Journal of
PERIODONTAL RESEARCH

J Periodont Res 2009; 44: 622–627
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JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01170.x

Stretching stimulates fibulin-5 expression and controls microfibril bundles in human periodontal ligament cells

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Nakashima K, Tsuruga E, Hisanaga Y, Ishikawa H, Sawa Y. Stretching stimulates fibulin-5 expression and controls microfibril bundles in human periodontal ligament cells. J Periodont Res 2009; 44: 622–627. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: The elastic fiber system comprises oxytalan, elaunin and elastic fibers, differing in their relative microfibril and elastin contents. Human periodontal ligaments contain oxytalan fibers (pure microfibrils). Periodontal ligaments are continuously exposed to various functional forces, such as tooth movement and occlusal loading. We have reported that bundles of microfibrils coalesce in response to mechanical strain in cultured periodontal ligament fibroblasts, as assessed in terms of their positivity for fibrillin-1 (the major component of microfibrils). However, the mechanism of microfibril coalescence is unclear. We hypothesized that the fibrillin-1-binding molecule, fibulin-5, contributes to oxytalan fiber formation under mechanical strain.

Material and Methods: We subjected periodontal ligament fibroblasts to stretching in order to examine the effects of fibulin-5 on the formation of oxytalan fibers in cell/matrix layers. We transfected periodontal ligament cells with small interference RNA for fibulin-5, then examined oxytalan fibers using immunofluorescence and electron microscopy.

Results: Immunofluorescence showed that fibrillin-1-positive microfibrils coalesced as a result of stretching, compared with cells that were not subjected to stretching. Fibulin-5 colocalized on fibrillin-1-positive microfibrils. Stretching increased fibulin-5 gene expression and protein deposition. Immunofluorescence and immunogold electron microscopy analysis revealed that fibulin-5 suppression inhibited the coalescence of microfibrils under stretching conditions.

Conclusion: These results suggest that fibulin-5 up-regulated in response to tension strain may control the formation of microfibril bundles in periodontal ligament.

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Key words: fibulin-5; oxytalan fiber; microfibrils; periodontal ligament; tension strain

Accepted for publication August 21, 2008

The elastic fiber system that gives tissue flexibility and extensibility has two components: an amorphous core of cross-linked elastin and a peripheral mantle of microfibrils (1). Elastic fiber formation is thought to begin with the formation of a scaffold of microfibrils on which the elastin is deposited. Depending on the relative proportions of microfibrils and elastin, the elastic fiber system can be classified into three types: oxytalan fibers, elaunin fibers and elastic fibers. Interestingly, in the periodontal ligaments, only oxytalan fibers, which are pure microfibrils, can be identified, whereas all three types of fibers are present in the gingiva (2,3). In the periodontal ligament, oxytalan

fibers are thought to have some functional roles, such as support of vascular walls and regulation of vascular flow. In our series of studies (4–6), we have demonstrated that human periodontal ligament fibroblasts express fibrillin-1 (the major component of oxytalan fibers) and form oxytalan fibers in cell/matrix layers. However, precisely how oxytalan fibers develop remains unclear.

The oxytalan fibers in periodontal ligament are arranged in a vertically oriented interlacing network enclosing the molar root apex (7), and are constantly exposed to mechanical stimulation. Recently, using immunofluorescence and immunogold electron microscopy analysis, we demonstrated that under stretching conditions, bundles of oxytalan fibers coalesce and thicken, although stretching did not affect the gene expression level of fibrillin-1 (6). This may have been because of the up-regulation of some fibrillin-binding molecules.

Fibulins are extracellular glycoproteins that are associated with elastic fibers (8,9). Among them, fibulin-5, a 55-kDa glycoprotein, has been reported to have a relationship with the elastic fiber system (10,11). Fibulin-5 is able to interact with fibrillin-1 (12), and is expressed even in elastin-free tissues (9). Previously, we showed that human periodontal ligament fibroblasts express not only fibrillin-1 but also fibulin-5 (13). However, any contribution of fibulin-5 to the formation of oxytalan fibers in periodontal ligament is not known. Therefore, in the present study, we examined fibulin-5 expression under stretching conditions and investigated the role of fibulin-5 in the appearance of oxytalan fibers.

Material and methods

Cells and culture

The protocol for these experiments was reviewed and approved by the Fukuoka Dental College Research Ethics Committee, and informed consent was obtained from the tissue donors. Briefly, connective tissues were obtained surgically from the periodontal ligament of molar teeth extracted for orthodontic reasons from three different donors. After washing in phosphate-buffered saline supplemented with 100 units/mL of penicillin and 100 μg/mL of streptomycin, the periodontal ligament tissues were cut into small pieces, plated in petri dishes and incubated in minimal essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% newborn calf serum (Invitrogen) at 37°C in humidified air containing 5% CO_2. When outgrowth of the cells reached confluence, they were harvested with 0.025% trypsin (Invitrogen) in phosphate-buffered saline and then transferred to plastic culture dishes at a 1:4 split ratio. For experiments, the cells were used from the third to the sixth passages to retain their phenotypes, as described previously (4).

Mechanical loading apparatus and cyclic stretching conditions

Periodontal ligament fibroblasts were stretched using a STB-140 STREX cell stretch system (Strex Co., Osaka, Japan) in accordance with the manufacturer's instructions. Cells were transferred to an elastic silicone chamber (4 mL) at a density of 1×10^5 cells/mL. The chamber was precoated with 50 μg/mL of type I collagen (C8919) (Sigma, St Louis, MO, USA). After 72 h, the periodontal ligament fibroblasts were found to be confluent. The medium was changed to fresh medium and the cells were subjected to stretching (the first day was set as day 0). The amplitude and frequency of stretching were controlled by a programmable microcomputer at 5% of the original strength and 1/60 Hz, respectively, for the times indicated. The stretch chamber with its frames was placed in an incubator. The silicone chamber was kept uniformly stretched over the whole membrane area during the incubation.

Small interference RNA design and transient transfection

Small interference RNA for human fibulin-5 (accession no. NM_006329) was designed and synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The synthesized small interference RNA corresponded to the 1165–1190 nucleotide coding region of fibulin-5. The small interference RNA sequence for fibulin-5 was: sense 5′-GGCAGAGAAUUUUACAUGCGGCAAAAG-3′ and antisense 3′-UACCGUCUCUUAAAAUGUACGCCGUUU-5′. Negative control (scrambled order) was: sense 5′-CGGUCUAGACUAGCGAGAUAAUAGAAG-3′ and antisense 3′-UAGCCAGAUCUGAUCGCUCUAUUAUCU-5′. BLAST searches of the database indicated that this small interference RNA is specific for fibulin-5 and has no homology with other proteins. Transfection was performed at days 1 and 4 of culture in a continuous manner, as follows. The small interference RNA was transfected into periodontal ligament fibroblasts using X-treme GENE small interference RNA transfection reagent (Roche, Mannheim, Germany). First, 237.5 μL of Opti-MEM medium (Invitrogen) and 12.5 μL of the transfection reagent were pre-incubated for 10 min at room temperature 23°C. During this time, 748 μL of OptiMEM medium was mixed with 2 μL of 100 μM small interference RNA. The two mixtures were then combined and incubated for 20 min at room temperature 23°C to allow formation of their complex. Then, the entire mixture was added to the cells in one dish, resulting in a final concentration of 200 nM for the small interference RNAs. After 12 h of incubation, the transfection medium was replaced with fresh complete medium (minimal essential medium containing 10% fetal calf serum). Mock transfection of cultures with the transfection reagent alone was used as a control. Periodontal ligament fibroblasts were transfected twice with the small interference RNA duplex (0, 50 and 200 nM), with a 72-h intervening interval (i.e. on days 1 and 4), and harvested at 7 d.

Northern blot analysis

Total RNA was prepared from the cultured periodontal ligament fibroblasts at 7 d using an RNeasy Mini Kit

(Qiagen, Hilden, Germany). One microgram of RNA was subjected to northern blot analysis, using a probe for recognition of human fibulin-5, generated as described previously (13). A probe for human tropoelastin was generated as described previously (4). The RNA probe for β-actin was from Roche Molecular Biochemicals (Mannheim, Germany).

Western blot analysis

The cell/matrix lysates (10 μg) at 7 d of culture were subjected to western blot analysis, as described previously (5). The antibodies used were polyclonal anti-(human fibulin-5) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal anti-(β-actin) (Sigma). Densitometric semiquantitative analysis of the signals was performed using the IMAGE J program (National Institutes of Health, Bethesda, MD, USA) after finding the linear portion by sequential dilution of the proteins, as described previously (6). Small variations in protein loading were corrected by normalization relative to the intensity of β-actin. Student's *t*-tests were used to determine the statistical significance of the difference. A *p*-value of ≤ 0.05 was considered significant.

Immunofluorescence

At 7 d of culture, periodontal ligament fibroblasts were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline for 15 min. The culture dishes were rinsed with phosphate-buffered saline and then treated with 6 M guanidine–HCl, 20 mM Tris and 50 mM dithiothreitol (pH 8.0) for 5 min. Nonspecific immunoreactivity was blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature 23°C. The cell layers were then incubated for 2 h at room temperature 23°C with the appropriate primary antibodies [polyclonal goat antibody against human fibulin-5 diluted 1:500 (Santa Cruz Biotechnology), rabbit antibody

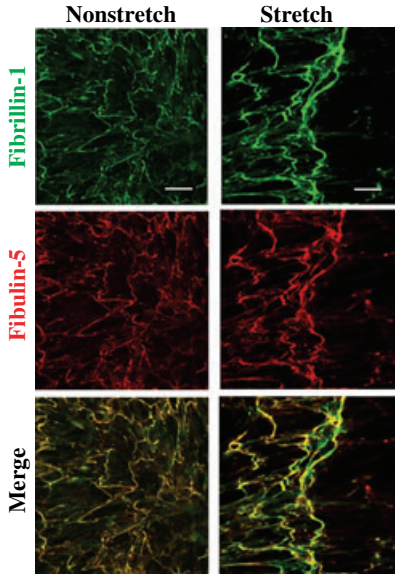

Fig. 1. Stretching coalesces microfibril bundles, and immunolocalization of fibulin-5 to microfibrils in periodontal ligament fibroblast cultures was observed. Periodontal ligament fibroblasts subjected to nonstretching (control) (left panels) and stretching (right panels) were labeled for fibrillin-1 (upper panels) and fibulin-5 (middle panels) on day 7. Bars indicate 100 μm.

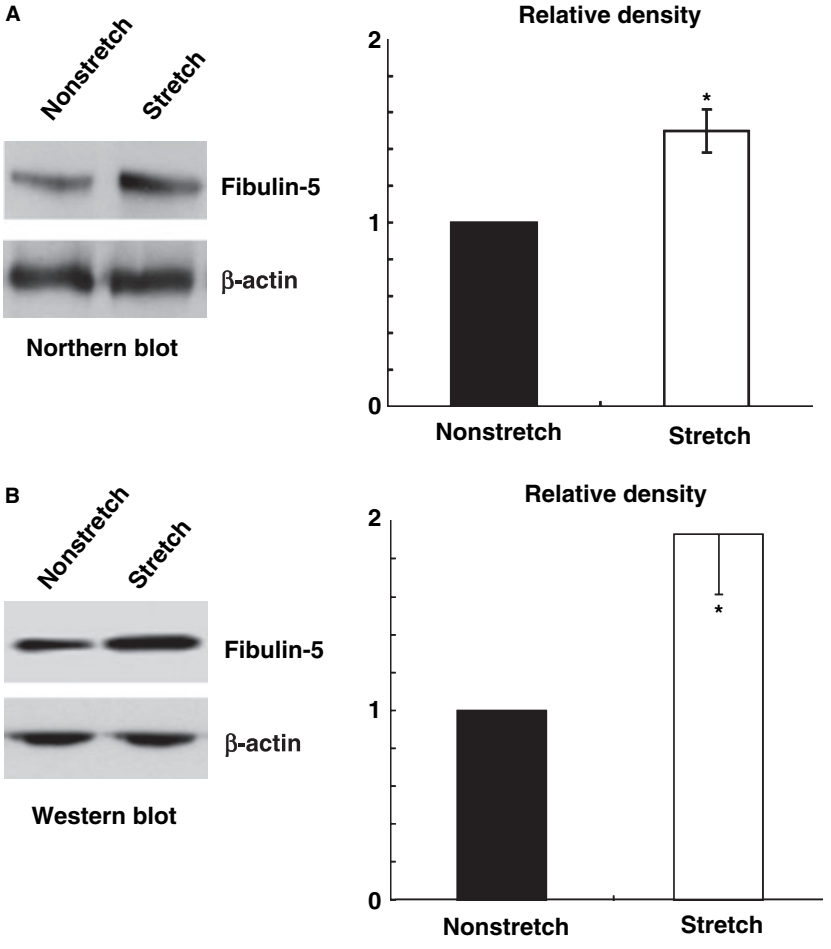

Fig. 2. Stretching stimulates fibulin-5 expression in periodontal ligament fibroblast cultures. (A) Northern blots of RNA samples (1 μg) extracted from periodontal ligament fibroblasts cultured for 7 d under nonstretch and stretch conditions. Stretching induced gene expression of fibulin-5 (left panel). The intensity of each band was normalized relative to that of β-actin and was quantified using the IMAGE J program. The intensity obtained for nonstretch was set as 1. Data represent the mean ± standard deviation of three independent experimental determinations. Student's *t*-tests were used to determine the statistical significance of the difference. *A *p*-level of ≤ 0.05 was considered significant (right panel). (B) Western blots of cell lysates (10 μg) of periodontal ligament fibroblasts cultured for 7 d under nonstretch and stretch conditions. Stretching induced deposition of fibulin-5 (left panel). Densitometric analysis of changes in the levels of fibulin-5 was conducted using IMAGE J software, normalized relative to the level of β-actin (right panel).

against human fibrillin-1 diluted 1:100 (Elastin Products Co., Owensville, MO, USA) and monoclonal antibody against tropoelastin (BA4; Santa Cruz Biotechnology)]. Controls included the use of pre-immune normal goat or rabbit immunoglobulin G for the primary antibody incubation. After rinsing in phosphate-buffered saline, the cells were incubated with Alexa Fluor® 568-labeled donkey anti-goat immunoglobulin G1 or Alexa Fluor® 488-labeled goat anti-rabbit immunoglobulin (H + L) (Molecular Probes, Eugene, OR, USA), diluted 1:100 with blocking buffer, for 1 h at room temperature 23°C. After the final wash, cells were viewed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

Immunoelectron microscopy

Periodontal ligament fibroblast layers at 7 d of culture were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline for 30 min. The cells were then incubated with primary antibody (fibrillin-1; diluted 1:100) for 2 h, followed by incubation with gold particle-conjugated anti-rabbit immunoglobulin (BB International, Cardiff, UK) at 4°C. Controls included the use of pre-immune normal rabbit immunoglobulin G for the primary antibody incubation. The cells were then treated with silver solution for silver intensification, followed by dehydration in a graded ethanol series and embedded in EPON 812 resin (TAAB, Berkshire, UK). Ultrathin sections were observed using a JEOL JEM 1400 transmission electron microscope (JEOL Ltd, Tokyo, Japan). The results shown in all figures are representative of at least three independent analyses.

Results

Stretching induces coalescence of microfibril bundles, and fibulin-5 localizes on microfibrils in periodontal ligament fibroblast cultures

We examined the appearance of oxytalan fibers on day 7 using immunofluorescence. The immunofluorescence images revealed that stretching appeared to induce the coalescence of fibrillin-1-positive microfibril bundles, in comparison with the nonstretched samples (Fig. 1, green). Control

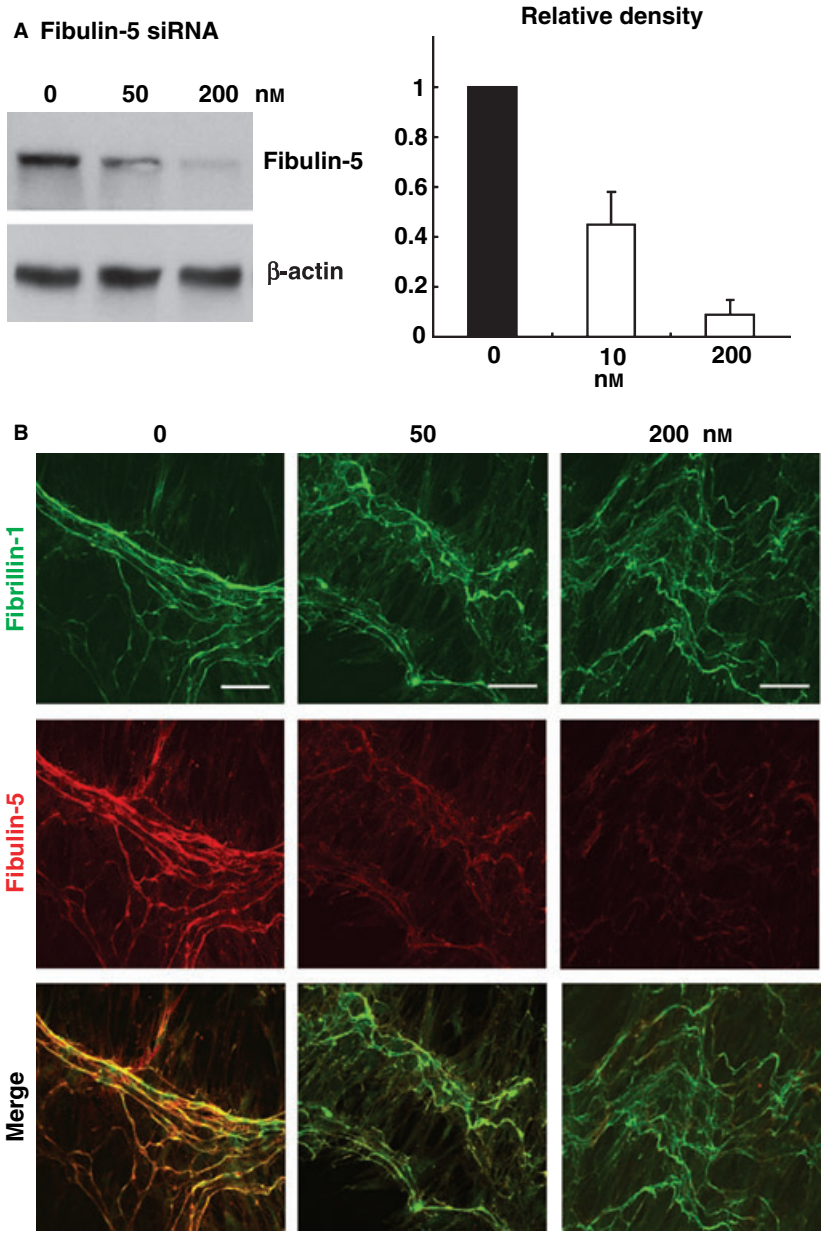

Fig. 3. Fibulin-5 small interference RNA suppresses the coalescence of fibrillin-1-positive microfibrils under stretching conditions. Periodontal ligament fibroblasts were transiently transfected with 0–200 nM fibulin-5 small interference RNA for 7 d under stretching conditions. (A) One microgram of RNA was analyzed by northern blotting. Mock-transfected (vehicle only) cultures were used as a control (first lane). βeta-actin was used as an internal control in each lane (left panel). The intensity of each band was normalized relative to that of β-actin and quantified using the IMAGE J program. The intensity obtained for mock-transfected cultures was set as 1. Data represent the mean ± standard deviation of three independent experimental determinations. Student's *t*-tests were used to determine the statistical significance of the difference. A *p*-level of ≤ 0.05 was considered significant (right panel). siRNA, small interference RNA. (B) Periodontal ligament fibroblasts were immunolabeled with anti-fibrillin-1 (all in green) and anti-fibulin-5 (all in red). Bar, 100 μm.

immune serum produced no labeling (results not shown). We then examined whether fibulin-5 was localized on microfibrils. Fibulin-5 was clearly identified on fibrillin-1-positive microfibrils of human periodontal ligament fibroblasts cultured for 7 d (Fig. 1, red). The signals of tropoelastin were not detectable before and after stretching stimulation (data not shown).

Stretching increases fibulin-5 expression

We examined gene expression and protein deposition of fibulin-5 to compare stretched with nonstretched samples. Figure 2A shows fibulin-5 gene expression revealed by northern blot analysis. Densitometric analysis was performed by normalizing each expression level to the corresponding level of β-actin. Fibulin-5 signals in stretched samples showed a significant 1.5-fold increase in comparison with the nonstretched samples. Similarly, the amounts of fibulin-5 in the periodontal ligament cell/matrix layers in stretched samples were significantly increased 1.9-fold in comparison with the nonstretched samples by western blotting, in agreement with the northern blot data (Fig. 2B). The gene expressions of tropoelastin were not detectable before and after treatment with fibulin-5 small interference RNA (data not shown).

Fibulin-5 small interference RNA inhibits coalescence of microfibril bundles

Next, in order to investigate the function of fibulin-5, we used small interference RNA to suppress fibulin-5 expression. Densitometric analysis based on the northern blots showed that when 200 nM small interference RNA was used for transfection, the level of fibulin-5 expression was reduced to less than 10% of the control (vehicle only) level at 7 d (Fig. 3A). In contrast, scrambled small interference RNA had no effect on fibulin-5 expression, and no difference from the control was evident (data not shown), proving that this small interference RNA was specific for fibulin-5. Immunolabeling data additionally supported the efficacy of the small interference RNA (Fig. 3B).

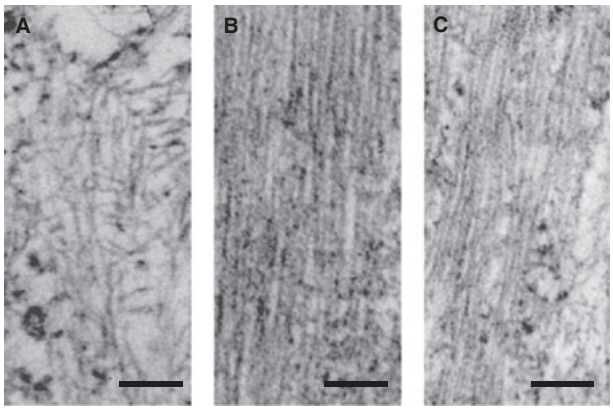

Fig. 4. Immunogold electron microscopy of the microfibrils formed by periodontal ligament fibroblasts. Unstretched periodontal ligament fibroblasts showed fibrillin-1-positive microfibrils arranged irregularly at low density (A). Under stretching conditions, periodontal ligament fibroblasts transfected with mock (vehicle only) culture showed microfibrils arranged regularly at high density (B), whereas periodontal ligament fibroblasts transfected with small interference RNAs (200 nM) for fibulin-5 showed fewer microfibrils arranged in irregular directions (C). Bar, 100 nm.

To investigate the effect of fibulin-5 on microfibril formation, we transfected stretched periodontal ligament fibroblasts with 200 nM fibulin-5 small interference RNA. Immunofluorescence images revealed that the greater the suppression of fibulin-5, the looser the appearance of the fibrillin-1-positive microfibril bundles (Fig. 3B). In addition, we examined the effect of fibulin-5 knockdown on the microfibril ultrastructure. Nonstretched periodontal ligament fibroblasts showed fibrillin-1-positive microfibrils arranged irregularly at low density (Fig. 4A). The fibrillin-1-positive microfibrils were arranged in parallel with a high fiber density in stretched fibroblasts transfected with vehicle only (Fig. 4B), whereas the periodontal ligament fibroblasts treated with 200 nM fibulin-5 small interference RNA contained far fewer microfibrils arranged in relatively irregular directions (Fig. 4C).

Discussion

In the present study using small interference RNA, we demonstrated, for the first time, that stretching increases fibulin-5 expression and that the up-regulated fibulin-5 controlled the coalescence of oxytalan fibers positive for fibrillin-1 in periodontal ligament fibroblast cultures. Previously, fibulin-5 was shown to be localized on fibrillin-1-positive fibers (14,15), and their molecular interactions have been demonstrated (12). This study provides new insight into the contribution of fibulin-5 to the formation of oxytalan fibers.

Oxytalan fibers, which consist of collections of microfibrils, were first described in periodontal ligament by Fullmer & Lillie (16). In periodontal ligament tissue, oxytalan fibers as well as collagen fibers are important matrix components for maintaining periodontal ligament homeostasis. Recently, we demonstrated that stretching increases the density of microfibril bundles in periodontal ligament fibroblast cultures without an increase of fibrillin-1 expression (6). Therefore, we speculated that some fibrillin-1-binding molecules might play a role in the coalescence of microfibril bundles. Fibulin-5 is the best candidate for this role because it can bind to fibrillin-1 (12) and has been shown to colocalize with fibrillin-1-positive microfibrils by immunofluorescence analysis, as described above (14,15). Originally, fibulin-5 was thought to be an adaptor for binding tropoelastin to microfibrils for the formation of elastic fibers, as

fibulin-5 can bind tropoelastin as well as fibrillin-1 (12). Fibulin-5 is thought to be a critical component for elastic fiber formation. Therefore, the role of fibulin-5 in pure microfibril formation has not yet been clarified. Under stretched conditions, the present study showed that up-regulated fibulin-5 controlled the density of microfibril bundles, thus demonstrating another role of fibulin-5 in the homeostasis of the elastic fiber system. The expression and distribution of fibulin-5 at sites of stretching and compression in the periodontal ligament *in vivo* are currently being investigated.

Fibulin-5 expression in fibroblasts is reported to be regulated by transforming growth factor-β (17) and tropoelastin (13). In addition, fibulin-5 exerts some effects on cellular functions such as migration and proliferation (17). Thus, fibulin-5 appears to have various functions, and future studies are needed to determine the signaling pathway that leads to the up-regulation of fibulin-5 by stretching.

In the present study, the oxytalan fibers in cells with fibulin-5 knockdown appeared to be similar to those in nonstretched samples that were detected using immunofluorescence. If physical elongation of periodontal ligament fibroblasts were a critical factor for that, it would be contradictory. This suggests that the increase of microfibril density may be caused by the up-regulation of fibulin-5. However, there is no available evidence that fibulin-5 directly controls microfibril bundles. Some fibulin-5-binding molecule may control the microfibril bundles directly by forming a complex with fibulin-5. Further analysis will be necessary to clarify this issue.

Acknowledgements

We wish to thank Mr K. Morishita for technical assistance. This work was supported by Grants-in-Aid for Scientific Research (no. 19592391) from the Ministry of Education, Science, Sports and Culture of Japan.

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