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Modulation of extracellular matrix synthesis and alkaline phosphatase activity of periodontal ligament cells by mechanical stress

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Background: Loss of occlusal function has been reported to induce atrophic changes in the periodontal ligament. It is likely that mechanical stress triggers the biological response of periodontal ligament. However, there have been few reports studying the correlation between mechanical stress of varying magnitude and periodontal ligament cell activities such as extracellular matrix (ECM) synthesis.

Objective: The objective of this study is to clarify the influence of the mechanical stress on changes in mRNA expression levels of type I collagen and decorin genes, as well as alkaline phosphatase (ALP) activity in response to mechanical stress of varying magnitude.

Methods: Bovine periodontal ligament cells were cultured on flexible-bottomed culture plates and placed on the BioFlex Loading Stations™. Cells were elongated at 6 cycles/min (5 s on and 5 s off) at each of six levels of stretch (0.2, 1.0, 2.0, 3.0, 10, 18% increase in the surface area of the bottom) for 48 h. We measured mRNA expression levels of type I collagen and decorin genes using quantitative reverse transcription–polymerase chain reaction (RT–PCR), and ALP activity in periodontal ligament cell culture under cyclic mechanical stretching.

Results: Mechanical tensional stress of low magnitude induced the increase of both type I collagen and decorin mRNA expression without changing ALP activity in periodontal ligament cells. Mechanical tensional stress of high magnitude induced the increase of type I collagen and decorin mRNA expression while decreasing ALP activity.

Conclusion: These results suggest that different magnitude of tensional force induces different responses from periodontal ligament cells, and that mechanical stress plays an important role in remodeling and functional regulation of periodontal ligament.

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The periodontal ligament is the connective tissue localized between the hard tissues of the tooth cementum and the alveolar bone. The periodontal ligament is composed of cellular and extracellular components. The cellular components, i.e. the periodontal ligament cells, actively synthesize and degrade extracellular matrix (ECM) molecules such as collagens or proteoglycans, and play important roles in the remodeling of periodontal tissues.

These cells include those that differentiate into cementoblasts to synthesize cementum of the teeth and into osteoblasts to synthesize alveolar bone for the skeletal support of the tooth. The major function of periodontal ligament is to form a shock absorbing system mitigating the mechanical forces such as mastication and orthodontic forces (1–3). Accordingly, periodontal ligament cells respond to mechanical forces and result in outcomes such as cell proliferation, differentiation, ECM synthesis and degradation (4, 5).

A previous study demonstrated that the periodontal ligament cells have morphological characteristics similar to those of fibroblast, but they show higher alkaline phosphatase (ALP) activity than any other connective tissue cells (6). It is generally accepted that ALP is involved in the process of calcification in various mineralizing tissues (7). Indeed, ALP activity has been widely used as an indicator of osteoblastic activity in bone tissues and cell culture systems (8, 9).

ECM components, which fill the space around cells in periodontal ligament, are mainly composed of fibrous molecules and the ground substance. The major fibrous molecules are collagens (type I and type III), which play a main role in resisting tensional forces and holding teeth in the alveolar socket. The major components of the ground substance of the periodontal ligament include proteoglycans and glycoproteins. Proteoglycans are molecules composed of a core protein and one or more covalently bound long chains of glycosaminoglycans (10, 11). They are considered to contribute to viscoelastic properties of periodontal ligament. Moreover, proteoglycans are involved in various interactions with other ECM molecules, and are one of the factors that modulated the function of periodontal ligament. Decorin belongs to a class of proteoglycans referred as small leucine-rich proteoglycans, which interact with matrix molecules, including various types of collagens and growth factors (12, 13), and is expressed at high level in the periodontal tissues (14). In previous studies based on targeted disruption of the decorin gene, abnormal morphology as well as organization of the collagen fibrils and tissue fragility in the tendon, skin and periodontal ligament have been reported (15). Several studies have demonstrated that decorin dissociates from collagen or is degraded prior to matrix mineralization *in vitro* (16), suggesting its role in temporal regulation of mineralization.

Loss of occlusal function has been reported to induce atrophic changes in the periodontal ligament, such as narrowing of the periodontal space, disorientation of collagen fibers and alteration in the production and distribution of proteoglycans (14, 17–19). As mechanical stress triggers periodontal responses, it is likely that the nature of mechanical stress, such as occlusal and orthodontic force, may modulate the biological response of periodontal ligament. Indeed, it has been reported that cellular responses are affected by type, frequency, strength and duration of mechanical stress (20). Additional studies showed that varying magnitudes of mechanical stresses influence the expression patterns of inflammatory cytokines in periodontal ligament cells (21). Therefore, it is likely that not only the nature but also the magnitude of mechanical stress affect the regulation of homeostasis in periodontal ligament. However, there have been few reports studying the correlation between mechanical stress of varying magnitude and periodontal ligament cell activities such as ECM synthesis.

The objective of this study is to clarify the influence of the mechanical stress on the periodontal ligament homeostasis. We focus on changes in mRNA expression levels of type I collagen and decorin genes, as well as ALP activity in response to mechanical stress of varying magnitude.

Materials and methods

Isolation of bovine periodontal ligament

Fresh bovine periodontal ligament was isolated from four mandibles of approximately 1.5-year-old cattle obtained at a local slaughterhouse. The periodontal ligament from fully erupted teeth was removed as follows: after sterilizing oral surface by wiping with povidone iodine, fully erupted permanent central incisors were extracted. Periodontal ligament attached to the tooth was scraped off from the middle third of the root with surgical blades. The coronal and apical portions of the root were not used to avoid contamination by the cells from other tissues such as gingiva and dental pulp.

Cell culture

The periodontal ligament tissue was washed twice with phosphate-buffered saline supplemented with 10 μl Antibiotic-Antimycotic (Gibco BRL, Tokyo, Japan) containing penicillin, streptomycin and fungizone. Pieces of tissue were placed in 35-mm diameter tissue culture dishes and covered with sterilized glass coverslips. Cultures were maintained in Minimum Essential Medium Eagle/Nutrient Mixture F-12 HAM (1 : 1) medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and 50 μl/ml gentamycin sulfate (Gibco BRL) and 10 μl Antibiotic-Antimycotic at 37°C in a humidified incubator in the presence of 95% air and 5% CO_2. When the periodontal ligament cells that grew out from the periodontal ligament explants had reached confluence, they were detached with 0.05% trypsin-EDTA (Gibco BRL) in serum-free medium and subcultured. Medium was refreshed after 24 h, then every 48 h.

Application of tensional force to the cell culture

A cyclic tensional force was applied on periodontal ligament cell cultures using the BioFlex Loading Stations™ (Flexcell International Co., McKeesport, PA, USA) by a procedure described by Banes *et al.* (22). Briefly, cells at the third or fourth passages were cultured (1×10^5 cells/well) on flexible-bottomed culture plates (BioFlex: Flexcell International Co.) for 48 h until they became subconfluent. The fresh medium was added every 24 h until the application of the mechanical stress. In

order to apply a tensional force to the cells, the flexible bottom of the plate was deformed at various extents by a computer operated vacuum system manufactured by the BioFlex Loading Stations™. Cells were elongated at 6 cycles/min (5 s on and 5 s off) at each of the six levels of stretch (0.2, 1.0, 2.0, 3.0, 10, 18% increase in surface area of the bottom) for 48 h as the experimental groups. Non-stretched cells were used as the control groups.

Quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from cells at 0, 24 and 48 h after application of the mechanical stress, using RNA STAT-60 (TELL-TEST, Friendswood, TX, USA) according to the manufacturer's instruction. The total RNA (2 μg) was reversibly transcribed using SuperScript™ II reverse transcriptase (Gibco BRL). Primer cDNAs were designed based on the published DNA sequence of human and bovine decorin, type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, and summarized in Table 1. Quantitative RT–PCR was done with the external standard DNA prepared according to the method of Tamarina *et al*. (23). Briefly, the wild-type DNA purified by 2.0% Tris-Acetate-EDTA (TAE) agarose gel (Agarose H14 TAKARA: TaKaRa, Tokyo, Japan) electrophoresis was amplified with sense primer and external standard synthesis primer. The resulting intermediate product was purified by 2.0% TAE agarose gel electrophoresis and further reamplified with a set of sense and antisense primers. External standard DNA was purified from the 2.0% TAE agarose gel using QIAEX II Agarose Gel Extraction Kit (QIAGEN, Tokyo, Japan). In order to confirm that the external standard DNA could be amplified competitively with wild-type DNA, 1×10^4 copies of the decorin external standard DNA was co-amplified with 0.5, 1, 2, 4 and 8 μg of sample DNA. Similarly, 5×10^8 copies of the type I collagen external standard DNA was coamplified with 0.5, 1, 2, 4 and 8 μg of sample DNA. PCR products were separated by 2.0% agarose gel electrophoresis in Tris–Borate–EDTA buffer, stained with ethidium bromide, and photographed. The images were then analyzed using the Scion Image program for Windows (Scion Co., Frederick, MD, USA). Peak areas for both amplified products, wild-type DNA and external standard DNA, were quantitated.

ALP activity assay

ALP activity in the cell was measured by the method of Lowry *et al*. (24) with *p*-nitrophenyl phosphate as a substrate and normalized by the total protein content of the cell determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Briefly, after finishing the application of tensional forces, the medium was removed and the cells were washed twice with Tris-buffered saline. The cells were detached from the culture dish with a cell scraper after the addition of Tris-buffered saline, 0.1% Triton X-100 and a proteinase inhibitor cocktail (Complete™ Protease Inhibitor Cocktail Set: Roche, Tokyo, Japan). ALP activity was then assayed using Alkaline Phosphate Substrate Kit (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

The significance of differences between mean values of experimental and control groups were determined by the analysis of variance (one-way ANOVA).

Results

Morphology of cultured periodontal ligament cells

Periodontal ligament cells before application of the tensional force at the third or fourth passage are found to have a spindle-like shape, characteristic to fibroblast-like cells, and aligned randomly. Cells of other shapes were not found, suggesting little, if any, contamination with surrounding tissues such as gingival and dental pulp.

Detection of decorin and type I collagen mRNAs in periodontal ligament cells by RT-PCR

Quantitative RT–PCR analysis was done for type I collagen, decorin and GAPDH using the cDNA primers shown in the Table 1. The PCR products of the expected sizes, 427 bp for type I collagen and 279 bp for decorin mRNA, were detected in cultured periodontal ligament cells (Fig. 1A). The DNA sequences of amplified PCR products were found to be identical to those reported in GeneBank (data not shown). The levels of type I collagen and decorin mRNA in all experimental groups were analyzed similarly (Fig. 1A and B).

Changes of expression levels of type I collagen and decorin mRNAs in periodontal ligament cells after receiving tensional forces

Periodontal ligament cells were exposed to various magnitudes of

Table 1. Oligonucleotide primers used for competitive polymerase chain reaction and corresponding annealing temperatures

Gene	Primer sequences		Annealing temperature
Type I collagen	Forward	TACGATGGAGACTTCTACAGGGCT	56°C
	ES	TTCCTTGGAAGCTTGGAGCTCCTATACC	
	Reverse	GTTGGGTAGCCATTTCCTTGGAAG	
Decorin	Forward	ATGCCCAAAACTCTTCAGGAGCTG	56°C
	ES	ACTCTGCTGATTGGTATCAGCAATGCGG	
	Reverse	GCTAGCTGCATCAACTCTGCTGAT	
GAPDH	forward	TGCCTCCTGCACCACCAACTGC	56°C
	ES	CAGCGTCAAAGCAGGTCCACCACTGACA	
	Reverse	AATGCCAGCCCCAGCGTCAAAG	

Primers are presented in a 5′ to 3′ orientation from left to right.
ES: used for the synthesis of external standard cDNA.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

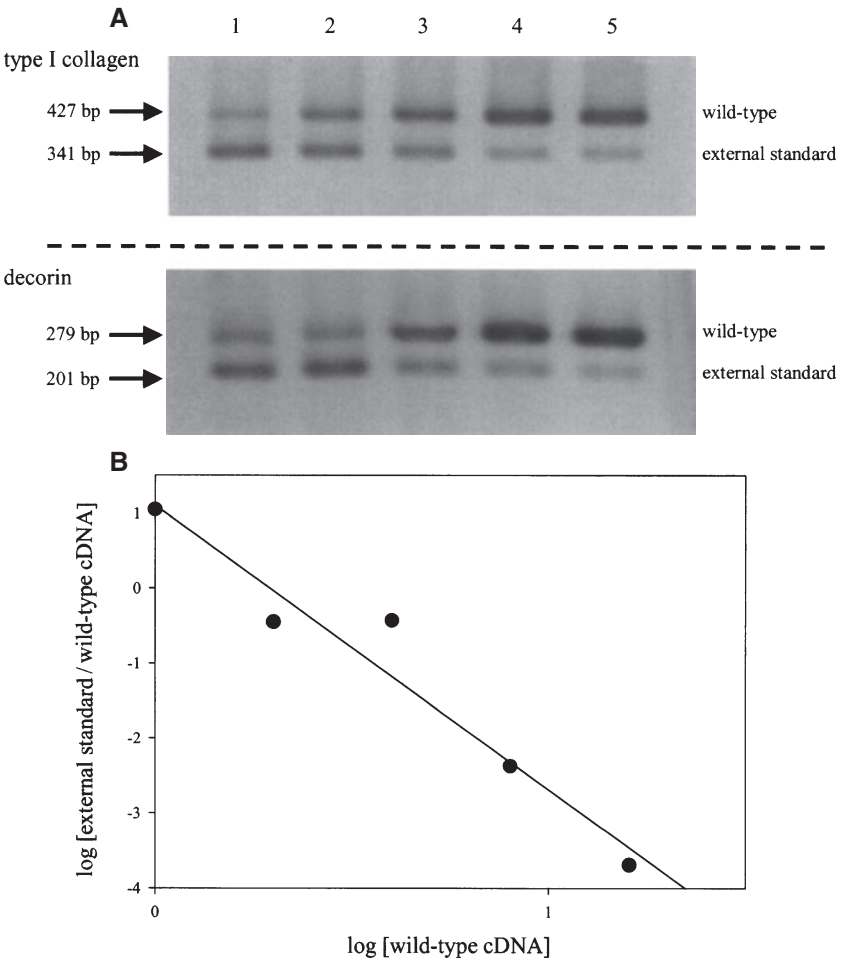

Fig. 1. Detection of type I collagen and decorin mRNA in periodontal ligament cells by competitive reverse transcription–polymerase chain reaction (RT–PCR) analysis. (A) Lanes 1–5 represent coamplification of the competitor (external standard DNA) at a fixed quantity with serially diluted sample cDNA (wild-type cDNA). The relative band intensities corresponding to the wild-type and external standard PCR products were quantified using Scion Image program for Windows. (B) The logarithm of the ratios of the wild-type cDNA relative to the external standard PCR products were plotted as a function of the logarithm of the amounts of wild-type same added. GADPH, glyceraldehyde-3-phosphate dehydrogenase.

tensional forces for 0, 24 or 48 h, and analyzed for type I collagen and decorin mRNA expression (Fig. 2A and B). Competitive PCR analyses revealed a time-dependent increase in mRNA levels for type I collagen in all experimental groups except the control (Fig. 2A). The highest value occurred in 10% and 18% stretch groups, reaching approximately 2.9-fold control after 48 h stimulation. Both 10% and 18% stretch groups showed a rapid increase during the first 24 h of treatment with a blunted increase in the second 24 h. Both 2.0% and 3.0% stretch groups continued to increase throughout the experimental period. The expression of type I collagen mRNA in 0.2% and 1.0% stretch groups did not change until 24 h of the treatment, then showed a slight increase during the second 24 h.

The expression of decorin mRNA showed a similar time-dependent increase in experimental group compared to the control group (Fig. 2B). The highest increase in decorin mRNA level was observed in the 10% and 18% stretch groups, reaching approximately threefold of the control at 48 h (Fig. 2*B*). In 10% and 18% stretch groups, an increase was clearly detected at 24 h, and the mRNA levels remained unchanged in the next 24 h. The 2.0% and 3.0% stretch groups showed a constant increase of decorin mRNA expression throughout the experimental period. Stimulation of periodontal ligament cells with the 0.2% and 1.0% stretch caused little change during the first 24 h, with a slight increase during the next 24 h treatment.

Comparison of changes in type I collagen and decorin mRNA levels after mechanical stress

Magnitudes of type I collagen and decorin mRNA increases at each stretch level after 48 h treatment were compared (Fig. 3). The highest rate of increment of type I collagen and decorin mRNA expression was observed between 1.0% and 10% stretch after 48 h treatment.

Time-dependent changes in ALP activity in periodontal ligament cells in response to tensional forces

Cellular ALP activity was measured after applying tensional forces of various magnitudes. ALP activity remained unchanged in the groups of cells exposed for 48 h to tensional forces of 0.2%, 1.0%, 2.0% and 3.0% stretch when compared with the control group. On the other hand, the ALP activity decreased gradually in the cells that received 10% and 18% stretch (Fig. 4).

The effects of various magnitudes of tensional forces on ALP activity

The measurement of ALP activity revealed the effects of tensional forces on the enzyme activity after 48 h treatment (Fig. 5). The highest rate of decrement of ALP activity was detected in cells that received greater than 10% stretch after 48 h stimulation. Stretch less than or equal to 3.0% did not affect ALP activity in periodontal ligament cells. The result indicated that the decrease of ALP activity might be caused by relatively high magnitudes of tensional forces.

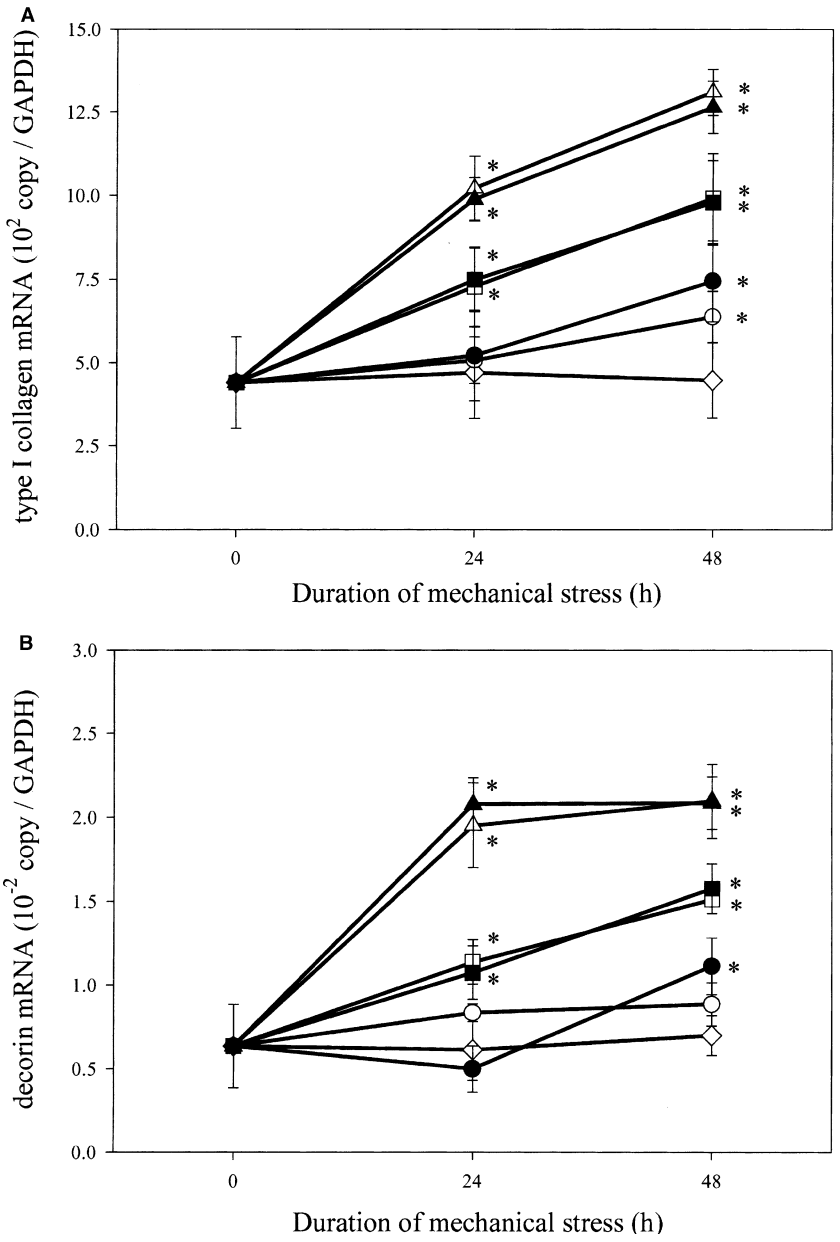

Fig. 2. Time-dependent changes in expression levels of type I collagen (A) and decorin (B) mRNAs in periodontal ligament cells subjected to tensional forces of various magnitudes. Data presented are means of four samples. Error bars represent standard deviation. ◇ , 0% (control); ○, 0.2%; ●, 1.0%; □, 2.0%; ■, 3.0%; △ , 10%; ▲, 18% stretch. *Significantly different from corresponding control by one-way ANOVA ($p < 0.01$).

Discussion

Mechanical stress induces inflammatory responses in the tissue (25–27). Such changes have been reported also in the periodontal ligament tissue both *in vivo* and *in vitro* (28, 29). As in orthodontic tooth movement, mechanical stress loads to the periodontal ligament, it does not induce any pathological inflammatory changes in case of an appropriate tooth movement, maintaining tissue homeostasis. However, the mechanism of this appropriate tooth movement has not been cleared in details yet. The present study was therefore designed to clarify the influence of mechanical stress such as occlusal force and orthodontic force on the homeostasis of periodontal ligament, with the focus on: (i) periodontal ligament cells, because of their crucial roles in the biological responses in periodontal ligament (3); (ii) tensional force, mimicking closely the strain that the periodontal ligament is subjected to during mastication or tooth movement (30); (iii) type I collagen, because it is one of the major fibrous elements comprising periodontal ligament (31); (iv) decorin, because it has been postulated to modulate functions of type I collagen through molecular interactions (13); (v) ALP activity, involved in the process of calcification in various mineralizing tissues, as an indicator to evaluate function of periodontal ligament cells (8).

The present findings show that type I collagen and decorin mRNA expression increased, whereas ALP activity decreased, with increase in the duration and strength of experimental tensional force. Thus, these results suggest that tensional forces affect type I collagen and decorin mRNA expression levels as well as ALP activity; moreover, different magnitude of tensional force induces different responses from periodontal ligament cells. Another study showed that the meniscus responds to compression with the changes of mRNA levels for the extracellular matrix proteins; decorin, types I and II collagen decreased by compressive force in osteocytes (32, 33). We speculate that in periodontal ligament cells levels of decorin may decrease in response to compressive force. Compressive force may also induce periodontal ligament abnormal organization of collagen fibrils, leading to changes in morphology and tissue fragility in the periodontal ligament, due to either loss of decorin–collagen interaction or reduction of decorin synthesis.

Several experimental models of mechanical stress using cell culture systems have been developed (34–36). However, most models have some limitations; for example, it is sometimes difficult to adjust precise conditions of mechanical stress such as type, frequency, strength and duration (37). In our system, these parameters are controlled by a computer, thus a set of experimental conditions studying bio-

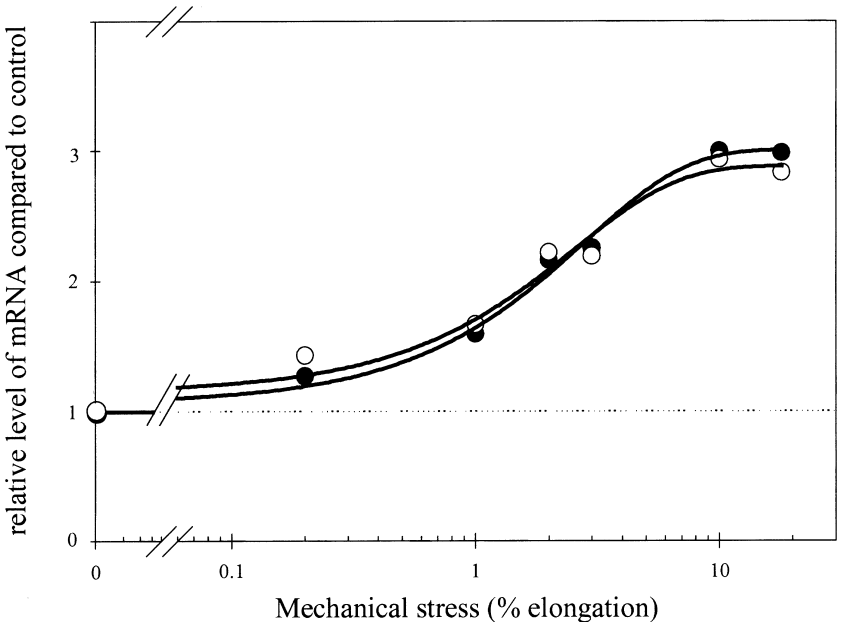

Fig. 3. The effects of tensional forces on relative amounts of type I collagen and decorin mRNA compared to their basal expression level in non-stretched cells after 48 h treatment. ○, type I collagen; ●, decorin. All data points were fitted by four parameter logistic curves. ALP, alkaline phosphatase.

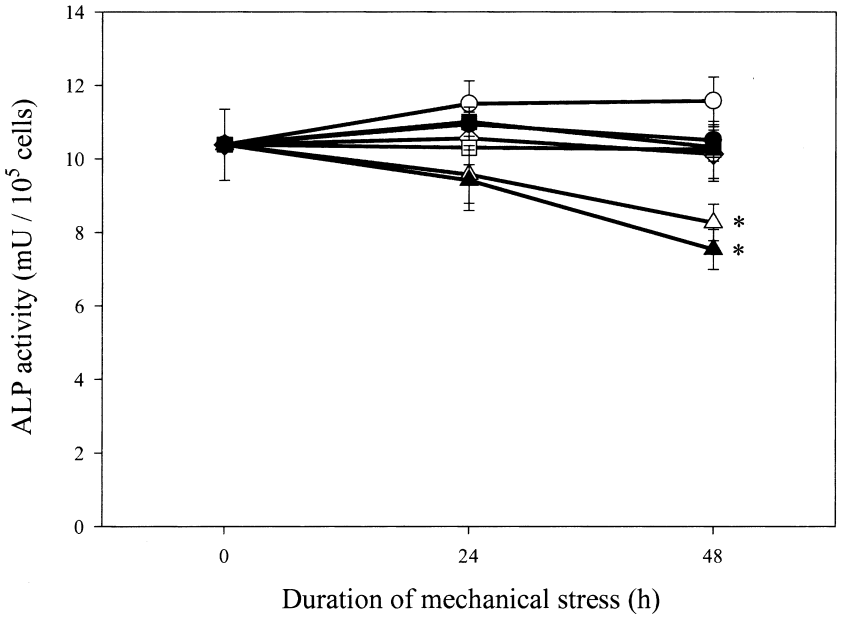

Fig. 4. Time-dependent changes in alkaline phosphatase (ALP) activity in periodontal ligament cells in response to various magnitudes of tensional forces. Data presented are means of four samples. Error bars represent standard deviation based on four samples. ◇, 0% (control, overlapped and hidden); ○, 0.2%; ●, 1.0% (partially overlapping); □, 2.0% (partially overlapping); ■, 3.0%; △, 10% (partially overlapping); ▲, 18%. *Significantly different from corresponding control by one-way ANOVA ($p < 0.01$).

logical responses of periodontal ligament cells to mechanical stress *in vitro* can be defined. Based on previous reports, in our study, we also used the frequency of 6 cycles/min to stimulate cultured cells (38, 39).

The setting of the magnitudes of tensional forces in our experiments was administered extensively compared to previous studies (38, 40). For example, application of 500 gf orthodontic force to human upper incisors can be calculated as about 23% stretch of periodontal ligament on the tension side (41). In this case of the tensional forces ranging 0–18% stretch would correspond to approximately 0–400 gf orthodontic force *in vivo*. Therefore the range of mechanical force employed was wider than most previous studies. If the mechanical stresses are dampened by fluids and connective tissues *in vivo*, the range of forces *in vivo* which periodontal ligament cells are receiving could be even narrower (42). Decorin plays important biological roles through its ability to interact with the fibrous proteins in the connective tissue matrix, including collagen, fibronectin, and fibrillin (12). There have been reports that decorin inhibited collagen fibril formation *in vitro* (13). It has been also reported that decorin acts as an inhibitor of matrix mineralization (16, 43). In immunohistochemical studies, the dominant expression of decorin appears at tensional side of periodontal ligament compared to compressional side in experimental tooth movement, and the dominant expression of decorin also appears in periodontal ligament of functional teeth while recessive expression in hypofunctional periodontal ligament (14), similarly our present findings. Although precise molecular mechanism are still elusive, regulatory roles of decorin in collagen fiber formation have been clearly demonstrated. When the connective tissue of periodontal ligament is reorganized or newly formed under mechanical stress, biosynthesis of collagen and decorin, as the major structural proteins, would be a part of the principal event. The observation in the present study was consistent with this idea. In summary, the present findings suggested that mechanical stress play an important role in remodeling and functional regulation of periodontal ligament through the modulation of expression levels of ECM molecules and ALP activity in periodontal ligament cells.

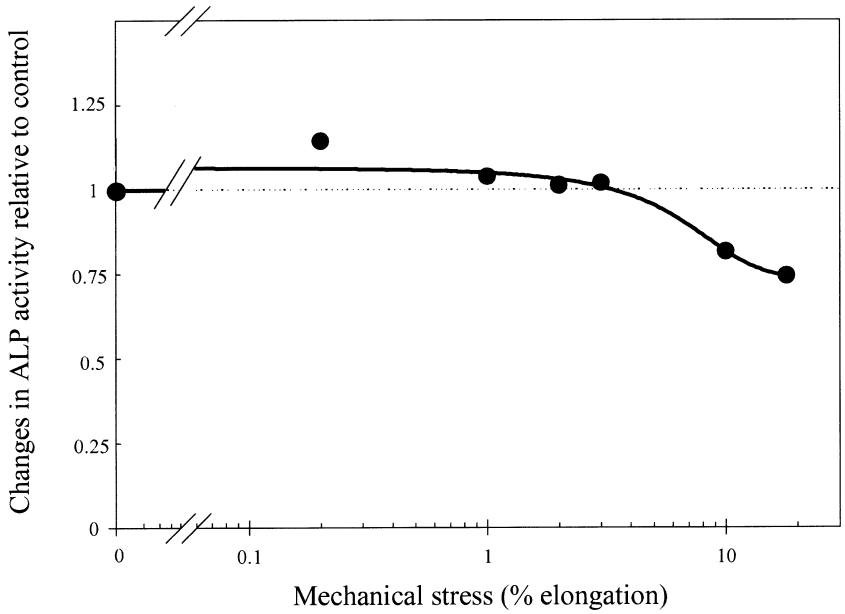

Fig. 5. The effect of tensional forces on alkaline phosphatase (ALP) activity compared to their basal activity level in non-stretched cells after 48 h treatment. All data points were fitted by a four parameter logistic curve.

Periodontal ligament cells have been observed to produce prostaglandin E_2 and interleukin-1β in response to mechanical stress (25). Both prostaglandin E_2 and interleukin-1β are important chemical mediators related to inflammatory reactions that stimulate bone resorption (44, 45). Several studies have suggested that prostaglandin E_2 and interleukin-1β inhibit ALP activity in fibroblasts and osteoblasts (46, 47). On the contrary, some other reports demonstrated that an intermittent compressive force enhanced ALP activity in mouse calvaria cells (33). These conflicting data seem to suggest that responses of ALP activity differ depending on the cell type and the nature of mechanical stress. Our present findings support the hypothesis that a strong mechanical stress could decrease ALP activity, resulting in acceleration of the production of inflammatory cytokines. Since it has been pointed out that periodontal ligament cells are heterogeneous (48), it is unclear whether the ALP activity of all periodontal ligament cells decreased or a subpopulation of periodontal ligament cells possessing high ALP activity diminished. Further studies are required to clarify this question.

In conclusion, the present study revealed that mechanical stress such as occlusal force and orthodontic forces modulates type I collagen and decorin synthesis and ALP activity in periodontal ligament, and suggested that its stress affects maintenance of periodontal ligament and adjacent alveolar bone metabolism and formation. Moreover, as a result that the different magnitudes of mechanical stress induce different responses from periodontal ligament cells, it is suggested that periodontal ligament cell has the mechanism to sense the different natures of mechanical stresses. Further studies are required to elucidate the precise cellular mechanism responding to various mechanical stresses.

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