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Stretching modulates oxytalan fibers in human periodontal ligament cells

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Background and Objective: Oxytalan fibers, as well as collagen fibers, are the structural components of periodontal ligaments. Periodontal ligaments are continuously exposed to various functional forces. However, the behavior of oxytalan fibers under mechanical strain has not been investigated. We hypothesized that strain would alter the amount and appearance of oxytalan fibers in terms of positivity for their major components, fibrillin-1 and fibrillin-2.

Material and Methods: We subjected periodontal ligament fibroblasts to stretching strain to examine the effects on their formation of oxytalan fibers in cell/matrix layers.

Results: Stretching increased the levels of fibrillin-1 and fibrillin-2 by 25% relative to the control, but did not affect the gene expression level of either type of fibrillin. Immunofluorescence and immunogold electron microscopy analysis revealed that bundles of oxytalan fibers became thicker under stretching conditions.

Conclusion: These results suggest that tension strain functionally regulates microfibril assembly in periodontal ligament fibroblasts and thus may contribute to the homeostasis of oxytalan fibers in periodontal ligaments.

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The elastic system fibers that give tissue resilience and flexibility comprise three types – oxytalan, elaunin and elastic – differing in their relative proportions of microfibrils and elastin (1). Oxytalan fibers are composed of pure microfibrils. Among the microfibrillar molecules, fibrillin-1 and fibrillin-2 are the best characterized (2) and are known to interact to form homotypic and heterotypic dimers (3,4). Fibrillins are assembled pericellularly into microfibril arrays that appear to undergo maturation into transglutaminase-crosslinked microfibrils (2). The microfibrils act as a structural template on which elastin is deposited during elastic fiber formation.

Oxytalan fibers were first described in periodontal ligaments by Fullmer & Little (5). Oxytalan fibers in periodontal ligaments are arranged in a vertically oriented interlacing network enclosing the molar root apex (6). The oxytalan fibers of periodontal ligaments are constantly exposed to mechanical stimulation. To study the metabolism of periodontal ligaments, the Flexercell system was applied to examine the expression of extracellular matrix components and matrix metalloproteinases (MMPs) of periodontal ligament fibroblasts (7,8). However, very little data are currently available on the functional metabolism of oxytalan fibers in periodontal ligaments.

In the 1970s, Sims proposed that oxytalan fibers in periodontal ligaments may support vascular orientation and regulate vascular flow (6,9). Oxytalan fibers in dog incisor periodontal ligaments subjected to orthodontic force were observed to be increased in both number and size (10). We have previously examined the degradation of oxytalan fibers in human periodontal ligament fibroblasts under normal culture conditions (11–13). To date, however, little information is available on the behavior of oxytalan fibers subjected to mechanical strain. The goal of the present study was to test the hypothesis that mechanical loading by application of

strain to periodontal ligament fibroblasts influences the amount and 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 minimum essential medium (ICN Biomedicals Inc., Aurora, OH, USA) supplemented with 10% newborn calf serum (Life Technologies, Inc., Grand Island, NY, USA) at 37°C in humidified air containing 5% CO_2. When outgrowth of the cells reached confluence, they were harvested with 0.025% trypsin (Life Technologies, Inc. Grand Island, NY, USA) in phosphate-buffered saline and transferred to plastic culture dishes at a 1:4 split ratio. For experiments, the cells were used from passages 3 to 6 to retain their phenotypes, as described previously (11).

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 then 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.

Gelatin zymography

On day 2 of culture, MMP-2 in the conditioned medium was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis zymography. Also on day 2 of culture, periodontal ligament fibroblasts were exposed to minimal essential medium for 12 h. Then, the collected medium (5 μg) was electrophoresed, without reduction, on sodium dodecyl sulfate – polyacrylamide gels prepared with 10% acrylamide containing 0.1% gelatin. The sodium dodecyl sulfate was removed by a 1-h incubation in 2.5% Triton X-100, then the gels were incubated in 30 mM Tris–HCl (pH 7.4), 200 mM NaCl, 5 mM $CaCl_2$ and 1 mM $ZnCl_2$ at 37°C for 24 h prior to staining with Coomassie Blue. Matrix metalloproteinase-2 at 68 kDa molecular weight was visualized as zones of gelatin clearance within the gels.

Western blot analysis

The cell/matrix lysates (5 μg) at 7 d of culture were subjected to western blot analysis, as described previously (13). The antibodies we used were monoclonal (anti-human MMP-2) IgG (Fuji Chemical Ind., Ltd., Toyama, Japan), polyclonal (anti-human fibrillin-1, -2) IgG (Elastin Products Co., Owensville, MO, USA) and polyclonal anti-(β-actin) polyclonal IgG (Sigma). We had already confirmed the specificity of the fibrillin-1 and fibrillin-2 antibodies, as reported previously (11). Densitometric semiquantitative analysis of the signals was performed using the NIH IMAGE program (National Institutes of Health, Bethesda, MD, USA) after finding the linear portion by sequential dilution of the proteins.

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, which was performed as described previously (12). The probes for recognition of human fibrillin-1 and fibrillin-2 were generated as described previously (12). The RNA probe for β-actin was from Roche Molecular Biochemicals (Mannheim, Germany).

Immunofluorescence

At 7 d of culture, periodontal ligament fibroblasts were fixed in ice-cold 4% paraformaldehyde for 15 min, then washed with phosphate-buffered saline. Nonspecific immunoreactivity was blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Then, the cell layers were incubated for 1 h at room temperature with the primary antibodies against polyclonal anti-(human fibrillin-1, -2) IgG (Elastin Products Co.). Controls included the use of pre-immune normal rabbit IgG for the primary antibody incubation. After washing three times with phosphate-buffered saline, the cells were incubated with secondary antibodies (goat anti-rabbit-labeled IgG with Alexa 488; Invitrogen, Eugene, OR, USA). After the final wash, cells were viewed using a confocal microscope (MRC-1024; Bio-Rad, Hemel Hempstead, UK).

Immunoelectron microscopy

At 7 d of culture, periodontal ligament fibroblast layers were fixed for 30 min in phosphate-buffered saline containing 2% paraformaldehyde and 0.1% glutaraldehyde. The cells were then incubated with primary antibodies (fibrillin-1 and fibrillin-2; 1:80 dilution) for 2 h, followed by incubation with gold particle-conjugated anti-rabbit IgG (BB International, Cardiff, UK) at 4°C. Controls included the use of pre-immune normal rabbit IgG for the primary antibody

incubation. Then the cells were 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 the JEOL JEM 1400 transmission electron microscope, Tokyo, Japan. Results shown in all figures are representative of at least three independent analyses.

Results

Stretching does not stimulate gene expression of fibrillins-1 and -2, but increases their deposition in cell/matrix layers

We first examined MMP-2 secretion and deposition at 2 d of culture by using gelatin zymography and western blot analysis, respectively (Fig. 1). The gelatin zymography showed that the 72-kDa pro-MMP-2 was present in the stretch sample than in the control sample, as shown by a more intense band in the stretch sample than in the control (Fig. 1A). Immunoblotting demonstrated that 2.5 times more pro-MMP-2 was present in the stretch samples than in the control samples (Fig. 1B). These results show that this culture system is consistent with the Flexercell Strain system.

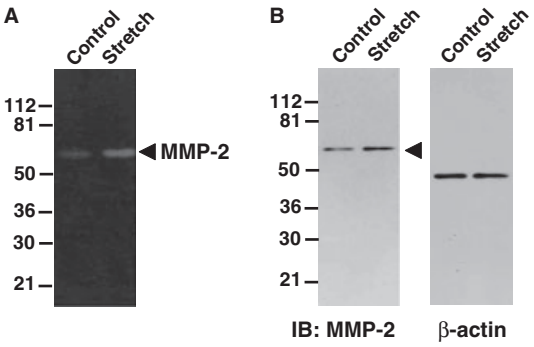

Fig. 1. Stretching stimulates matrix metalloproteinase (MMP)-2 secretion and deposition. (A) Serum-free culture medium was collected from control and stretched periodontal ligament fibroblasts at 2 d of culture. The harvested media (5 μg) were subjected to gelatin zymography. (B) Immunoblotting of MMP-2. Periodontal ligament fibroblasts were cultured for 2 d. Five micrograms of cell lysate was electrophoresed on 10% polyacrylamide gels and immunoblotted for MMP -2 analyses. Equivalent protein loading was confirmed by β-actin. The position of the prestained molecular weight markers are indicated on the left-hand side of the panel. IB, immunoblot.

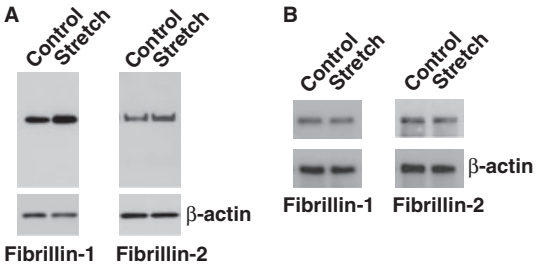

Fig. 2. Stretching stimulates oxytalan fiber deposition. (A) Western blots of cell lysates of periodontal ligament fibroblasts cultured for 7 d under nonstretch (control) and stretch conditions. Stretching induced the deposition of both fibrillin-1 and fibrillin-2. Densitometric analysis of changes in the levels of fibrillins-1 and -2 was conducted using National Institutes of Health imaging software, normalized relative to the level of β-actin. (B) Northern blots of RNA samples (1 μg) extracted from periodontal ligament fibroblasts subjected to stretching stress. Stretching did not induce gene expression of either fibrillin-1 or fibrillin-2.

We next compared, by western blot analysis, the effects of stretching on the deposition of fibrillins-1 and -2 in the cell/matrix layers at 7 d of culture (Fig. 2A). Changes in the intensities of the signals were compared, using the signal for β-actin as an internal control. Densitometric analysis of the western blots identified significant increases in the expression of fibrillins-1 and -2, by 27 ± 5% and 23 ± 6%, respectively, in the stretched samples compared with the nonstretched samples (control). To examine whether these effects were caused by a difference in the gene expression level, we performed northern blotting. The northern blots showed that stretching did not induce gene expression of either type of fibrillin compared with the control (Fig. 2B).

Stretching induces accumulation of microfibril bundles

Next, we examined the appearance of oxytalan fibers using immunofluorescence and immunogold electron microscopy. Immunofluorescence images revealed that stretching appeared to induce gathering of fibrillin-1-positive and fibrillin-2-positive microfibril bundles (Fig. 3). No labelling was detected in the control immune serum (data not shown). Immunoelectron microscopy analysis provided further support for thickening of microfibril bundles positive for fibrillin-1 and fibrillin-2 in the stretched samples, in comparison with the nonstretched samples (Fig. 4). No labelling was detected in the control immune serum (data not shown).

Discussion

In the present study, by subjecting periodontal ligament fibroblasts to stretch stimulation, we demonstrated, for the first time, that the extracellular deposition of fibrillin-1 and fibrillin-2 is increased by this type of mechanical stress. The fact that cell stretching did not affect the gene-expression levels of either type of fibrillin suggests that fibrillin deposition may be controlled intracellularly or extracellularly in the process of deposition into oxytalan fibers.

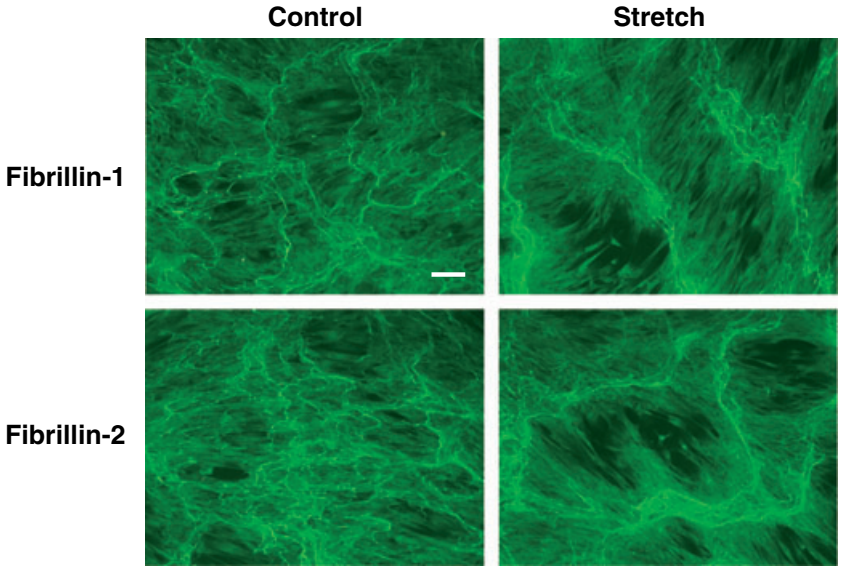

Fig. 3. Immunofluorescence of fibrillin-1 and fibrillin-2 in nonstretched and stretched periodontal ligament fibroblasts. Periodontal ligament fibroblasts subjected to nonstretching (control) (left panels) and stretching (right panels) treatments are labeled for fibrillin-1 (upper panels) and fibrillin-2 (lower panels). The scale bar corresponds to 10 μm.

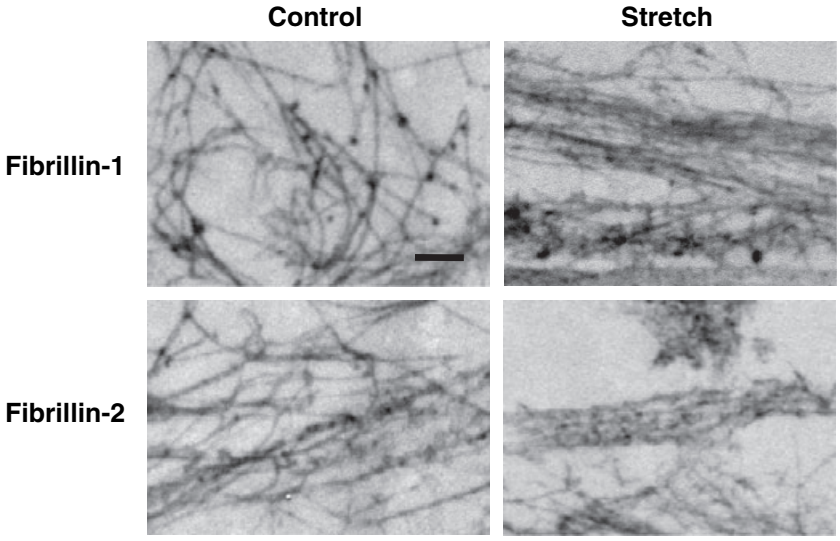

Fig. 4. Immunogold labeling of fibrillin-1 and fibrillin-2 in periodontal ligament cultures under nonstretching and stretching conditions. Periodontal ligament fibroblasts subjected to nonstretching (control) (left panels) and stretching (right panels) conditions were immunolabeled and examined by transmission electron microscopy. Clusters of gold particles indicate the presence of bound anti-fibrillin-1 (upper panels) and anti-fibrillin-2 (lower panels). The scale bar corresponds to 200 nm.

The method we used to apply stretching to the cells has been used by many researchers (14,15) and allows the stretch tension to be applied to the cells uniformly over the whole membrane area. Therefore, stable biochemical and histological results can be obtained with minimal variation. In the present study we found that a greater amount of MMP-2 was secreted by in the stretched cells than in the control cells, in agreement with the finding by Bolcato-Bellemin *et al*. (8). Therefore, this stretch system is thought to act similar manner to the Flexercell system. The appearance of periodontal ligament fibroblasts in the stretched chambers seemed to be different from the random uniform appearance evident in the nonstretched chambers. Immunofluorescence analysis showed that oxytalan fibers positive for fibrillins-1 and -2 were arranged along the stretched cells and that the bundles of microfibrils appeared to gather together. On the other hand, in the nonstretched cells, the microfibrils appeared to be relatively separate from each other. Immunoelectron microscopy images supported these observations. These results suggest that stretch stimulation may control the assembly of microfibrils.

A number of previous studies have investigated the expression of extracellular matrix components and MMPs in periodontal ligament fibroblasts under stretch conditions (7,8,16). However, there are little data on oxytalan fibers in periodontal ligaments subjected to such stress. This may be a result of the difficulty in determining the role of oxytalan fibers in periodontal ligament, where they are oriented vertically in an interlacing network enclosing the molar root apex (6). Some oxytalan fibers extend from the dentino–cemental junction to the vessels adjacent to the alveolar walls. Therefore, some researchers believe that oxytalan fibers may support vascular vessels and regulate blood flow. Sims (6) also observed that, under occlusal loads, oxytalan fibers in humans tend to relax in a similar way to collagen fibers in the tensioned periodontal ligament. Moreover, oxytalan fibers in dog periodontal ligaments have been observed to increase in both size and number under orthodontic force (10). In the present study, we demonstrated, for the first time, that the amount of oxytalan fibers increased significantly under stretched conditions *in vitro*, whereas the gene expression of fibrillins was unaffected. This increase may be a result of the upregulation of fibrillin-binding molecules, such as fibulin-5 (17) and versican (18), etc. Further work will be required to identify the factors that control the

formation of oxytalan fibers under stretching stress.

In conclusion, we have obtained evidence that under stretch conditions the extracellular deposition of both fibrillin-1 and fibrillin-2 increases in human periodontal ligament fibroblast cultures, and that microfibrils appear to thicken. These results demonstrate that tension strain upregulates microfibril assembly in periodontal ligament fibroblasts and thereby may contribute to the homeostasis of oxytalan fibers in periodontal ligaments.

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References

1. Mecham RP, Davis EC. Elastic fiber structure and assembly. In: Yurchenco PD, Birk DE, Mecham RP, eds. *Extracellular Matrix Assembly and Structure*. New York: Academic Press, 1994:281–314.
2. Kielty CM. Elastic fibres in health and disease. *Expert Rev Mol Med* 2006;**8:**1–23.
3. Lin G, Tiedemann K, Vollbrandt T *et al*. Homo- and heterotypic fibrillin-1 and -2 interactions constitute the basis for the assembly of microfibrils. *J Biol Chem* 2002;**277:**50795–50804.
4. Charbonneau NL, Dzamba BJ, Ono RN *et al*. Fibrillins can co-assemble in fibrils, but fibrillin fibril composition displays cell-specific differences. *J Biol Chem* 2003;**278:**2740–2749.
5. Fullmer HM, Lillie RD. The oxytalan fiber: a previously undescribed connective tissue fiber. *J Histochem Cytochem* 1958;**6:**425–430.
6. Sims MR. Oxytalan fiber system of molars in the mouse mandible. *J Dent Res* 1973;**52:**797–802.
7. Howard PS, Kucich U, Taliwal R, Korostoff JM. Mechanical forces alter extracellular matrix synthesis by human periodontal ligament fibroblasts. *J Periodontal Res* 1998;**33:**500–508.
8. Bolcato-Bellemin AL, Elkaim R, Abehsera A *et al*. Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. *J Dent Res* 2000;**79:**1712–1716.
9. Sims MR. Oxytalan-vascular relationships observed in histologic examination of the periodontal ligaments of man and mouse. *Arch Oral Biol* 1975;**20:**713–716.
10. Fullmer HM, Sheetz JH, Narkates AJ. Oxytalan connective tissue fibers: a review. *J Oral Pathol* 1974;**3:**291–316.
11. Tsuruga E, Irie K, Sakakura Y, Yajima T. Expression of fibrillins and tropoelastin by human gingival and periodontal ligament fibroblasts in vitro. *J Periodontal Res* 2002;**37:**23–28.
12. Tsuruga E, Irie K, Yajima T. Gene expression and accumulation of fibrillin-1, fibrillin-2, and tropoelastin in cultured periodontal fibroblasts. *J Dent Res* 2002;**81:**771–775.
13. Tsuruga E, Irie K, Yajima T. Fibrillin-2 degradation by matrix metalloproteinase-2 in periodontium. *J Dent Res* 2007; **86:**352–356.
14. Danciu TE, Adam RM, Naruse K, Freeman MR, Hauschka PV. Calcium regulates the PI3K-Akt pathway in stretched osteoblasts. *FEBS Lett* 2003;**536:**193–197.
15. Sasamoto A, Nagino M, Kobayashi S *et al*. Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch. *Am J Physiol Cell Physiol* 2005;**288:**C1012–C1022.
16. Pavlin D, Gluhak-Heinrich J. Effect of mechanical loading on periodontal cells. *Crit Rev Oral Biol Med* 2001;**12:**414–424.
17. Freeman LJ, Lomas A, Hodson N *et al*. Fibulin-5 interacts with fibrillin-1 molecules and microfibrils. *Biochem J* 2005; **388:**1–5.
18. Isogai Z, Aspberg A, Keene DR *et al*. Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* 2002;**277:**4565–4572.