Degradation of Filamin Induces Contraction of Vascular Smooth Muscle Cells in Type-I Collagen Matrix Honeycombs

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Key Words

Smooth muscle cell • Three-dimensional culture • Filamin • Cytoskeleton

Abstract

Background: Dedifferentiated rabbit vascular smooth muscle cells (SMCs) exhibit similar features to differentiated SMCs when cultured in three-dimensional matrices of type-I collagen called "honeycombs," but the mechanism is unknown. The role of filamin, an actin-binding protein that links actin filaments in SMCs, was investigated. **Methods:** Filamin and other related proteins were detected by western blot analysis and immunofluorescence staining. Honeycomb size was measured to confirm the contraction of SMCs. **Results:** Full-length filamin was expressed in subconfluent SMCs cultured on plates; however, degradation of filamin, which might be regulated by calpain, was observed in confluent SMCs cultured on plates and in honeycombs. While filamin was co-localized with β -actin in subconfluent SMCs grown on plates, filamin was detected in the cytoplasm in SMCs cultured in honeycombs, and degraded filamin was mainly detected in the cytoplasmic fraction of these cells. In addition, β -actin

expression was low in the cytoskeletal fraction of SMCs cultured in honeycombs compared with cells cultured on plates, and the size of the honeycombs used for culturing SMCs was significantly reduced. **Conclusion:** These data suggest that degradation of filamin in SMCs cultured in honeycombs induces structural weakness of β -non-muscle actin filaments, thereby permitting SMCs in honeycombs to achieve contractility.

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Introduction

Vascular smooth muscle cells (SMCs) undergo phenotypic changes upon differentiation and dedifferentiation. A shift from a dedifferentiated to a differentiated phenotype is a crucial event in the regression of atherosclerosis [1, 2]. In normal aortas, SMCs can contract when they differentiate, but in atherosclerotic lesions, SMCs undergo proliferation and lose the ability to contract after dedifferentiation [3]. SMCs in the normal arterial media are surrounded by components of the extracellular matrix (ECM), which is thought to maintain SMCs in the differentiated phenotype.

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If SMCs are isolated from the ECM and cultured in medium containing 5% serum, they maintain the dedifferentiated phenotype [4].

Dedifferentiated rabbit SMCs displayed unique features when cultured in three-dimensional matrices of type-I collagen called “honeycombs” [5]. They stopped proliferating despite the presence of sufficient serum in the medium, showed morphological changes and expressed caldesmon heavy chain (a marker of mature differentiated SMCs), all of which are features that are similar to those observed in aortic SMCs *in vivo*. SMCs in honeycombs are attached across the lumens of the honeycombs by a few adhesion points and have a more spindly shape than do SMCs cultured on plastic plates. We have also reported that formation of cellular cross-bridges in honeycombs is important for changes that occur in SMCs grown in honeycombs [6]. These results suggest that relationships exist between the structural rearrangement of the cytoskeleton and specific features of SMCs cultured in honeycombs.

It has been demonstrated that SMCs derived from freshly isolated gizzard smooth muscle strips possess a structural lattice that is composed of a contractile apparatus and a cytoskeleton (β -non-muscle actin filaments, intermediate filaments and dense bodies) [7, 8]. The main components of the contractile apparatus are known to be α -smooth muscle actin (α -actin), myosin, tropomyosin, calponin, caldesmon and myosin light chain kinase, while the cytoskeleton is mainly constructed of β -non-muscle actin (β -actin) filaments, filamin, calponin and vimentin. Dense bodies are composed of β -actin, α -actinin and calponin [9]. Of these proteins, α -actinin, an actin-binding protein localized to dense bodies and adherens junctions that is involved in bundling of actin filaments [8, 10, 11], and filamin, an actin cross-linking protein, are thought to affect the arrangement of actin filaments.

Three filamin genes (FLNA, FLNB and FLNC) have been identified in humans [12]. Filamin A has a molecular weight of 280 kDa and a molecular length of approximately 160 nm [13]. Filamin A consists of an N-terminal actin-binding domain (ABD) and a rod-like domain of 24 repeats, which is interrupted by 30-amino-acid long flexible loops, H1 (between repeats 15 and 16) and H2 (between repeats 23 and 24). The last repeat, repeat 24, represents the self-association domain of the molecule. Filamin dimers link actin filaments into orthogonal networks or parallel bundles, and an *in vitro* study has shown that the manner in which actin filaments are organized depends on the ratio of filamin to actin [14]. It

has also been reported that filamin co-localizes with the non-muscle actin cytoskeleton and intermediate filaments, but not with the contractile apparatus, in SMCs derived from freshly isolated gizzard smooth muscle strips [7, 8]; these findings indicate that filamin mainly interacts with the β -non-muscle actin filaments in SMCs.

In the present study, we hypothesized that filamin would be expected to influence the arrangement of the cytoskeletal structure in SMCs. Filamin is known to be degraded at H1 and H2 sites by Ca^{2+} -dependent proteases known as calpains [15], including μ -calpain and m-calpain, which are ubiquitously expressed [16, 17]. We therefore investigated whether filamin in SMCs was degraded by calpain and whether SMCs cultured in honeycombs could contract. We also discuss the relationship between filamin and phenotypic changes in SMCs.

Materials and Methods

Honeycomb collagen tubes

Collagen sponges called honeycombs that consisted of type-I collagen were obtained from Koken Co. Ltd. (Japan). The structure of the honeycomb was porous and consisted of many tubes aligned side-by-side, similar to a beehive [5]. The pore diameters of the tubes were 200–300 μm . The honeycombs were cut vertically into cubes of dimensions $4 \times 4 \times 2 \text{ mm}$ or $6 \times 6 \times 2 \text{ mm}$.

Cell culture

Animal experiments were carried out according to the “Principles of Laboratory Animal Care” (NIH publication number 85-23, revised 1985) and the Guidelines of the Animal Investigation Committee, Chiba University.

SMCs from the medial layer of the thoracic aorta of male Japanese white rabbits weighing $\sim 2 \text{ kg}$ were prepared using the explant method. Nembutal (Dainippon Sumitomo Pharma, Japan) was administered (25 mg kg^{-1} , i.v.) before the artery was extirpated. SMCs were cultured as described by Ishii et al. [5]. SMCs were treated with trypsin–EDTA (Sigma–Aldrich) for 1–2 minutes, and released SMCs were subcultured at 1×10^6 cells per dish (diameter, 10 cm) in 8 ml of 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium (10% FBS-DMEM). Biochemical analyses (expression of α -actin and doubling time in proliferation) were conducted on SMCs prior to use. SMCs were subcultured every 2–4 days, and the process was repeated when cells reached confluence. SMCs used in this study were passages 4–7.

NIH3T3 cells were cultured in 10% FBS-DMEM under standard cell culture conditions (37°C , 5% CO_2). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured under standard cell culture conditions (37°C , 5% CO_2) in endothelial basal medium 2 (EBM2, Lonza) supplemented with human recombinant fibroblast growth factor-B (hFGF-B), human recombinant vascular endothelial growth factor (VEGF), human

recombinant epidermal growth factor (hEGF), hydrocortisone, heparin, ascorbic acid, R3-insulin-like growth factor-1, gentamicin/amphotericin B (GA) and 2% FBS, as suggested by the supplier.

The following procedure was used to prepare cells in honeycombs. First, cells cultured on plates were released by incubation with trypsin–EDTA and collected by centrifugation at $200 \times g$ for 10 minutes at 25°C. Next, cells were suspended in 300–400 μ l of culture medium and incubated with honeycombs ($4 \times 4 \times 2$ or $6 \times 6 \times 2$ mm, 2×10^6 cells per 30 honeycombs) on a dish (diameter, 6 cm) for 3 hours at 37°C. Then, 3–4 ml of culture medium was added. The medium was changed every 2–3 days. ALLN was obtained from Calbiochem and added to 10% FBS–DMEM for experiments.

Cell number measurements

Cells were washed twice with PBS and released from plates by incubation with trypsin–EDTA at 37 °C for 1 minute or from honeycombs by incubation with collagenase-I (Sigma–Aldrich) at 37 °C for 30 minutes. After the cells were collected, cell number was determined using an improved Neubauer deep chamber hemocytometer. Cell viability was assessed by trypan blue exclusion.

Determination of honeycomb area

Honeycombs ($6 \times 6 \times 2$ mm) were incubated with or without cells for 0–7 days in culture medium. At days 0, 1, 3 and 7, pictures were taken of the honeycombs, with or without cells, immersed in medium in dishes (diameter, 6 cm) using a digital camera (Canon). The photographs were enlarged, and the areas (length $\times$ width) of the honeycombs were calculated using Image J 1.41.

Subcellular fractionation

Triton X-100-soluble (cytoplasmic) and -insoluble (cytoskeletal) fractions were prepared as previously described [18].

Western blotting

Detection of filamin, μ -calpain, m-calpain, phospho-filamin A (Ser²¹⁵²), calpastatin, vimentin, myosin heavy chain (MHC), tropomyosin, α -actinin, α -actin or β -actin was carried out by western blotting analysis.

SMCs were washed twice with PBS and released from plates by incubation with trypsin–EDTA at 37°C for 1 minute or from honeycombs by incubation with collagenase-I at 37°C for 30 minutes. SMCs were collected and washed twice with cold PBS. Cells were lysed in IP buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% sodium cholate, 0.5% Triton X-100, 5 mM NaF, 0.5 mM Na₃VO₄, and 1% protease inhibitor cocktail (Sigma–Aldrich)] for 20 minutes on ice. Cell lysates were centrifuged at $12,000 \times g$ for 20 minutes, and protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce). Bovine serum albumin was used as a standard in this assay. After lysates were separated on 6%, 8%, or 12% SDS–PAGE gels, proteins were transferred to an Immobilon membrane (Millipore) and immunoblotted with specific antibodies. Immunoblots were visualized using electrochemiluminescence (ECL) western blotting detection

reagents (GE Healthcare) or Immobilon™ Western (Millipore). The primary antibodies used for western blotting were monoclonal anti-filamin antibody (1.BB.804; Abcam), polyclonal anti-calpain 1 (μ -calpain) antibody (H-65; Santa Cruz Biotechnology), polyclonal anti-calpain II (m-calpain) antibody (Chemicon International), polyclonal anti- α -actinin antibody (H-300; Santa Cruz Biotechnology), polyclonal anti- β -actin antibody (I-19; Santa Cruz Biotechnology), polyclonal anti-phospho-filamin A (Ser²¹⁵²) (Cell Signaling), monoclonal anti-calpastatin antibody (PI-11; Santa Cruz Biotechnology), monoclonal anti-vimentin antibody (V9; Sigma–Aldrich), monoclonal anti- α -actin antibody (1A4; DAKO), monoclonal anti-myosin (smooth) antibody (HSM-V; Sigma–Aldrich) and monoclonal anti-tropomyosin antibody (TM311; Sigma–Aldrich). Membranes were incubated with antibody for 1–3 hours. Membranes were treated with anti-mouse or anti-rabbit IgG (horseradish-peroxidase-linked species-specific antibody; GE Healthcare) as the secondary antibody for 30 minutes.

Northern blotting

Total RNA was isolated from SMCs using Isogen reagent (Isogen International). Ten micrograms of total RNA was fractionated by electrophoresis in a 1% agarose gel containing 400 mM formaldehyde. RNA was transferred to a Hybond N⁺ membrane (Amersham) by capillary transfer overnight and fixed to the membrane by UV crosslinking. Polymerase chain reaction (PCR)-derived probes for filamin A were prepared from the total cDNA of SMCs using a DIG DNA Labeling Kit (Roche). The primer pairs for probes were as follows: forward primer, 5'-GGG TCA CCT ACT GCC CCA C-3'; reverse primer, 5'-GGA TCT TGA GGC TGA GGT CG-3'. DIG Easy Hyb (Roche), DIG Wash and Block Buffer Set (Roche), DIG Luminescent Detection Kit (Roche) and CDP-Star ready-to-use (Roche) were used for hybridization. Detection was carried out according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SMCs using Isogen reagent. cDNA was synthesized using 1 μ g RNA in 20 μ l of reaction mixture with Omniscript® Reverse Transcription (QIAGEN). One microliter of reverse-transcribed cDNA was used in a total reaction volume of 25 μ l for PCR amplification using specific primers for filamin A. Amplification of glycerol-3-phosphate dehydrogenase (GAPDH) was used as a control. The following primer pairs were used: forward and reverse primers for filamin A were the same as those used for northern blotting; forward primer for GAPDH, 5'-G ATG CCC CCA TGT TTG TGA T-3'; reverse primer for GAPDH, 5'-AAG GCC ATG CCA GTG AGT TT-3'. Reaction products were analyzed by electrophoresis on 2% agarose gels and observed with ultraviolet transillumination after ethidium bromide staining. PCR cycling was as follows: initial denaturation at 94°C for 1 minute (one cycle), denaturation at 94°C for 10 seconds, annealing by Taq polymerase (Takara Bio Incorporated) at 69.3°C for filamin A or at 66.2°C for GAPDH for 10 seconds and elongation at 72°C for 30 seconds. The number of amplification cycles performed for filamin A and GAPDH were 27 and 22 respectively.

Fig. 1. Change in the molecular weight of filamin in SMCs cultured under different conditions. Honeycomb: SMCs cultured on a dish (diameter, 10 cm) were treated with trypsin-EDTA, and isolated cells (0 hour) were cultured in honeycombs for 6, 12, 24, and 48 hours (hrs in figures). Plate: SMCs were plated at 5×10^5 cells per dish (diameter, 10 cm) and cultured for 3, 7 and 14 days. (A) Western blot analysis of filamin and related proteins. (B) Cell growth of SMCs cultured in honeycombs and on plates. ●, SMCs cultured in honeycombs; ○, SMCs cultured on plates.

Immunofluorescence
 SMCs on coverslips or in honeycombs were fixed in 3.7% formaldehyde in PBS for 10 minutes. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and pre-incubated with 10% normal goat serum (Wako) for 10 minutes to block non-specific binding. These cells were then incubated with monoclonal anti-filamin antibody (1.BB.804; Abcam) or monoclonal anti- α -actin antibody (1A4; DAKO) and polyclonal anti- β -actin antibody (1-19; Santa Cruz Biotechnology) for 3 hours. This was followed by incubation with FITC-conjugated anti-mouse IgG (Sigma-Aldrich) and TRITC-conjugated anti-rabbit IgG (Sigma-Aldrich) for 1 hour. Immunofluorescence was observed using a Fluoview FV500 (Olympus) laser-scanning microscope with a 60 x 1.00 NA water immersion objective. Images were digitally processed using Adobe Photoshop Elements 4.0 software (Adobe).

Statistical analysis
 The statistical significance of differences was assessed by Tukey's test in Fig. 8B and D, and Dunnett's test in Fig. 8C. Data are mean $\pm$ S.E.

Results

Changes in the molecular weight of filamin in SMCs cultured under different conditions
 Changes in the molecular weight of filamin in SMCs cultured under different conditions were investigated (Fig. 1). At 0 hours, the molecular weight of filamin was mainly 280 kDa. After SMCs were cultured for 6 hours in honeycombs, however, filamin with molecular weights of both 280 and 180 kDa were observed (Fig. 1A). Moreover, SMCs in honeycombs were spindle-shaped, adhered across the lumen of the honeycombs and stopped

Fig. 2. Expression of filamin in aortic media. M, aortic media of Japanese white rabbits; H, SMCs cultured in honeycombs for 12 hours.

proliferating (Fig. 1B). The viability of these cells at 12, 24 and 48 hours was 85, 85 and 86%, respectively. These data are consisted with our previous study [5]. SMCs cultured on plates proliferated and became confluent on day 7 (Fig. 1B). The viability of the SMCs grown on plates on day 3, 7 and 14 was 88, 78 and 84%, respectively. Subconfluent SMCs cultured on plates mainly expressed filamin with a molecular weight of 280 kDa, but two types of filamin were apparent after these cells became confluent (Fig. 1A). Subconfluent SMCs were widely spread on plates, while confluent SMCs had a spindly shape. The aortic media of Japanese white rabbits expressed filamin of both 280 and 180 kDa (Fig. 2), and this pattern was identical to that of filamin in SMCs

Fig. 3. Filamin degradation. (A) Western blot analysis of filamin in SMCs. SMCs were plated on a dish (diameter, 10 cm) and cultured until subconfluent or confluent. (B) Northern blot analysis of filamin A in SMCs cultured on plates. SMCs were plated on a dish (diameter, 10 cm) and cultured until subconfluent or confluent. Total RNA was isolated, northern-blotted and probed with filamin A. (C) Western blot analysis of filamin in SMCs. SMCs were plated at 2×10^5 cells per dish (diameter, 6 cm) and cultured for 24 hours. Next, SMCs were incubated with 2 μ M ALLN (added at day 0) for 1 or 2 days. (D) Western blot analysis of filamin in SMCs. SMCs were plated at 2×10^5 cells per dish (diameter, 6 cm) and cultured for 8 days to reach confluence. Two micromolar ALLN was then added, and SMCs were incubated for 1 or 2 days. (E) Western blot analysis of filamin in SMCs. SMCs cultured on dishes (diameter, 10 cm) were incubated with 2 μ M ALLN for 24 hours. SMCs were then treated with trypsin-EDTA, and isolated SMCs were cultured in honeycombs in the presence of 2 μ M ALLN for 1 or 2 days.

cultured in honeycombs. The filamin antibody used in this experiment does not distinguish between filamin isoforms, but matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) analysis using an AB4700 mass spectrometer (Applied Biosystems) confirmed that the 280 and 180 kDa forms of filamin in SMCs were the filamin A isoform (data not shown). μ -Calpain and m-calpain were expressed in SMCs under each of the culture conditions described above, and expression of α -actinin and β -actin was not altered by different culture conditions.

Degradation of filamin

To begin investigating how the molecular weight of filamin changed in SMCs, we initially examined whether this process involves regulation at the transcriptional level.

Filamin A mRNA was detected by northern blot analysis of total RNA isolated from subconfluent and confluent SMCs cultured on plates (Fig. 3A and B). Results from this study indicated that both the subconfluent and confluent SMCs expressed filamin A mRNA of only one size (~8.1 kb). Filamin transcripts in humans and mice are reported to be 8–9.5 kb and 9.5–10 kb, respectively [15, 19, 20]. Filamin A mRNA therefore appears to encode the entire filamin molecule; thus, it is likely that the change

in the molecular weight of filamin observed in SMCs occurs after translation.

We next examined whether filamin was degraded by calpain in SMCs. Filamin expression was not altered upon incubated with 2 μ M ALLN (N-Acetyl-Leu-Leu-Nle-CHO), an inhibitor of calpain, in subconfluent SMCs cultured on plates (Fig. 3C). In contrast, the change in the molecular weight of filamin was suppressed by 2 μ M ALLN when confluent SMCs on plates at day 8 were incubated with 2 μ M ALLN (Fig. 3D). These results indicate that filamin is degraded by calpain. When SMCs were cultured in honeycombs, filamin degradation was not suppressed by ALLN, even though SMCs cultured in honeycombs were incubated with 2 μ M ALLN from day 1 (data not shown). The lack of an effect of ALLN on filamin degradation in this study might be due to the fact that ALLN was not incorporated into SMCs cultured in honeycombs. Next, SMCs were pre-incubated with 2 μ M ALLN for 24 hours while still cultured on plates and then transferred to honeycombs with 2 μ M ALLN; however, filamin degradation by calpain was still not suppressed by ALLN (Fig. 3E). When the concentration of ALLN was increased to 50 μ M, most SMCs died, but living cells in honeycombs expressed filamin with molecular weights of both 280 and 180 kDa (data not shown).

Phosphorylation of filamin A and the presence of calpastatin

Next, we examined the regulation of filamin degradation in SMCs. Filamin is known to be degraded by calpain at residues 1761-1762 in the H1 region. As a result, filamin is divided into 2 fragments of 180 kDa and 100 kDa [15]. Phosphorylation of the Ser²¹⁵² residue of filamin A has been reported to be responsible for the degradation of filamin A by calpain in platelets [21, 22]. Thus, the phosphorylation status of filamin A in SMCs cultured on plates and in honeycombs was investigated. The monoclonal antibody for filamin detects both full-length filamin and a degraded filamin fragment of 180 kDa. Conversely, the anti-phospho-filamin A (Ser²¹⁵²) antibody detects the full-length and 100 kDa fragment of phosphorylated filamin A because the Ser²¹⁵² residue is in the 100 kDa fragment. As shown in Fig. 4, the 280 kDa form of filamin A and a degraded filamin A fragment of 100 kDa were detected in SMCs. Phosphorylation of the 280 kDa filamin A was not changed when filamin degradation occurred in SMCs cultured on plates (days 3, 6 and 14) or in honeycombs (days 0, 1 and 2). These results suggest that phosphorylation of the Ser²¹⁵² residue of filamin A is not responsible for the degradation of filamin A by calpain in SMCs under the conditions used in this study.

Calpastatin is known to be an endogenous inhibitor of calpain [23]; therefore, the relationship between presence of calpastatin and appearance of the 180 kDa filamin was investigated in SMCs. Calpastatin was present when 180 kDa filamin was absent in SMCs cultured on plates and in honeycombs (Fig. 4). Moreover, when the 180 kDa filamin appeared, calpastatin disappeared. Calpastatin mRNA levels were almost the same in subconfluent SMCs cultured on plates and those cultured in honeycombs, which did and did not express calpastatin protein, respectively (data not shown). These results suggest that filamin degradation in SMCs might be regulated by calpastatin expression.

Localization of filamin and β -non-muscle actin filaments in SMCs

Previous studies have shown that filamin mainly interacts with β -non-muscle actin filaments in SMCs [7, 8]. To clarify the intracellular distribution of filamin after degradation, we examined filamin and β -actin expression by immunofluorescence microscopy in SMCs. Filamin immunoreactivity was observed along fibers and co-localized with β -actin immunoreactivity in subconfluent SMCs cultured on plates (Fig. 5A and B). When SMCs

Fig. 4. Expression of phosphorylated filamin A and calpastatin in SMCs. Plate: SMCs were plated at 1.5×10^5 cells per well (6-well plate) and cultured for 3, 6 and 14 days. Honeycomb: SMCs cultured on a dish (diameter, 10 cm) were treated with trypsin-EDTA, and isolated cells (day 0) were cultured in honeycombs for 1 and 2 days.

were incubated with 0.5 μ M cytochalasin D, this co-localization was disrupted, and both filamin and β -actin were widely dispersed in the cytoplasm (data not shown), suggesting that filamin interacts with β -non-muscle actin filaments. In honeycombs, filamin staining was evenly detected in the cytoplasm and was not stained as fibers in most cells (Fig. 5C and D). A small amount of filamin was co-localized with β -actin in a few cells (data not shown).

As shown in Fig. 6, only the 280 kDa form of filamin was detected in whole cell lysates of subconfluent SMCs cultured on plates, and it was mainly detected in the Triton X-100-insoluble (cytoskeletal) fraction. In honeycombs, both the 280 and 180 kDa forms of filamin were detected in whole cell lysates. Expression of the 180 kDa form of filamin in the Triton X-100-soluble (cytoplasmic) fraction was greater than that in the insoluble fraction, whereas the 280 kDa form of filamin was mainly detected in the insoluble fraction. Although β -actin expression was greater in the Triton X-100-insoluble fraction than in the soluble fraction in subconfluent SMCs grown on plates, expression of β -actin in SMCs cultured in honeycombs was almost the same in the Triton X-100-soluble and -insoluble fractions. μ -Calpain was detected only in the Triton X-100-soluble fraction. Vimentin, a major component of intermediate filaments, was mainly seen in the Triton X-100-insoluble fraction, as expected [24]. Based on the results presented in Figs. 5 and 6, it can be assumed that degraded filamin, which does not bind to β -non-muscle

Fig. 5. Localization of filamin and β -actin in SMCs. (A, B) SMCs were cultured to subconfluence on a plate. (C, D) SMCs were cultured in a honeycomb for 3 days. Green indicates filamin, and red indicates β -actin. Scale bar, 20 μ m (A, C); 5 μ m (B, D).

Fig. 6. Subcellular localization of filamin and β -actin between cytoplasmic and cytoskeletal fractions in SMCs. P, SMCs cultured to subconfluence on plates; H, SMCs cultured in honeycombs for 3 days; whole, whole cell lysates; s, Triton X-100-soluble fraction; i, Triton X-100-insoluble fraction.

actin filaments, is present in the cytoplasmic fraction in SMCs cultured in honeycombs.

Fig. 7. Expression and localization of filamin and β -actin in ODC-SMCs. The rat ODC gene was stably overexpressed by transfection of a pTracer-CMV plasmid encoding the rat ODC gene into SMCs using electroporation (ODC-SMCs). (A) Western blot analysis of filamin and β -actin in ODC-SMCs. SMCs and ODC-SMCs were cultured on plates to subconfluence. Lane 1, SMC; lane 2, molecular marker; lane 3, ODC-SMC. Localization of filamin and β -actin in SMCs (B, C) and ODC-SMCs (D, E). SMCs and ODC-SMCs were cultured on plates to subconfluence. Green indicates filamin, and red indicates β -actin. Scale bar, 20 μ m (B, D); 5 μ m (C, E).

Recently, we stably overexpressed the rat ornithine-decarboxylase gene in SMCs (termed 'ODC-SMCs') to study the function of polyamine and ODC-antizyme in SMCs (unpublished data). Interestingly, expression of filamin was dramatically decreased in ODC-SMCs compared to SMCs (Fig. 7A). In ODC-SMCs cultured on plates, β -non-muscle actin filaments were thinner than those of SMCs, although expression of β -actin, as detected

Fig. 8. Changes in honeycomb size of various cultured cells. (A) Observation of honeycombs in a dish (diameter, 6 cm). (B) Cell growth in honeycombs. ●, SMC; ■, NIH 3T3 cell; ▲, HUVEC. (C) Changes in the area of honeycombs. The size of the honeycomb before culturing with cells was 6 x 6 x 2 mm. A change in the area of the honeycombs (length x width) was observed. ○, honeycombs without cells; ●, honeycombs with SMCs; ■, honeycombs with NIH 3T3 cells; ▲, honeycombs with HUVECs. * indicates $p < 0.05$ for the area of honeycombs cultured with a specific cell compared to the area of honeycombs without cells on each day. (D) Reduction of the area of honeycombs by one cell. Closed bar, SMC; shaded bar, NIH3T3 cell; open bar, HUVEC. Calculations were performed as follows: {(average of the area of honeycombs with each type of cells on day 0) – (the area of honeycombs with each type of cells on day 1, 3 or 7)} / the cell number of each type of cell on day 1, 3 or 7. * indicates $p < 0.05$. (E) Western blot analysis of proteins involved in the contractile apparatus and cytoskeleton in SMCs cultured in honeycombs for 0, 1, 3 and 7 days.

by western blotting analysis, was almost the same in SMCs and ODC-SMCs (Fig. 7B, C, D and E). These results suggest that the 280 kDa form of filamin is responsible for bundling of β -non-muscle actin filaments in SMCs. It has been reported that filamin lacking a self-association domain cannot induce bundling of actin filaments *in vitro* [13]. These data suggest that degradation of filamin in SMCs cultured in honeycombs induces relative structural weakness of β -non-muscle actin filaments.

Shrinkage of honeycombs used for culturing SMCs

Changes in the size of the honeycombs used for culturing SMCs and other non-contractile cells (NIH3T3 cells and HUVECs) were investigated to determine whether SMCs cultured in honeycombs can contract. SMCs mainly cross perpendicular to the pore of the honeycombs; therefore, it was expected that the height of honeycombs would not be dramatically changed by SMCs. Honeycomb size (length x width) was measured as honeycomb area (Fig. 8A). NIH3T3 cells were attached across the lumens of the honeycombs, while HUVEC were attached to the walls of honeycombs and maintained a round shape, similar to that seen when they

are grown on plates. NIH3T3 cells proliferated rapidly in honeycombs, reaching confluence on day 3, while the number of SMCs and HUVEC in the honeycombs did not change (Fig. 8B). The area of the honeycombs was reduced after culturing each cell-type (Fig. 8C). Moreover, the honeycomb areas of SMC cultures were reduced to a greater extent each day than the honeycomb areas of the other two cell-types (Fig. 8D). This suggests that the reduced honeycomb area in SMCs is caused by the tension of SMCs attached across the lumen of the honeycombs or that these SMCs have contractile activity. Expression of proteins involved in both the contractile apparatus and the cytoskeleton were detected in SMCs cultured in honeycombs (Fig. 8E). Expression of α -actin, smooth muscle myosin heavy chain (MHC), and tropomyosin (components of the contractile apparatus) was almost the same in SMCs cultured in honeycombs as in SMCs at day 0. Among components of the cytoskeleton, expression of β -actin and α -actinin were unchanged. SMCs at day 0 expressed the 280 kDa form of filamin, whereas SMCs cultured in honeycombs expressed both the 280 and 180 kDa forms of filamin. In subconfluent SMCs cultured on plates or in SMCs cultured in honeycombs, α - and β -actin were co-localized (Fig. 9), suggesting that the contractile apparatus was

Fig. 9. Localization of α -actin and β -actin in SMCs. (A, B) SMCs were cultured on a plate to subconfluence. (C, D) SMCs were cultured in a honeycomb for 3 days. Green indicates α -actin, and red indicates β -actin. Scale bar, 20 μ m (A, C); 5 μ m (B, D).

aligned with β -non-muscle actin filaments. These findings suggest that degradation of filamin reduces the cellular tension maintained by β -non-muscle actin filaments, indicating that SMCs in honeycombs achieve contractility.

Continuous expression of filamin mRNA in SMCs under different culture conditions

SMCs cultured in honeycombs for 14 days immediately started to proliferate when they were transferred to plates (Fig. 10A), and their growth rate was almost the same as that of SMCs prepared from media. Filamin A mRNA expression was almost identical in SMCs cultured in honeycombs and on plates (Fig. 10B), and SMCs moved to plates mainly expressed the 280 kDa form of filamin after 12 hours (Fig. 10C). These observations indicate that filamin might contribute to the phenotypic changes seen in SMCs.

Fig. 10. Continuous expression of filamin mRNA in SMCs under different culture conditions. SMCs were cultured in honeycombs for 14 days and treated with collagenase. Isolated SMCs were plated at 1×10^5 cells per well (6-well plate) (A) Cell growth of SMCs cultured on plates after culturing in honeycombs for 14 days. (B) RT-PCR analysis of filamin A. (C) Western blot analysis of filamin.

Discussion

In this study, we focused on the regulation and role of filamin in SMCs because the molecular weight of filamin changed in SMCs cultured in honeycombs. Our findings indicate that the change in the molecular weight of filamin might be related to rearrangement of the cytoskeletal structure. Finally, our results suggest that SMCs cultured in honeycombs exhibit contraction.

When SMCs were cultured on plates, a 180 kDa form of filamin could be produced by degradation of the 280 kDa form of filamin, and this degradation was almost completely suppressed by the calpain inhibitor ALLN (Fig. 3D). Other enzyme candidates that might also degrade filamin are caspase 3 and granzyme B, two key mediators of apoptosis [25, 26]; however, the pattern of filamin degradation produced by calpain is different from that

produced by caspase 3 and granzyme B, and there was no indication that apoptosis was induced in SMCs when the 280 kDa filamin was degraded to the 180 kDa filamin. As a result, we concluded that the 180 kDa form of filamin was a product of degradation of the 280 kDa form of filamin by calpain.

Although the degradation pattern of filamin was identical in SMCs cultured in honeycombs and SMCs cultured on plates (Figs. 1, 3), ALLN did not work in SMCs cultured in honeycombs (Fig. 3E). A possible explanation for this discrepancy is that ALLN might be released from cells by multidrug resistance protein (MRP)-1, which is known to transport ALLN, and MRP-1-overexpressing cells show distinct resistant to ALLN [27, 28]. Aorta and vascular SMCs have been reported to express MRP-1 abundantly [29]. On the other hand, incorporation activity, such as thymidine and amino acid incorporation into SMCs, was quite low in honeycombs [5], suggesting that SMCs cultured in honeycombs may have difficulty with ALLN uptake. This suggests that SMCs cultured in honeycombs might release ALLN through MRP-1, while barely taking up any ALLN. We therefore suggest that calpain could contribute to degradation of filamin in SMCs cultured in honeycombs in the current study; however, additional studies are needed.

In this study, we identified a clear relationship between calpastatin expression and filamin degradation. Specifically, when calpastatin was expressed in SMCs, the 180 kDa form of filamin was absent, whereas calpastatin was not detected when the 180 kDa form of filamin was present. Although calpastatin is a well-known inhibitor of calpain [30], other studies have shown that calpastatin can be cleaved by μ -calpain and m-calpain *in vitro* when sufficient Ca^{2+} is provided [31, 32]. Despite being cleaved, calpastatin fragments were still potent inhibitors of calpains, though the inhibitory activity was lower [31, 32]. It has been reported that calpain activity is dependent on Ca^{2+} -concentration [23, 33, 34]. When SMCs contract, Ca^{2+} is provided by Ca^{2+} influx from the extracellular space and Ca^{2+} release from the sarcoplasmic reticulum into the cytoplasm [35]. Thus, it can be assumed that an increase in calpain activity induced by higher concentrations of Ca^{2+} might induce cleavage of calpastatin, resulting in calpain-mediated degradation of filamin due to the reduced inhibitory activity of the degraded calpastatin.

Examination of the effects of filamin degradation on the cytoskeleton also yielded interesting data. It is known that degraded filamin can no longer dimerize, and

the avidity of degraded filamin binding to actin filaments is reduced compared with that of non-degraded filamin [15]. Our data suggest that degradation of filamin reduces the interaction between filamin and β -non-muscle actin filaments, which results in disruption of their co-localization (Figs 5, 6). Our data also suggest that the 280 kDa form of filamin is responsible for bundling of β -non-muscle actin filaments in SMCs (Fig. 7). This hypothesis is supported by the observation that stress fibers in filamin-deficient human melanoma (M2) cells were compared with A7 cells that were stably reconstituted with filamin [36]. A7 cells spread on fibronectin and formed well-developed focal adhesions and stress fibers, whereas filamin-deficient M2 cells attached, but remained retracted with only a few small focal adhesions and rare short actin bundles. It has also been reported that filamin lacking a self-association domain cannot induce bundling of actin filaments *in vitro* [13]. These data suggest that degradation of filamin in SMCs cultured in honeycombs induces relative structural weakness of β -non-muscle actin filaments compared with the contractile apparatus.

Honeycombs with SMCs shrank significantly over time (Fig. 8). This shrinkage of honeycombs could be the result of the tension within SMCs attached across their lumens or due to contraction of the SMCs. In the latter case, SMCs in honeycombs could achieve contractility because cellular tension maintained by β -non-muscle actin filaments is reduced in these cells due to filamin degradation. The expression levels of α -actin, MHC, tropomyosin, β -actin and α -actinin were almost identical in SMCs cultured on plates and in honeycombs, but filamin in SMCs cultured in honeycombs was degraded (Fig. 8E). In subconfluent SMCs cultured on plates, or SMCs cultured in honeycombs, α - and β -actin were co-localized (Fig. 9), which suggests that the contractile apparatus is aligned with β -non-muscle actin filaments. SMCs cultured on plates do not contract under normal culturing conditions [37]. Our results could explain why contraction, which is a result of shortening of the contractile apparatus, was not observed in SMCs cultured on plates. SMCs attach to rigid plastic plates via focal adhesion, and the resulting cytoskeletal tension, which is maintained by β -non-muscle actin filaments, inhibits shortening of the contractile apparatus. In honeycombs, SMCs can achieve contractility because filamin degradation reduces the cytoskeletal tension and allows shortening of the contractile apparatus. Features of dedifferentiated SMCs are large amounts of rough endoplasmic reticulum, free ribosomes and mitochondria, and proliferation in response to PDGF-BB [4, 38]. In our model, the number of

ribosomes and mitochondria present in a certain area of SMCs cultured in honeycombs was almost identical with the amount of ribosomes and mitochondria in aortic SMCs, and the number of ribosomes and mitochondria in a single cell in honeycombs was 20% to 25% of that in SMCs cultured on plates. SMCs cultured in honeycombs also stopped proliferating and did not respond to PDGF-BB anymore [5]. Additionally, SMCs cultured in honeycombs expressed caldesmon heavy chain (a marker protein of mature differentiated SMCs) [5, 6]. Although expression levels of α -actin and MHC (a marker of differentiated SMCs) in SMCs cultured on plates and in honeycombs were almost identical, SMCs in honeycombs might be able to achieve contractility. Based on these results, we believe that SMCs cultured in honeycombs have the functional ability to contract.

When SMCs were released from honeycombs and moved to plates, they immediately began to proliferate (Fig. 10). This response could be regarded as similar

in some respects to the change to a proliferative phenotype of SMCs *in vivo*. It is assumed that SMCs can adopt a suitable cytoskeletal structure for their surrounding environment by rearrangement of actin filaments, and filamin can regulate this rearrangement. The results presented herein suggest that constant expression of filamin mRNA and regulation of filamin function by degradation, which is a post-translational regulatory mechanism, might at least partially contribute to the immediate and reversible phenotypic changes in SMCs.

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References

- 1 Armstrong ML, Warner ED, Connor WE: Regression of coronary atherosclerosis in rhesus monkeys. *Circ Res* 1970;27:59-67.
- 2 Corti R, Osende JI, Fallon JT, Fuster V, Mizsei G, Jneid H, Wright SD, Chaplin WF, Badimon JJ: The selective peroxisomal proliferator-activated receptor-gamma agonist has an additive effect on plaque regression in combination with simvastatin in experimental atherosclerosis: *in vivo* study by high-resolution magnetic resonance imaging. *J Am Coll Cardiol* 2004;43:464-473.
- 3 Chamley-Campbell J, Campbell GR, Ross R: The smooth muscle cell in culture. *Physiol Rev* 1979;59:1-61.
- 4 Chamley-Campbell JH, Campbell GR, Ross R: Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *J Cell Biol* 1981;89:379-383.
- 5 Ishii I, Tomizawa A, Kawachi H, Suzuki T, Kotani A, Koshushi I, Itoh H, Morisaki N, Bujo H, Saito Y, Ohmori S, Kitada M: Histological and functional analysis of vascular smooth muscle cells in a novel culture system with honeycomb-like structure. *Atherosclerosis* 2001;158:377-384.
- 6 Suzuki T, Ishii I, Kotani A, Masuda M, Hirata K, Ueda M, Ogata T, Sakai T, Ariyoshi N, Kitada M: Growth inhibition and differentiation of cultured smooth muscle cells depend on cellular crossbridges across the tubular lumen of type I collagen matrix honeycombs. *Microvasc Res* 2009;77:143-149.
- 7 Small JV, Fürst DO, De Mey J: Localization of filamin in smooth muscle. *J Cell Biol* 1986;102:210-220.
- 8 North AJ, Gimona M, Lando Z, Small JV: Actin isoform compartments in chicken gizzard smooth muscle cells. *J Cell Sci* 1994;107:445-455.
- 9 Small JV, Gimona M: The cytoskeleton of the vertebrate smooth muscle cell. *Acta Physiol Scand* 1998;164:341-348.
- 10 Worth NF, Rolfe BE, Song J, Campbell GR: Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins. *Cell Motil Cytoskeleton* 2001;49:130-145.
- 11 Sjöblom B, Salmazo A, Djinnoviae-Carugo K: Alpha-actinin structure and regulation. *Cell Mol Life Sci* 2008;65:2688-2701.
- 12 van der Flier A, Sonnenberg A: Structural and functional aspects of filamins. *Biochim Biophys Acta* 2001;1538:99-117.
- 13 Nakamura F, Osborn TM, Hartemink CA, Hartwig JH, Stossel TP: Structural basis of filamin A functions. *J Cell Biol* 2007;179:1011-1025.
- 14 Dabrowska R, Goch A, Osinińska H, Szpacenko A, Sosinski J: Dual effect of filamin on actomyosin ATPase activity. *J Muscle Res Cell Motil* 1985;6:29-42.
- 15 Gorlin JB, Yamin R, Egan S, Stewart M, Stossel TP, Kwiatkowski DJ, Hartwig JH: Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J Cell Biol* 1990;111:1089-1105.
- 16 Huff-Lonergan E, Mitsunashi T, Beekman DD, Parrish FC Jr, Olson DG, Robson RM: Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. *J Anim Sci* 1996;74:993-1008.
- 17 Kwak KB, Chung SS, Kim OM, Kang MS, Ha DB, Chung CH: Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. *Biochim Biophys Acta* 1993;1175:243-249.

- 18 Carragher NO, Levkau B, Ross R, Raines EW: Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J Cell Biol* 1999;147:619-630.
- 19 Chiang W, Greaser ML, Lyons GE: Filamin isogene expression during mouse myogenesis. *Dev Dyn* 2000;217:99-108.
- 20 Takafuta T, Wu G, Murphy GF, Shapiro SS: Human beta-filamin is a new protein that interacts with the cytoplasmic tail of glycoprotein Ibalpha. *J Biol Chem* 1998;273:17531-17538.
- 21 Chen M, Stracher A: *In situ* phosphorylation of platelet actin-binding protein by cAMP-dependent protein kinase stabilizes it against proteolysis by calpain. *J Biol Chem* 1989;264:14282-14289.
- 22 Jay D, Garcia EJ, Lara JE, Medina MA, de la Luz Ibarra M: Determination of a cAMP-dependent protein kinase phosphorylation site in the C-terminal region of human endothelial actin-binding protein. *Arch Biochem Biophys* 2000;377:80-84.
- 23 Goll DE, Thompson VF, Li H, Wei W, Cong J: The calpain system. *Physiol Rev* 2003;83:731-801.
- 24 Aoyama K, Matsuda T, Aoki N: Characterization of newly identified four isoforms for a putative cytosolic protein tyrosine phosphatase PTP36. *Biochem Biophys Res Commun* 1999;266:523-531.
- 25 Umeda T, Kouchi Z, Kawahara H, Tomioka S, Sasagawa N, Maeda T, Sorimachi H, Ishiura S, Suzuki K: Limited proteolysis of filamin is catalyzed by caspase-3 in U937 and Jurkat cells. *J Biochem* 2001;130:535-542.
- 26 Browne KA, Johnstone RW, Jans DA, Trapani JA: Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. *J Biol Chem* 2000;275:39262-39266.
- 27 Inoue S, Sharma RC, Schimke RT, Simoni RD: Cellular detoxification of tripeptidyl aldehydes by an aldo-keto reductase. *J Biol Chem* 1993;268:5894-5898.
- 28 de Jong MC, Slootstra JW, Scheffer GL, Schroeijers AB, Puijk WC, Dinkelberg R, Kool M, Broxterman HJ, Meloen RH, Scheper RJ: Peptide transport by the multidrug resistance protein MRP1. *Cancer Res* 2001;61:2552-2557.
- 29 Mueller CF, Wassmann K, Widder JD, Wassmann S, Chen CH, Keuler B, Kudin A, Kunz WS, Nickenig G: Multidrug resistance protein-1 affects oxidative stress, endothelial dysfunction, and atherogenesis via leukotriene C4 export. *Circulation* 2008;117:2912-2918.
- 30 Hanna RA, Campbell RL, Davies PL: Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature* 2008;456:409-412.
- 31 Nakamura M, Inomata M, Imajoh S, Suzuki K, Kawashima S: Fragmentation of an endogenous inhibitor upon complex formation with high- and low-Ca²⁺-requiring forms of calcium-activated neutral proteases. *Biochemistry* 1989;28:449-455.
- 32 Doumit ME, Koohmaraie MJ: Immunoblot analysis of calpastatin degradation: evidence for cleavage by calpain in postmortem muscle. *J Anim Sci* 1999;77:1467-1473.
- 33 Edmunds T, Nagainis PA, Sathe SK, Thompson VF, Goll DE: Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. *Biochim Biophys Acta* 1991;1077:197-208.
- 34 Arthur JS, Crawford C: Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. *Biochim Biophys Acta* 1996;1293:201-206.
- 35 Ganitkevich V, Hasse V, Pfitzer G: Ca²⁺-dependent and Ca²⁺-independent regulation of smooth muscle contraction. *J Muscle Res Cell Motil* 2002;23:47-52.
- 36 Mammoto A, Huang S, Ingber DE: Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts. *J Cell Sci* 2007;120:456-467.
- 37 Han M, Wen JK, Zheng B, Cheng Y, Zhang C: Serum deprivation results in redifferentiation of human umbilical vascular smooth muscle cells. *Am J Physiol Cell Physiol* 2006;291:C50-58.
- 38 Chamley-Campbell JH, Campbell GR: What controls smooth muscle phenotype? *Atherosclerosis* 1981;40:347-357.