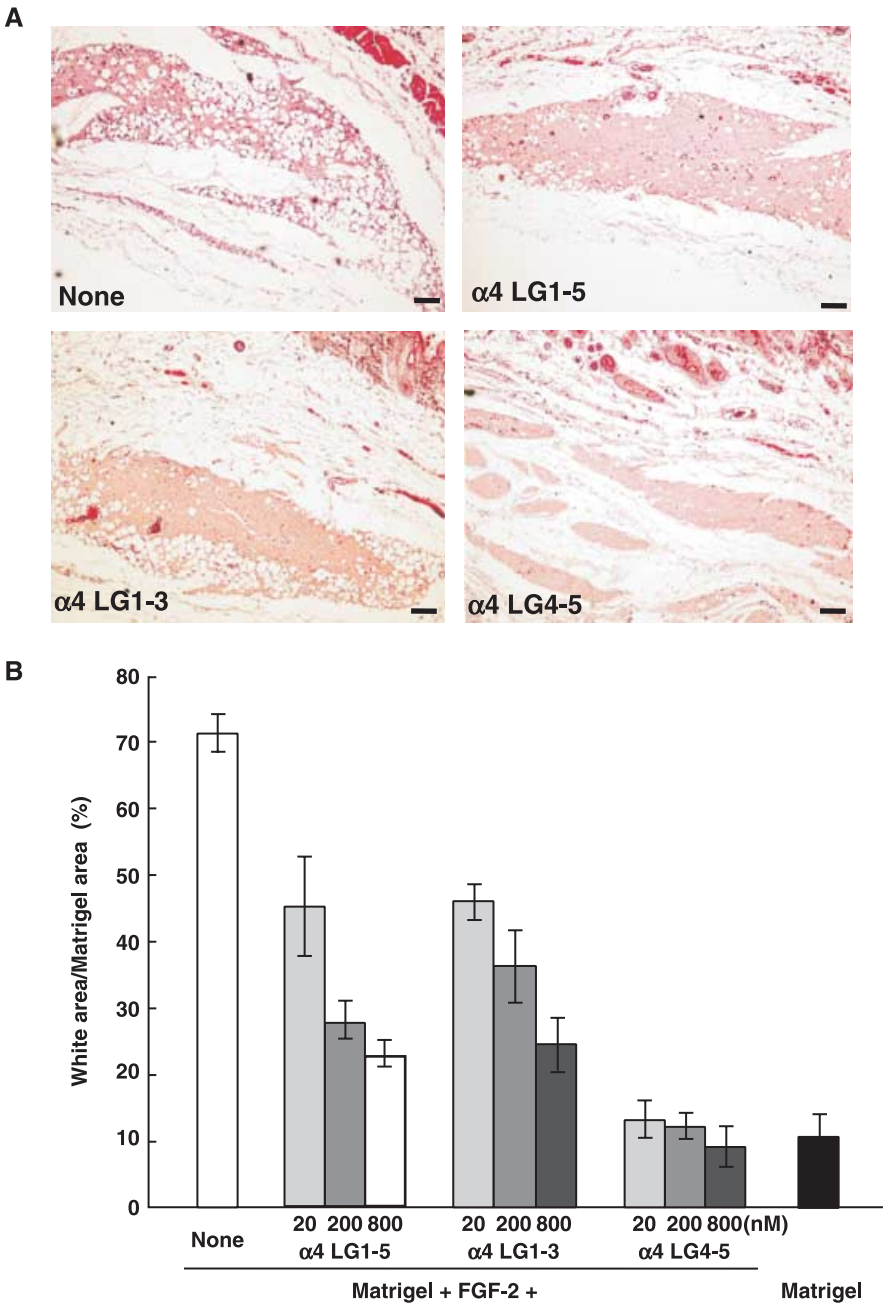

Fig. 2. Suppression of de novo adipogenesis at the site of injection of Matrigel plus fibroblast growth factor (FGF)-2 by laminin α4 chain G domain fragments. Matrigel with or without FGF-2 was injected subcutaneously into Balb/c nude mice after adding the indicated concentration of laminin α4 G domain fragments. Two weeks later, Matrigel plugs were excised, fixed, and sectioned for staining with hematoxylin-eosin. (B) Representative color images of the sections of Matrigel plugs with added FGF-2 (1 μg) alone (none), FGF-2 plus α4 laminin G (LG1-5) (800 nM), FGF-2 plus α4 LG1-3 fragment (800 nM), and FGF-2 plus α4 LG4-5 fragment (800 nM) are shown. The scale bars are 100 μm (B) The white area of Matrigel space was semiquantified as detailed in the Materials and methods. The data are expressed as the mean values ± standard deviation of at least four separate Matrigel plugs.

the α4 LG4-5 fragment was reduced to 20 nM, the white area increased, but remained far below that in the positive control (Fig. 2B). The α4 LG1-3 fragment was also suppressive, but the suppression at 800 nM α4LG1-3 was weaker than that seen with α4 LG4-5 fragment injection at 20 nM (Fig. 2B). Interestingly, the suppressive effect of α4 LG1-5 was as weak as that of the α4 LG1-3 fragment, in spite of α4 LG1-5 containing an LG4-5 region. This suggested that the strong suppressive activity of the α4 LG4-5 fragment was cryptic in the structure of whole α4 LG1-5.

Suppression of de novo adipogenesis by laminin α4 LG4-5 fragment is due to heparin-binding activity of the LG4 module

Using GST-fused laminin α4 LG1, LG2, LG4 and LG5 modules (GST-fused LG3 module was unstable), we have previously shown that only the LG4 module has adhesive activity for several cell lines by direct interaction with cell surface syndecans (Yamashita *et al.* 2004). Furthermore, using a series of GST-fused α4 LG4 mutants, in which each of the 27 basic residues were

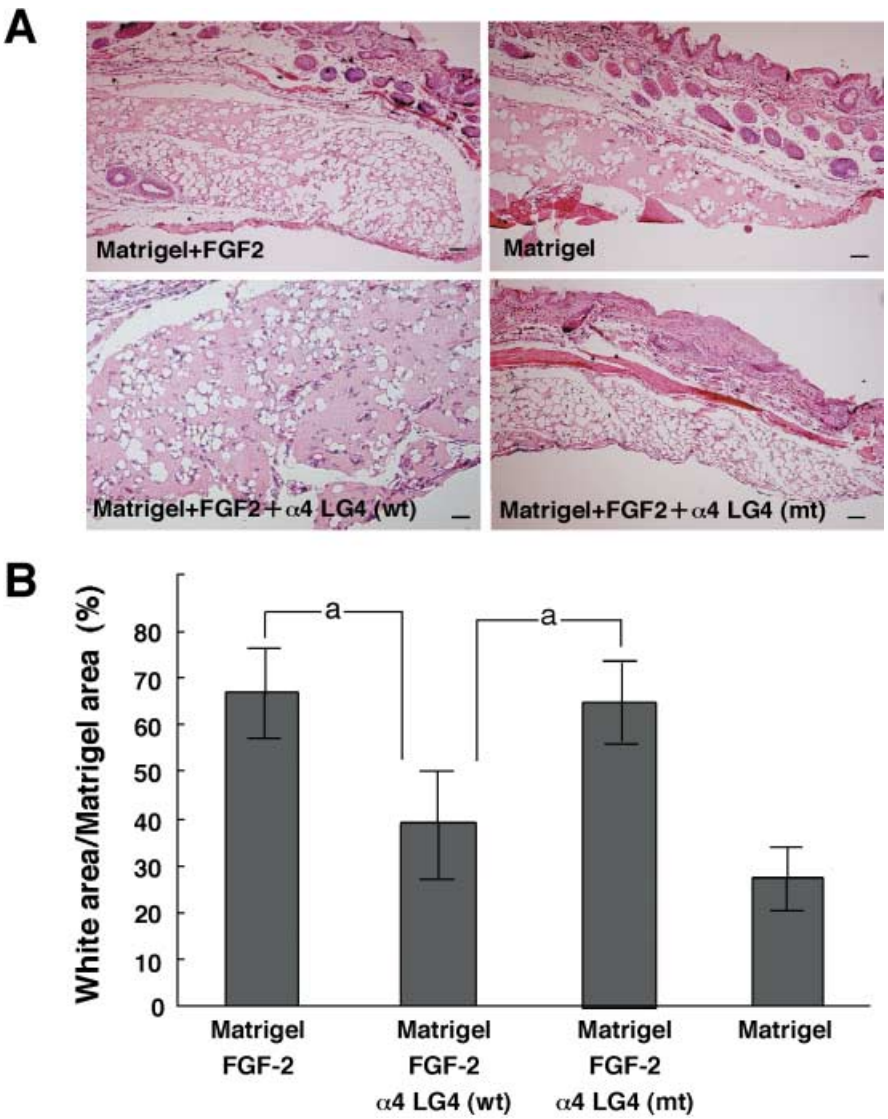

Fig. 3. Suppression of de novo adipogenesis by glutathione s-transferase (GST)-fused laminin α4 chain LG4 module and heparin-binding mutant. Recombinant GST-fused α4 LG4 module (wt) or its mutant (mt) was added to Matrigel plus fibroblast growth factor (FGF)-2 at 800 nM, and injected into mice in parallel with Matrigel plus FGF-2 or Matrigel alone. Other details are as in Figure 2. The values connected by 'a' were statistically different at $P < 0.01$.

replaced one by one with alanine, we specified R1520, K1531, K1533 and K1539 (R: arginine; K: lysine) to be the crucial amino acids for high affinity heparin-binding of the α4 LG4 module (Yamashita *et al.* 2004).

Taking advantage of these GST-fused proteins, we examined whether the suppression of de novo adipogenesis by the laminin α4 LG4-5 fragment is due to the heparin-binding activity of the LG4 module. Although the suppressive effect of α4 LG4 module was weaker than that of the α4 LG4-5 fragment, the addition of the GST-fused α4 LG4 module at 800 nM to Matrigel plus FGF-2 caused clear suppression of adipogenesis (Fig. 3). When a mutant of GST-fused α4 LG4 module (K1539A; A: alanine) with reduced heparin-binding activity was added, the suppression was diminished (Fig. 3). A similar result was obtained with another mutant (K1533A) (data not shown). Further addition of heparin at 50 μg/mL counteracted the suppressive effect of the GST-fused α4 LG4 module (Fig. 4). Since the addition of heparin to Matrigel plus FGF-2 or to Matrigel alone showed no effect, we can conclude that the counteraction was not due to an indirect effect of heparin. The addition of antithrombin III, which is known to be one of the strongest heparin-binding proteins (Ersdal-Badju *et al.* 1997), at only 1 nM to Matrigel plus FGF-2, strongly suppressed adipogenesis; this effect was counteracted by further addition of heparin (Fig. 4). Taken together, these results suggested that the suppression of de novo adipogenesis by α4 G domain fragments was due to the heparin-binding activity of the LG4 module.

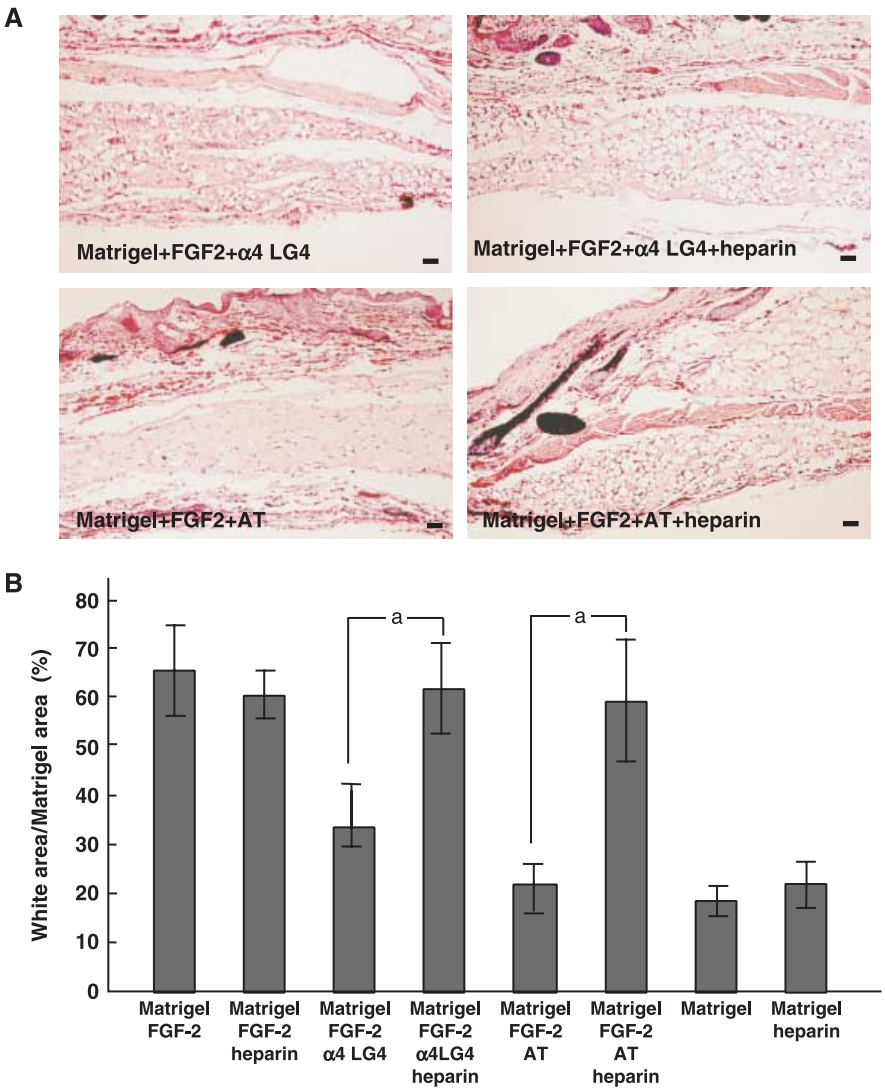

Fig. 4. Suppression of de novo adipogenesis by antithrombin III and its counteraction by heparin. Antithrombin III (AT; 1 nM), glutathione s-transferase (GST)-fused laminin α4 laminin G (LG4) (800 nM), and/or heparin were added to Matrigel plus fibroblast growth factor (FGF)-2 in the indicated combinations, and injected into mice in parallel with Matrigel plus FGF-2 or Matrigel alone. Other details are as in Figure 2. The values connected by 'a' were statistically different at $P < 0.01$.

Antibodies against the extracellular, but not the cytoplasmic domain of syndecan-2 and -4 suppress de novo adipogenesis

The relevance of the heparin-binding activity of α4 G domain fragments to the suppression of de novo adipogenesis suggests that these fragments bind to heparan sulfate (HS)/HSPGs in the ECM or at the cell surface. In the former case, the binding may affect the prevention of the degradation or sequestration of FGF-2, thereby altering its activity. In the latter case, it may compete with FGF-2 and consequently block FGF-2 signaling via its receptor. Therefore, we took advantage of antibodies against the extracellular domain of syndecan-2 or -4 and the cytoplasmic domain of syndecan-4 to investigate whether cell surface syndecans are involved in de novo adipogenesis. Addition of antisyndecan-2 or -4 extracellular domain to Matrigel plus FGF-2 weakly suppressed adipogenesis, whereas the addition of antisyndecan-4 cytoplasmic domain did not (Fig. 5). These results suggested that the free extracellular domain of syndecans was indispensable for de novo adipogenesis.

Discussion

The main proteases to cleave ECM molecules are secreted and membrane-type matrix metalloproteases (MMPs), tolloid (TLD) metalloproteinases and bone morphogenetic protein (BMP)-1 (Davis *et al.* 2000; Schenk & Quaranta 2003; Bix & Iozzo 2005). Additionally, cysteine, aspartic and serine protease families play a key role in ECM degradation during tumor invasion and tissue remodeling. According to our

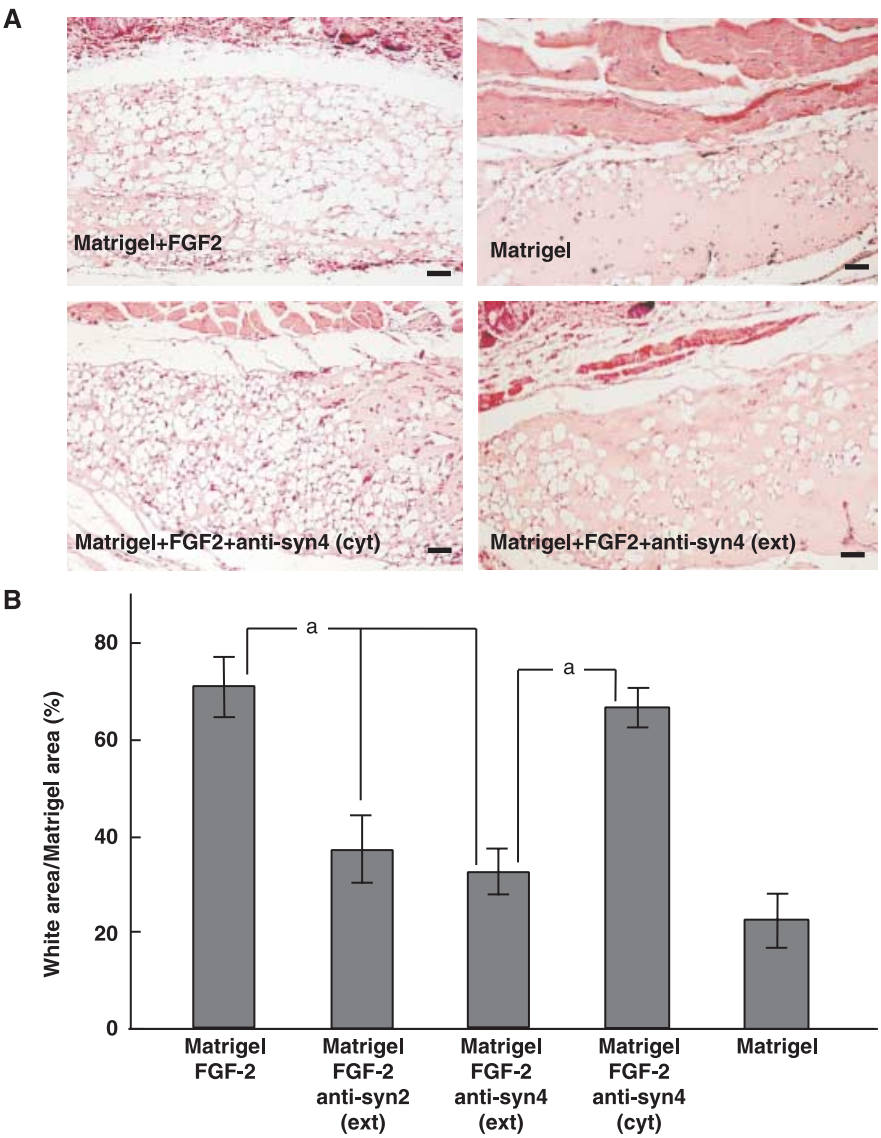

Fig. 5. Extracellular domain of syndecan-2 or -4 is required for de novo adipogenesis. Monoclonal antibodies against syndecan-2 extracellular [antisyn2 (ext)], syndecan-4 extracellular [antisyn-4 (ext)] or syndecan-4 cytoplasmic domain [antisyn-2 (cyt)] were added to Matrigel plus fibroblast growth factor (FGF)-2 at dilutions of 1 : 500, and injected into mice in parallel with Matrigel plus FGF-2 or Matrigel alone. Other details are as in Figure 2. The values connected by 'a' were statistically different at $P < 0.01$.

previous study, the laminin α4 LG4-5 region appeared to be processed by unidentified protease(s) from recombinant laminin α4 LG1-5 protein in cell culture medium (Yamaguchi *et al*. 2000). Another study also reported that the laminin α4 LG4-5 fragment could be proteolytically cleaved off *in vivo* from the main body of the laminin α4 chain (Talts *et al*. 2000). In the present study, we demonstrated that a laminin α4 LG4-5 fragment could be generated specifically by elastase, which is classified in the serine protease family, from laminin α4 G domain *in vitro*. Further studies are required to identify the protease(s) that is responsible for the cleavage of laminin α4 G domain *in vivo*.

De novo adipogenesis is induced reproducibly by subcutaneous injection of Matrigel and FGF-2 over the chest, lateral abdomen or head (Kawaguchi *et al*. 1998). In this system, it is thought that Matrigel can prevent FGF-2 from degradation, and stable FGF-2 can easily bind to the FGF receptor, thereby enhancing neovascularization and differentiation of endogenous adipose precursor cells and mesenchymal stem cells into mature adipocytes (Toriyama *et al*. 2002). Neubauer *et al*. (2004) showed that FGF-2 induces adipogenesis of mesenchymal stem cells by increasing peroxisome proliferator-activated receptor (PPAR)-γ, a key transcription factor involved in adipogenesis. Therefore, we examined laminin α4-derived fragments for the suppression of de novo adipogenesis. Based on semiquantification of adipogenesis, we can assign the following relative activities to these fragments: α4 LG4-5 > α4 LG1-5 = α4 LG1-3. Interestingly, the

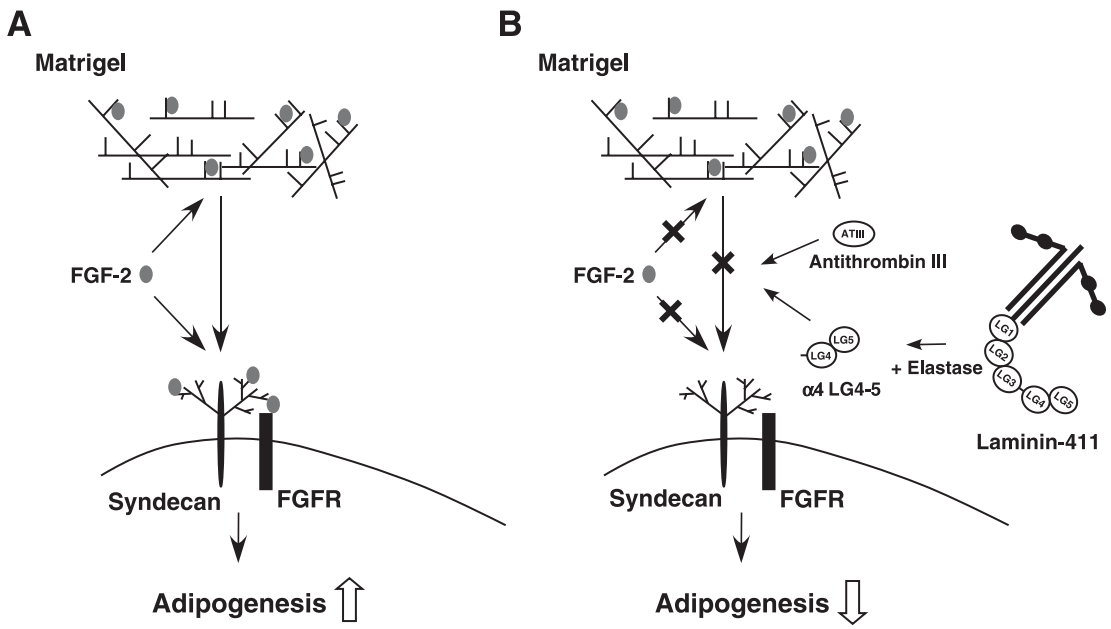

Fig. 6. Model of the inhibitory effect of laminin α4 LG4-5 on de novo adipogenesis. (A) Fibroblast growth factor (FGF)-2 released from Matrigel would bind to both cell surface *HSPG*, syndecan, and FGF receptor (FGFR) on preadipocytes, endothelial cells or mesenchymal stem cells to induce de novo adipogenesis. (B) The cryptic fragment α4 LG4-5, derived from the laminin α4 chain, blocks the interaction between FGF-2 and syndecan, and inhibits de novo adipogenesis.

suppressive activity of the α4 LG4-5 fragment was about threefold stronger than that of parental α4 LG1-5 and the α4 LG1-3 fragment. Although α4 LG1-5 and the α4 LG1-3 fragment also had some weak suppressive activity on adipogenesis, this effect required higher concentrations of 200 or 800 nM compared with the α4 LG4-5 fragment, which was effective even at a concentration of 20 nM. This suggests that the cryptic domain in α4 LG4-5 was exposed by elastase digestion of α4 LG1-5.

The suppressive effect of α4 LG4 was lower than that of the α4 LG4-5 fragment. This indicates the possibility that another region within α4 LG4-5, apart from the LG4 module, is also involved in the suppressive effect. In fact, there are two distinct differences between the α4 LG4-5 fragment and the α4 LG4 module used in this study. First, the α4 LG4-5 fragment contains an additional 13–15 amino acids derived from the linker region between LG3 and LG4 modules. Second, the α4 LG4-5 fragment has a structure arranged in a V-shaped fashion between LG4 and LG5 modules (Harrison *et al*. 2007). Interestingly, such a structure of α4 LG1-2 has been shown to interact with αVβ3 integrin (Gonzalez *et al*. 2002). Therefore, we can not rule out the possibility that the integrin-binding ability of the α4 LG4-5 fragment may be involved in the suppressive effect. Although αVβ3, α6β1 and α3β1 integrins are so far known to bind to the laminin α4 chain (Fujiwara *et al*. 2001), the binding sites for α6β1 and α3β1 integrins have not yet been clearly identified. Other cryptic fragments are known to bind to integrin families. For example, endostatin binds to α5β1 integrin to inhibit endothelial cell migration and adhesion (Sudhakar *et al*. 2003). Tumstatin binds to αVβ3 and αVβ5 integrins to inhibit endothelial cell growth and protein synthesis (Pedchenko *et al*. 2004). Thus, it remains to be elucidated if the α4 LG4-5 fragment binds to these integrins.

In this study, we suggest the potential model that α4 LG4-5 suppresses adipogenesis by blocking the interaction of FGF-2 with HSPG (Fig. 6). FGF-2 can induce adipogenesis by interacting with HSPGs in the Matrigel or at the cell surface. The cryptic α4 LG4-5 fragment generated by elastase competes with FGF-2 for binding to HSPGs, and leads to suppression of adipogenesis. Our preliminary experiment demonstrated that the distal administration of α4 LG4 suppressed de novo adipogenesis, with statistical difference from the intraperitoneal injection of phosphate-buffered saline, indicating that α4 LG4 can be delivered and remain stably at the sites of injection of Matrigel plus FGF-2 to suppress adipogenesis. Increasing evidence indicates that FGF proteins are key regulators in human adipogenesis (Hutley *et al*. 2004; Neubauer *et al*. 2004). Although it remains to

be determined whether the α4 LG4-5 fragment exists *in vivo* within adipose tissue, our study presents the possibility that this fragment functions as a modulator in adipogenesis by preventing FGF from working.

Acknowledgments

This paper is dedicated to the late Professor Yasuo Kitagawa who wrote the original version of the manuscript. This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan awarded to YK and TN (18108003). ATK was supported by the Academic Frontiers Student Exchange Promotion Program provided by the Ministry of Education, Science, Culture and Sports of Japan.

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