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Differential regulation of collagen, lysyl oxidase and MMP-2 in human periodontal ligament cells by low- and high-level mechanical stretching

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Background and Objective: Mechanical stretching modulates extracellular matrix (ECM) protein synthesis by periodontal ligament (PDL) cells. However, the mechanoregulation of lysyl oxidase (LOX), a key enzyme for collagen cross-linking, is not fully understood. In the present study, we hypothesized that low-level and high-level mechanical stretching differentially regulates collagen deposition and the expression of LOX and the enzymes responsible for ECM degradation, such as MMP-2 in PDL cells.

Material and Methods: Human PDL cells were cultured on flexible-bottom culture plates and subjected to cyclic mechanical stretching (3% and 10% elongation at 0.1 Hz) for 24 and 48 h in a Flexercell FX-4000 strain unit. The levels of expression of type I collagen alpha 1 (*COL1A1*), type III collagen alpha 1 (*COL3A1*), lysyl oxidase (*LOX*), *MMP2* and *TIMP2* mRNAs were analyzed using an RT-PCR technique. The cell layer and the culture medium were separately collected and processed for detection of the following ECM-related molecules: (i) total collagen content using a Sircol dye-binding method; (ii) LOX protein expression by western blotting; (iii) LOX activity using a fluorometric assay; and (iv) MMP-2 enzyme activity by gelatin zymography.

Results: Low-level (3%) mechanical stretching of PDL cells upregulated the expression of *COL1A1*, *COL3A1* and *LOX* mRNAs, enhanced the production of collagen and increased the LOX activity but did not change the level of expression of *MMP2* or *TIMP2* mRNA. The collagen content and LOX activity showed obvious elevation in the medium, but not in the cell layer. High-level (10%) mechanical stretching downregulated *COL1A1* mRNA but upregulated *COL3A1* mRNA; however, the effect on *COL3A1* was smaller, and occurred earlier, compared with the effect on the *COL1A1* gene. High-level mechanical stretching upregulated the expression of *MMP2* and *TIMP2* mRNAs but did not change collagen production or LOX activity. Moreover,

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high-level mechanical stretching increased the level of pro-MMP-2, especially in the cell layer.

Conclusions: This study substantiates the mechanoregulation of the expression of ECM-related molecules in PDL cells. High-level mechanical stretching upregulated the expression of *MMP2* and *TIMP2* mRNAs, but did not affect collagen production or LOX activity. In addition to increasing the transcription of *COL1A1*, *COL3A1* and *LOX* genes, low-level mechanical stretching enhanced total collagen production and LOX activity, which should favor ECM stabilization. As an effective regulator of ECM remodeling, mechanical stretching can be exploited in periodontal regeneration and ligament tissue engineering via application of appropriate mechanical stimulation.

The periodontal ligament (PDL) is a dense, cell-rich connective tissue capable of sustaining mechanical loading that occurs during mastication and orthodontic tooth movements. PDL cells, predominantly fibroblasts, are exposed to dynamic mechanical signals of different levels of force and mediate the homeostasis and remodeling of both the PDL and adjacent hard tissues (1,2). The production of extracellular matrix (ECM) proteins by PDL cells is regulated by mechanical stimulation, such as compressive, tensional (3) and gravitational forces (4). Moreover, PDL cells are differentially responsive to varying levels of mechanical strain associated with growth substrate deformation (3,5–7).

The major fibrous ECM components in the PDL are collagens (types I and III), which play major roles in resisting mechanical tensional forces and maintaining teeth in the alveolar socket. Collagen biosynthesis involves many intracellular and extracellular post-translational modifications. Collagen fibrils are assembled from tropocollagen and are secreted as procollagens, which consist of a large triple helical rod-shaped domain flanked by amino- and carboxy-terminal globular peptides. These two peptidic extensions ensure solubility in physiological conditions to prevent intracellular molecule assembly. The amino- and carboxy-terminal peptides are cleaved before assembly of collagen fibers in the extracellular space. The extracellular aggregation of fibrillar collagen molecules is followed by covalent intramolecular and intermolecular cross-linking between lysyl and hydroxylysyl residues after oxidative deamination by lysyl oxidase (LOX) (8,9). Treatment of rats with beta-aminopropionitrile (β-APN, a potent inhibitor of LOX) resulted in accelerated tooth movement, presumably because of decreased collagen cross-linking in the PDL (10). A tension–relaxation study on dermal fibroblasts revealed that mechanical tension may modulate ECM stiffness by regulating LOX expression (11). However, mechanoregulation of the expression of LOX in human PDL cells is not fully understood.

In contrast to the regulation of ECM cross-linking by LOX, ECM remodeling processes require the collaborative action of MMPs for ECM protein degradation (12). MMP-2 and MMP-9, also known as gelatinases A and B, share similar proteolytic activities and degraded gelatins (denatured collagens) and ECM molecules including native type IV, V and XI collagens (13). The latent form of MMP-2 (pro-MMP-2; 72kDa) is activated on the cell surface by membrane type 1 (MT1)-MMP, in a process regulated by TIMP-2 (14). A low level of TIMP-2 is involved in the MT1-MMP-dependent activation of MMP-2. In contrast, a high level of TIMP-2 blocks this activation of MMP-2 and the enzyme activity of the fully active form of MMP-2.

The effects of mechanical stress on cultured PDL cells depend on its type, magnitude, frequency and duration. Continuous mechanical stretching was reported to upregulate the level of *MMP2* mRNA in PDL cells (5). A cyclic tensional force also increased the level of *MMP2* mRNA, but not of MMP-2 protein expression in PDL cells (3). In the present study, we hypothesized that low-level and high-level mechanical stretching would differentially regulate the production of collagen, and the expression of LOX and of enzymes responsible for ECM degradation, such as MMP-2. Our study revealed that low-level and high-level mechanical stretching differentially modulated ECM proteins by controlling the expression of its major constituent (i.e. collagen) and of enzymes that participate in ECM stabilization and degradation.

Material and methods

Cell culture and mechanical stretching

The PDL cells were cultured from PDL tissues of premolar roots obtained from three healthy young orthodontic patients according to previously described methods (15). Institutional Review Board approval was obtained. The premolars were washed twice with phosphate-buffered saline (PBS) in a vortex mixer to remove blood, and the PDL tissue attached to the middle-third of the root was then removed using a surgical scalpel. To avoid contamination with cells of the gingiva, nerves and blood vessels, the coronal and apical portions of the root were not used. The PDL tissue was cut into 1-mm^2 chips, collected by centrifugation and placed in

culture dishes. The tissue fragments were then covered with sterilized glass coverslips. The medium used was Dulbecco's modified Eagle's medium supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal bovine serum. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. After the PDL cells had grown from explants and reached confluence, they were detached with 0.05% trypsin in EDTA and subcultured at a ratio of 1 : 3. Cells from passages four to eight were used in this study.

PDL cells were plated onto flexible-bottom six-well plates (coated with type I collagen, BioFlex-I; Flexcell International, McKeesport, PA, USA) at an initial density of 2×10^5 cells/well and allowed to achieve confluence. Then, the cells were cultured in low-serum medium (2% fetal bovine serum) to minimize the influence of serum and other growth factors. After 24 h, the medium was replaced with fresh low-serum medium, and the cells were subjected to cyclic mechanical stretching [3% and 10% elongation of the growth substratum at 0.1 Hz for 24 and 48 h using a Flexercell Strain Unit (FX-4000; Flexcell International)]. The strain unit is a computer-controlled device that subjects the cultured cells to mechanical strain by applying vacuum pressure to stretch the flexible membranes of the culture wells. Biaxial stretching was delivered with a sinusoidal-shaped waveform. The frequency was set to 0.1 Hz (six cycles/min) according to previous reports (16,17). Therefore, the duration of 10 s in a cycle consisted of 5 s of stretching and 5 s of relaxation. Control cells were cultured on similar plates and kept in the same incubator but were not exposed to mechanical stretching. After the experimental periods indicated, the culture medium and the cell layer were separately collected for further analysis, as described below.

RNA isolation and RT-PCR

The levels of mRNA expressed from type I collagen alpha 1 (*COL1A1*), type III collagen alpha 1 (*COL3A1*), *LOX*, *MMP2* and *TIMP2* were analyzed using RT-PCR, as described previously (18). Total cellular RNA was prepared from PDL cells using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). After lysis for 1 min, cells were transferred to centrifuge tubes. Chloroform was added and mixed with the lysed cells, which were incubated at room temperature for 5 min and then centrifuged at 12,000 ***g*** for 30 min at 4°C. The aqueous phase was transferred to new tubes, each containing 400 μL of isopropanol, and centrifuged at 12,000 ***g*** for 30 min at 4°C. The supernatant was removed and the RNA pellet was washed with 400 μL of 75% ethanol. The RNA pellet was then dissolved in 10 μL of water containing 0.01% diethylpyrocarbonate and stored in a −80°C freezer until required.

One microgram of RNA was reverse transcribed using an RT-PCR system from Promega (Madison, WI, USA). The reaction was carried out in a PTC-200 PCR machine (MJ Research, Watertown, MA, USA) with the following parameters: 25°C for 5 min, 42°C for 60 min, 70°C for 15 min and 4°C to complete the reaction. The complementary DNA target (2 λ) was mixed with 20 pmol each of the sense and antisense primers and polymerase, followed by amplification. The sequence of primers for each gene, the annealing temperature, the cycle number of the PCR and the expected size of the PCR product are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene. PCR products were identified and analyzed using ALPHAIMAGER 3300 software (Alpha Innotech, San Leandro, CA, USA) after electrophoretic separation on a 1.5% agarose gel and staining with 0.5 mg/mL of ethidium bromide.

Measurement of collagen content

After being subjected to mechanical stretching, as described above, collagen released into the culture medium and that recovered from newly formed ECM in the cell layer were individually measured using a Sircol™ Collagen Assay (Biocolor,

Table 1. Primers used for the PCR

Gene	PCR primer sequence	Annealing temperature (°C)	Cycle number	Product size (bp)
COL1A1	S 5′-CTGGCA AAGAAGGCGGCA AA-3′ (20) A 5′-CTCACCACGATCACCACTCT-3′ (20)	58	30	502
COL3A1	S 5′-CAGTATTCTCCACTCTTGAGTTCAG-3′ (25) A 5′-GGTGACAAAGGTGAAACAGGTGAAC-3′ (25)	60	30	555
LOX	S 5′-CCCCTACAAGTACTCTGACGACAA-3′ (24) A 5′-CGCCGCCATCTCAGGTTGTACAT-3′ (23)	66	30	263
MMP2	S 5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′ (25) A 5′-GTGCTGAAGGACACACTAAAGAAGA-3′ (25)	58	30	580
TIMP2	S 5′-AAACGACATTTATGGCAACCCTATC-3′ (25) A 5′-ACAGGAGCCGTCACTTCTCTTGATG-3′ (25)	56	30	405
GAPDH	S 5′-GTC TTCACCACCATGGAGAAGGCT-3′ (24) A 5′-CATGCCAGTGAGCTTCCCGTT-3′ (21)	58	30	392

COL1A, type I collagen alpha 1; COL3A, type III collagen alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LOX, lysyl oxidase; MMP2, matrix metalloproteinase 2; TIMP2, tissue inhibitor of metalloproteinase-2; S, sense; A, antisense.

Newtownabbey, UK). The mode of action of this quantitative dye-binding method is based on the reaction of the anionic dye reagent with the side-chain groups of basic amino acids present in collagen. The specific affinity of the dye for collagen under the assay conditions is a result of the elongated dye molecules becoming aligned in parallel with the long, rigid helical structure of native collagen.

To investigate the effect of mechanical stretching on collagen production by PDL cells, the contents of collagen in the cell layer and in the culture medium were separately assessed. The collagen coated on the blank Bioflex-I culture plate was subtracted from the content of collagen in the cell layer. Therefore, the difference between experimental and control groups should be attributed to mechanical stretching because cells in the control group were also cultured on BioFlex-I plates. The collagen content in the unstretched control group was set to 100%, and that of the experimental groups was expressed as multiples of the respective control.

Measurement of LOX activity

LOX activity was determined in both the culture medium and the cell layer by using a fluorescence assay that utilizes 1,5-diaminopentane as the substrate according to the method described by Palamakumbura and Trackman (19). The released hydrogen peroxide was detected using Amplex Red in horseradish peroxidase (HRP)-coupled reactions. In brief, 100 μL of samples with known protein concentrations were incubated in buffer containing 1.2 M urea, 0.05 M sodium borate (pH 8.2), 1 U/mL of HRP, 10 μM Amplex Red and 10 mM diaminopentane in a final volume of 200 μL at 37°C for 20 min. The fluorescence intensities were then recorded with respective excitation and emission wavelengths of 560 and 590 nm using a microplate reader (BMG Labtechnologies, Durham, NC, USA). Purified bovine LOX (1.5 μg/mL) was used as a positive control. Each assay was monitored in parallel with an identical assay solution containing 500 μM β-APN as a specific inhibitor of LOX. The difference in the fluorescence activity (with and without β-APN) was used to calculate the LOX activity. Then, the results of the stretched groups were presented as multiples over the respective control.

Electrophoresis and western blot analysis

The expression of LOX protein in stretched PDL cells and in the culture medium was assessed separately using western blot analysis. At the end of the experimental periods (24 and 48 h), whole-cell extracts were prepared by harvesting cells in ice-cold RIPA lysis buffer [150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM EDTA and 0.05% sodium dodecyl sulfate (SDS), pH 7.5] with sonication for 10 s. Then, the cell lysates were centrifuged at 15,000 ***g*** for 20 min at 4°C. The protein concentration of the resulting supernatant was determined using a bicinchoninic acid protein assay kit. To assess the amount of LOX protein released into the extracellular space, the culture medium was concentrated by a factor of 50 using Amicon Ultra-4 centrifugal filter units (Millipore, Billerica, MA, USA) and processed, as follows, to determine the LOX protein expression. In brief, 50–100 μg of the protein (whole-cell extract or concentrated medium) was diluted in 3× Laemmli sample buffer, denatured at 95°C for 5 min, resolved by electrophoresis on a 10% SDS polyacrylamide gel and electrophoretically transblotted onto polyvinylidene difluoride microporous membranes. The membranes were incubated overnight in blocking solution (10% nonfat dried milk in PBS) and were then probed with a primary antibody against human LOX (1 : 1000 dilution) and tubulin (1 : 10000 dilution) for 1 h at room temperature. After three washes with 0.5% Tween 20 in PBS (PBST), the membranes were incubated with HRP-conjugated secondary IgG (1 : 10000) for 1 h, followed by another three washes with PBST. Immunoreactive bands were detected using Immobilon Western Chemiluminescent HRP Substrate® (Millipore) and quantitatively analyzed in triplicate by normalizing the band intensities to the controls on scanned films using Alpha Image® software. The expected molecular mass was 37 kDa for LOX and 55 kDa for tubulin, which was used as an internal control.

Gelatin zymography

To evaluate whether 3% and 10% mechanical stretching differentially regulated the expression of pro-MMP-2 by PDL cells, both the cell lysate and the culture medium were separately analyzed by SDS-polyacrylamide gel electrophoresis gelatin zymography. Briefly, samples were normalized to an equal amount of protein (50 μg), denatured in the absence of a reducing agent and electrophoresed in an 8% SDS polyacrylamide gel containing 0.15% (weight by volume) gelatin. The gels were incubated in the presence of 2.5% Triton X-100 at room temperature for 2 h and subsequently at 37°C overnight in buffer containing 50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl and 0.02% NaN_3. Thereafter, the gels were stained with 0.25% Coomassie Blue, and proteolysis was detected as a white band against a blue background. Images of gels were acquired and analyzed using ALPHAIMAGER 3300 software.

Statistical analysis

Multiples of the stretched groups (3% or 10%) over the unstretched control were determined. Statistical analysis was performed using the unpaired Student's *t*-test. A p value of < 0.05 was considered statistically significant.

Results

Cell morphology and orientation

Morphological changes of PDL cells, especially the alignment, were noted irrespective of whether 3% or 10% stretching was applied for 48 h

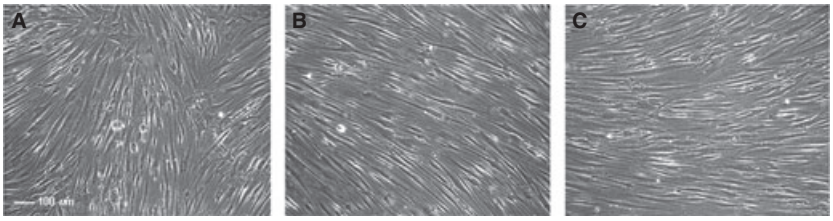

Fig. 1. Morphological changes and realignment of periodontal ligament cells subjected to cyclic mechanical stretching. (A) Unstretched control; (B) 3% stretching; and (C) 10% stretching.

(Fig. 1). Unstretched control cells remained randomly oriented (Fig. 1A). After being subjected to cyclic mechanical stretching, PDL cells in the peripheral region of the culture well became more slender and aligned (Fig. 1B and 1C).

Regulation of the expression of mRNA from *COL1A1*, *COL3A1*, *LOX*, *MMP2* and *TIMP2* genes by mechanical stretching

Expression of mRNAs from *COL1A1*, *COL3A1*, *LOX*, *MMP2* and *TIMP2* genes was examined using a semiquantitative RT-PCR for PDL cells subjected to 3% or 10% mechanical stretching. At 48 h, the expression of *COL1A1* mRNA was significantly upregulated in 3% stretched cells, but downregulated in 10% stretched cells (Fig. 2A). In contrast to *COL1A1*, the *COL3A1* gene was more responsive at an earlier time point and was significantly upregulated at 24 h by both 3% and 10% stretching; however, the response was lower than that of *COL1A1* at 48 h (Fig. 2B). Interestingly, the expression of mRNA from the *LOX* gene was upregulated by 3% stretching, but not by 10% stretching (Fig. 2C). The expression of mRNA from neither the *MMP2* gene nor the *TIMP2* gene was affected by 3% stretching, but both showed obvious upregulation by 10% stretching at 24 h (Fig. 3).

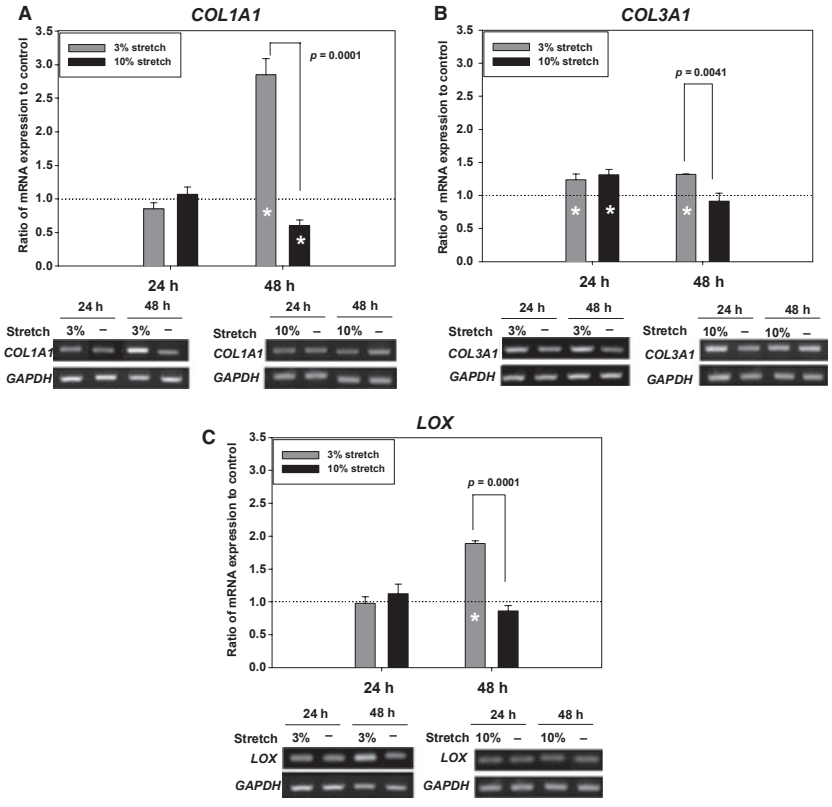

Fig. 2. Mechanical stretching differentially regulated the expression of mRNA from type I collagen (*COL1A1*), type III collagen (*COL3A1*) and lysyl oxidase (*LOX*) genes in periodontal ligament cells. After being subjected to 3% and 10% cyclic stretching for 24 and 48 h, the mRNA levels were analyzed using RT-PCR and were normalized to those of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), by band densitometry. (A) *COL1A1*. (B) *COL3A1*. (C) *LOX*. The level of expression of each mRNA in unstretched control cells was used as the denominator to calculate the relative expressions of mRNAs in the respective stretched group. Representative images of multiple repeated experiments are shown for each gene. Statistical significance in the comparison between stretched and unstretched control groups was checked using the Student's *t*-test (*$p < 0.05$). Differences between the 3% and 10% stretched groups were also checked, and $p < 0.05$ indicates statistical significance.

Collagen production in the cell layer and in the culture medium

Collagen, the most abundant ECM protein in the PDL, was measured in human PDL cells subjected to 3% and 10% stretching at 24 and 48 h. Figure 4A shows that the collagen contents of the cell layer in the stretched groups did not differ significantly from those of the unstretched controls, regardless of the duration of mechanical stretching. The content of collagen released into the culture medium was significantly increased by 3% stretching at 24 h, and the elevation had become more obvious at 48 h (Fig. 4B). However, 10% stretching did not affect collagen production at 24 or 48 h.

Stretching-induced LOX activity and expression of LOX protein

LOX is an extracellular enzyme required in the process of collagen cross-linking. Figure 5A shows the increased LOX activities in 3% stretched cells, regardless of the stretching duration (24 or 48 h). However, there was no significant change in LOX activities in cells subjected to 10% stretching. The results of the western blot analysis further revealed the stimulatory effect of 3% stretching

on LOX protein expression. In both the cell layer and the culture medium, LOX protein expression exhibited a small increase at 24 h, which had become more obvious at 48 h (Fig. 5B). In the group subjected to 10% stretching, LOX protein was not expressed in either the cell layer or the culture medium (data not shown).

Effects of mechanical stretching on MMP-2 expression

Gelatin zymography revealed that the expression of MMP-2 was enhanced more by 10% stretching than by 3% stretching. Figure 6A shows that 10% stretching induced pro-MMP-2 by approximately twofold to threefold in the cell layer at 24 h and at 48 h. A similar effect was also induced by 3% stretching, but only at the later time point (48 h). In the culture medium, pro-MMP-2 expression showed a small increase in the 10% stretching group, whereas no significant change in the pro-MMP-2 level was noted in the 3% stretching group (Fig. 6B). Moreover, an active intermediate form of MMP-2 (68 kDa) existed in the culture medium (Fig. 6C).

Discussion

The PDLs are functionally and metabolically active tissues and respond to various mechanical stresses associated with mastication and orthodontic forces. The present study clearly delineated that low-level (3%) and high-level (10%) mechanical stretching differentially regulated the transcription of mRNA from *COL1A1*, *COL3A1*, *LOX*, *MMP2* and *TIMP2* genes, collagen production, LOX enzyme activity and MMP-2 expression by human PDL cells. Specifically, low-level stretching collectively

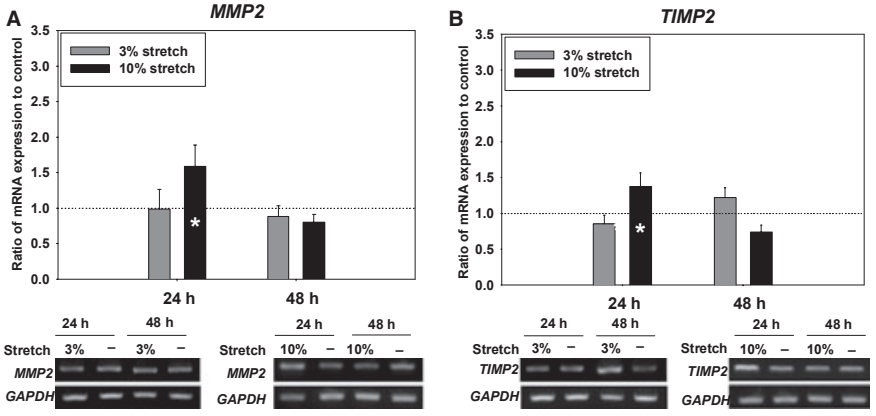

Fig. 3. Mechanical stretching differentially regulated the expression of mRNA from *MMP2* and *TIMP*2 genes in periodontal ligament cells. After being subjected to 3% and 10% cyclic stretching for 24 and 48 h, the mRNA levels were analyzed using RT-PCR and normalized to those of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), by band densitometry. (A) *MMP2*. (B) *TIMP*. The level of expression of each gene in unstretched control cells was used as the denominator to calculate the relative expression of mRNAs in the respective stretched group. Representative images of multiple repeated experiments are shown for each gene. Statistical significance in the comparison between stretched and unstretched control groups was checked using the Student's *t*-test (*$p < 0.05$). Differences between the 3% and the 10% stretched groups were also checked, and $p < 0.05$ indicates statistical significance.

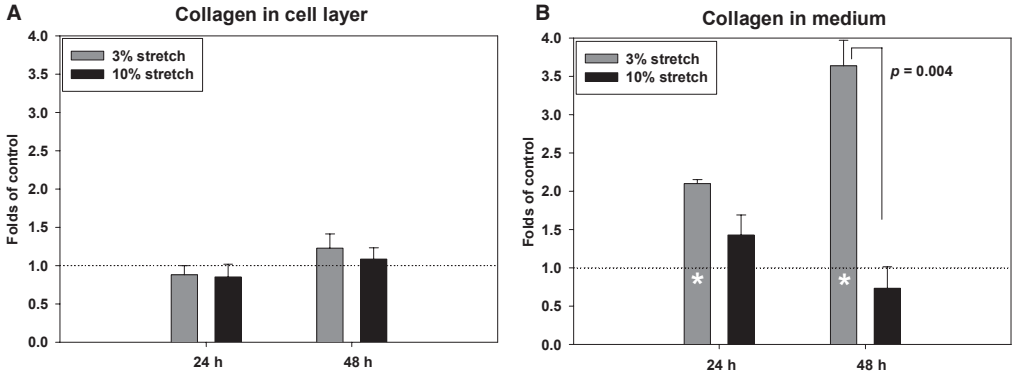

Fig. 4. Low-level (3%) mechanical stretching increased the collagen content in the culture medium of periodontal ligament cells. After cells were subjected to 3% and 10% cyclic stretching for 24 and 48 h, the collagen contents were measured using a Sircol collagen assay. (A) Collagen in the cell layer. (B) Collagen in the medium. Statistical significance of the comparison between the stretched and unstretched control groups was checked using the Student's *t*-test (*$p < 0.05$). Differences between the 3% and 10% stretched groups were also checked, and $p < 0.05$ indicates statistical significance.

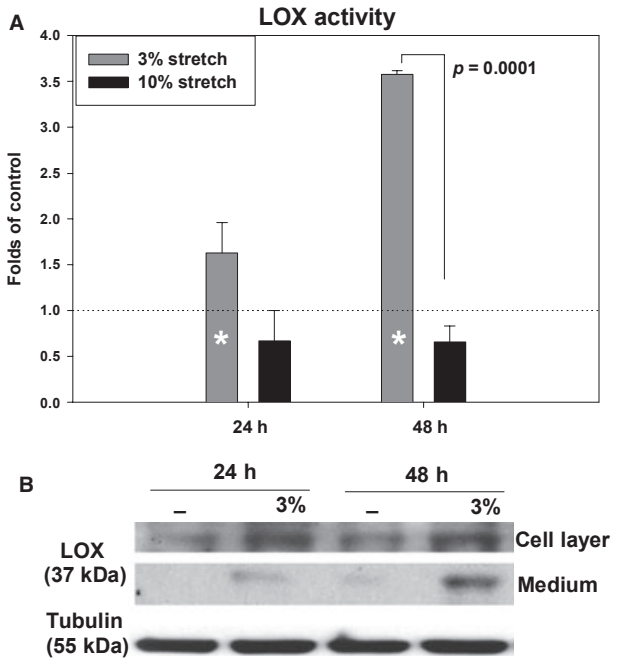

Fig. 5. Low-level (3%) mechanical stretching increased lysyl oxidase (*LOX*) enzyme activity and protein expression in periodontal ligament cells. After cells were subjected to 3% and 10% cyclic stretching for 24 and 48 h, *LOX* activities were determined using a fluorometric method. (A) *LOX* activity in the culture medium. Statistical significance in the comparison between the stretched and unstretched control groups was checked using the Student's *t*-test (*$p < 0.05$). Differences between the 3% and 10% stretched groups were also checked, and $p < 0.05$ indicates statistical significance. (B) Representative western blot showing the expression of *LOX* in the cell layer and the culture medium of periodontal ligament cells subjected to 3% stretching.

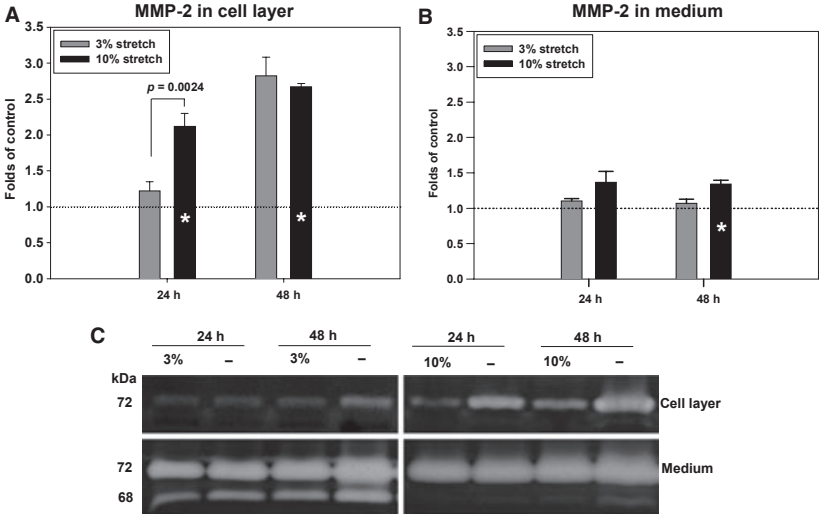

Fig. 6. Mechanical stretching activated MMP-2 expression in periodontal ligament cells. After cells were subjected to 3% and 10% cyclic stretching for 24 and 48 h, activation of MMP-2 was analyzed using gelatin zymography. (A) MMP-2 activity in the cell layer. (B) MMP-2 activity in the culture medium. (C) Representative zymographic image. Statistical significance of the comparison between the stretched and unstretched control groups was checked using the Student's *t*-test (*$p < 0.05$). The difference between the 3% and the 10% stretched groups was also checked, and $p < 0.05$ indicates statistical significance.

upregulated the expression of mRNA from *COL1A1*, *COL3A1* and *LOX* genes, enhanced total collagen synthesis and increased LOX activity by greater than threefold (Figs. 2, 4, 5). These results imply that the molecular responses induced by a low-level mechanical tensional force are orchestrated to favor ECM deposition and stabilization, which is considered crucial in periodontal tissue repair and regeneration. High-level mechanical stretching significantly downregulated the expression of mRNA from the *COL1A1* gene, upregulated the expression of mRNA from the *MMP2* and *TIMP2* genes and increased the MMP-2 level. It is conceivable that PDL cells may respond to excessive strain by expressing a proteolytic enzyme to promote ECM degradation for tissue remodeling.

The present study was designed to clarify the effects of mechanical tensional force on ECM synthesis and degradation by PDL cells. If the thickness of the PDL is taken to be 0.35 mm, a force of 500 ***g*** applied to the upper central incisor could produce tooth displacement of 0.28 mm at the alveolar crest level and that is about 23% stretching of the PDL on the tension side (20). In the case of 3% and 10% stretching, PDL stretching would correspond to a force of approximately 60 and 200 ***g***, respectively, *in vivo*. Therefore, we adopted 3% and 10% cyclic stretching to mimic low-level and high-level tension forces to which PDL cells are subjected during mastication and orthodontic tooth movements. Clearly, the forces to which PDL cells are subjected *in vitro* could be higher than the mechanical stress *in vivo* because of the presence of connective tissues *in vivo*, which may dampen the forces to which PDL cells are exposed. Our study demonstrated that 3% stretching promoted collagen production and LOX activity, and 10% stretching increased the expression of *MMP2* and *TIMP2* mRNAs without changing the collagen level or LOX activity. These results substantiate the concept that light orthodontic force should be used to avoid overstretching PDL tissues during orthodontic treatment.

Compared with type I collagen, type III collagen is described as less resistant to tensile forces, but more elastic and more flexible upon mechanical loading (21). It was reported that the synthesis of type III collagen increases in stressed PDL tissues (22). Our present study revealed that high-level mechanical stretching (10%) downregulated *COL1A1* mRNA but upregulated *COL3A1* mRNA, and that the effect on the *COL3A1* gene occurred earlier compared with the effect on the *COL1A* gene. It would be interesting to explore further if the expression of type I and III collagen proteins is similar to the mechanoregulation of the *COL1A1* and *COL3A1* genes.

Collagen cross-linking is essential to provide tissue with tensile strength, and it increases the resistance of collagen fibers against proteolysis. LOX catalyzes the first step in the cross-linking reaction of collagen and elastin and the subsequent accumulation of insoluble collagen (8,9). Treatment with β-APN, an inhibitor of LOX, inhibited collagen accumulation in the cell layer of an osteoblast culture (23). Relaxation of mechanical tension downregulated the expression of *LOX* mRNA in dermal fibroblasts (11). To our knowledge, the present study reports for the first time that low-level and high-level mechanical tensional forces differentially regulate LOX enzyme activity and protein expression in PDL cells. Low-level stretching increased the expression of LOX at the levels of transcription and translation, implying its crucial role in mechanically induced periodontal tissue remodeling.

Mechanical tension forces alter ECM protein synthesis by PDL cells (3,6). After secretion into the extracellular space, collagen is cross-linked and deposited as ECM proteins. In our study, the collagen contents in the culture medium and in the cell layer were measured separately. The results revealed no significant change in collagen deposition in the cell layer, irrespective of whether 3% or 10% stretching was applied. However, the collagen contents in the culture medium obviously increased in cells subjected to 3% stretching. In the present study, experiments were conducted over a time period of 48 h. It is likely that only a small extent of cross-linking could occur in this short period of time. If these experiments had been carried out for longer periods of time, increased levels of mature insoluble collagen would be expected in the cell layer.

ECM proteins in tissues have structural and functional roles, such as cell support, mechanical integrity and cell–matrix signaling. Mechanical signals regulate the expression of ECM protein in fibroblasts (24,25). LOX helps to form collagen cross-links within newly deposited ECM, and consequently enhances the mechanical performance of engineered tissue constructs (26). Thus, mechanical stretching-induced LOX expression and ECM cross-linking may be a promising means of improving the mechanical properties of engineered PDL tissues. It was recently reported that functional alignment of collagen fibers and PDL cells could be enhanced by applying shear stresses to engineered PDL constructs (23). In addition to stabilizing ECM molecules, the post-translational modification by LOX may trigger signal-transduction pathways responsible for mechanical stretching-induced ECM remodeling, as the interactions between cells and insoluble ECM proteins play important roles in cellular differentiation (27,28).

MMP-2, also known as gelatinase A, degrades gelatin and native type IV collagen, the major structural component of basement membranes (13). The stepwise activation of pro-MMP-2 is tightly regulated and involved with the balance of TIMP-2 and MT1-MMP (13). MT1-MMP leads to the cleavage of MMP-2, which generates an active intermediate form (64–68 kDa). Autocatalysis of the intermediate form results in the fully activated form of MMP-2 ($\sim$62 kDa) (14,29). It was reported that continuous stretching increased the expression of *MMP2* and *TIMP2* mRNAs, and also of *MMP1* and *TIMP1* mRNAs in PDL fibroblasts. However, transcription of the *MT1-MMP* gene did not change with stretching (5). Another study also reported increased transcription of *MMP-2* and *TIMP-2* genes in human PDL fibroblasts subjected to a 10% cyclic tensional force (3). In our present study, PDL cells were subjected to 3% and 10% cyclic stretching. Interestingly, our data showed that PDL cells could perceive these two different levels of mechanical stress and responded with differential levels of transcription of the *MMP2* and *TIMP2* genes. Stretching at 10% increased the expression of *MMP2* and *TIMP2* mRNAs at 24 h; by 48 h the upregulatory effect had diminished and the mRNA expression had returned to the level of the unstretched control. Moreover, no significant changes in the expression of *MMP2* and *TIMP2* mRNAs were induced by 3% stretching, irrespective of the stretching period (24 h or 48 h).

Mechanical stress plays an important role in modulating the balance between MMP-2 and TIMP-2. Our study revealed that 3% mechanical stretching increased the pro-MMP-2 levels in cell layers at the later time point (48 h), but did not change the level of expression of *MMP2* and *TIMP2* mRNAs, as described previously. In addition to upregulating the expression of the *MMP2* and *TIMP2* mRNAs, 10% mechanical stretching increased the level of pro-MMP-2, especially in the cell layer. Taken together, the level of MMP-2 was elevated more by 10% mechanical stretching than by 3% mechanical stretching (Fig. 6). As previously mentioned, 3% stretching enhanced collagen production and LOX enzyme activity, and therefore could be considered as an appropriate mechanical strain for collagen deposition and ECM maturation rather than for degradation. Further study is required to investigate whether low-level and high-level stretching differentially regulate the expression of TIMP-2 protein, which is tightly regulated with MMP2 in the complex ECM remodeling process.

In conclusion, the present study delineates that low-level and high-level mechanical stretching differentially regulates the expression of molecules responsible for collagen production, cross-linking and ECM degradation

by PDL cells. High-level stretching increased MMP-2 expression without changing collagen deposition or LOX activity. Low-level stretching acted as a positive regulator in promoting ECM formation and stabilization. Therefore, mechanical stretching can be exploited in periodontal regeneration and ligament tissue engineering via application of appropriate mechanical stimulation.

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References

1. Lekic P, McCulloch CA. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. *Anat Rec* 1996;**245**:327–341.
2. Mabuchi R, Matsuzaka K, Shimono M. Cell proliferation and cell death in periodontal ligaments during orthodontic tooth movement. *J Periodontal Res* 2002;**37**:118–124.
3. He Y, Macarak EJ, Korostoff JM, Howard PS. Compression and tension: differential effects on matrix accumulation by periodontal ligament fibroblasts *in vitro*. *Connect Tissue Res* 2004;**45**:28–39.
4. Theilig C, Bernd A, Leyhausen G, Kaufmann R, Geurtsen W. Effects of mechanical force on primary human fibroblasts derived from the gingiva and the periodontal ligament. *J Dent Res* 2001;**80**:1777–1780.
5. Bolcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. 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.
6. Howard PS, Kucich U, Taliwal R, Korostoff JM. Mechanical forces alter extracellular matrix synthesis by human periodontal ligament fibroblasts. *J Periodont Res* 1998;**33**:500–508.
7. Ozaki S, Kaneko S, Podyma-Inoue KA, Yanagishita M, Soma K. Modulation of extracellular matrix synthesis and alkaline phosphatase activity of periodontal ligament cells by mechanical stress. *J Periodont Res* 2005;**40**:110–117.
8. Ricard-Blum S, Ville G. Collagen cross-linking. *Int J Biochem* 1989;**21**:1185–1189.
9. Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* 2003;**88**:660–672.
10. Yamane A, Fukui T, Chiba M. *In vitro* measurement of orthodontic tooth movement in rats given beta-aminopropionitrile or hydrocortisone using a time-lapse videotape recorder. *Eur J Orthod* 1997;**19**:21–28.
11. Lambert CA, Colige AC, Lapiere CM, Nusgens BV. Coordinated regulation of procollagens I and III and their post-translational enzymes by dissipation of mechanical tension in human dermal fibroblasts. *Eur J Cell Biol* 2001;**80**:479–485.
12. Murray GI. Matrix metalloproteinases: a multifunctional group of molecules. *J Pathol* 2001;**195**:135–137.
13. Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med* 2008;**29**:290–308.
14. Bernardo MM, Fridman R. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP. *Biochem J* 2003;**374**:739–745.
15. Hou LT, Yaeger JA. Cloning and characterization of human gingival and periodontal ligament fibroblasts. *J Periodontol* 1993;**64**:1209–1218.
16. Myokai F, Oyama M, Nishimura F *et al.* Unique genes induced by mechanical stress in periodontal ligament cells. *J Periodontal Res* 2003;**38**:255–261.
17. Yamaguchi M, Shimizu N, Goseki T *et al.* Effect of different magnitudes of tension force on prostaglandin E2 production by human periodontal ligament cells. *Arch Oral Biol* 1994;**39**:877–884.
18. Chang HH, Wu CB, Chen YJ *et al.* MMP-3 response to compressive forces *in vitro* and *in vivo*. *J Dent Res* 2008;**87**:692–696.
19. Palamakumbura AH, Trackman PC. A fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem* 2002;**300**:245–251.
20. Muhlemann HR, Zander HA. Tooth mobility. (III) The mechanism of tooth mobility. *J Periodontol* 1954;**25**:128–137.
21. Riechert K, Labs K, Lindenhayn K, Sinha P. Semiquantitative analysis of types I and III collagen from tendons and ligaments in a rabbit model. *J Orthop Sci* 2001;**6**:68–74.
22. Duncan GW, Yen EH, Pritchard ET, Suga DM. Collagen and prostaglandin synthesis in force-stressed periodontal ligament *in vitro*. *J Dent Res* 1984;**63**:665–669.
23. Gerstenfeld LC, Riva A, Hodgens K, Eyre DR, Landis WJ. Post-translational control of collagen fibrillogenesis in mineralizing cultures of chick osteoblasts. *J Bone Miner Res* 1993;**8**:1031–1043.
24. Lambert CA, Soudant EP, Nusgens BV, Lapiere CM. Retranslational regulation of extracellular matrix macromolecules and collagenase expression in fibroblasts by mechanical forces. *Lab Invest* 1992;**66**:444–451.
25. Sarasa-Renedo A, Chiquet M. Mechanical signals regulating extracellular matrix gene expression in fibroblasts. *Scand J Med Sci Sports* 2005;**15**:223–230.
26. Elbjeirami WM, Yonter EO, Starcher BC, West JL. Enhancing mechanical properties of tissue-engineered constructs via lysyl oxidase crosslinking activity. *J Biomed Mater Res A* 2003;**66**:513–521.
27. Huang CH, Chen MH, Young TH, Jeng JH, Chen YJ. Interactive effects of mechanical stretching and extracellular matrix proteins on initiating osteogenic differentiation of human mesenchymal stem cells. *J Cell Biochem* 2009;**108**:1263–1273.
28. Chiquet M, Gelmn L, Lutz R, Mier S. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim et Biophys Acta* 2009;**1793**:911–920.
29. Shapiro S, Khodalev O, Bitterman H, Auslender R, Lahat N. Different activation forms of MMP-2 oppositely affect the fate of endothelial cells. *Am J Physiol Cell Physiol* 2010;**298**:C942–C951.

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