Periodontal ligament cells under intermittent tensile stress regulate mRNA expression of osteoprotegerin and tissue inhibitor of matrix metalloprotease-1 and -2.

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Running title: Tensile stress up-regulates OPG mRNA in PDL cells

Number of pages: 34
Number of pages for figure legends: 7
Number of figures: 8
Number of tables: 3

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Abstract

We studied the mRNA expression of osteoprotegerin (OPG), receptor activator of NF- kappa B ligand (RANKL), tissue inhibitor of matrix metalloprotease (TIMP) -1 and -2 and matrix metalloprotease (MMP) -1 and -2 by human periodontal ligament (PDL) cells under intermittent tensile stress using a Flexercell Strain Unit. Analysis by reverse transcriptase-polymerase chain reaction showed that mechanical force up-regulated OPG mRNA. We also demonstrated that the protein concentration of OPG in conditioned medium increased upon loading with tensile stress**,** as determined by enzyme-linked immunosorbent assay. TIMP-1 and -2 mRNA levels also increased, whereas levels of RANKL, MMP-1 and -2 mRNA were barely affected. We further examined the effect of loading with tensile stress and addition of *Salmonella abortus equi* lipopolysaccharide (LPS) on the mRNA expression of PDL cells. The amount of OPG mRNA induced by mechanical strain was found to decrease with the addition of LPS to cultures. The induction of OPG mRNA expression by stretching was inhibited in the presence of indomethacin or genistein, whereas TIMP-1 mRNA expression induced by

stretching was inhibited by the addition of cycloheximide, suggesting that tensile stress regulates cyclooxygenase activities, tyrosine phosphorylation and *de novo* protein synthesis in PDL cells through the induction of OPG and TIMP-1 mRNA expression. These results provide evidence that the mechanical stimulus of stretching is responsible for the observed regulation of bone resorption and tissue degradation in PDL tissue.

Introduction

The periodontal ligament (PDL) is always exposed to mechanical loading during occlusion and mastication in healthy periodontal tissue. Under normal physiologic conditions the health of PDL tissues is maintained by appropriate mechanical loading. However, excess mechanical force and/or tissue inflammation can become manifest as periodontal tissue damage including alveolar bone resorption and ultimately, tooth loss [1]. Obviously, many cell types are able to control their physiologic functions *in vivo* under constant mechanical stresses such as stretching, sheer stress and pressure. Several studies have reported that mechanical stimuli regulate various cellular functions and that the mechano-sensing system including ion channel receptors, soluble cytokines, the cytoskeleton or intracellular signaling pathways are likely to be involved in the signaling of and response to mechanical stresses in mammalian cells [2-4]. The regulatory role of mechanical force is believed to be critical in the remodeling of the skeleton. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. It is well known that molecular interactions between

receptor activator of NF-kappa B (RANK) and RANK ligand (RANKL) confirm the hypothesis that osteoblasts play an essential role in osteoclastic differentiation [5]. Osteoclast precursors express RANK and recognize RANKL through cell-to-cell interactions that activate intracellular signaling pathways leading to their differentiation into mature osteoclasts which actively resorb bone [5]. Simonet *et al.* [6] identified osteoprotegerin (OPG), a soluble circulating protein member of the tumor necrosis factor (TNF) receptor superfamily, as the key osteoclastogenesis inhibitory factor. OPG strongly inhibits bone resorption by binding with high affinity to RANKL. Not only osteoblasts but also PDL cells express both RANKL and OPG mRNA and protein, therefore we propose that PDL cells could stimulate osteoclastogenesis by RANKL through cell-to-cell contact and inhibit osteoclastogenesis by OPG [5]. These interactions could affect the condition of processes such as periodontitis and orthodontic tooth movement. It has been reported that expression of RANKL mRNA and protein in PDL cells increased with compressive force in parallel with changes in the number of osteoclasts in an *in vitro* osteoclastogenesis system [7]. In contrast, the regulation of OPG

mRNA expression in PDL cells by mechanical stress induced by cell stretching remains unknown.

In cases involving excessive mechanical force in combination with periodontitis, not only alveolar bone resorption but also degradation of periodontal tissue is observed. Matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloprotease (TIMPs) have been implicated in the degradation of many extracellular matrix (ECM) proteins [8,9]. MMP-1 and -2 catalyze the initial degradation of the major ECM proteins, collagen types I and III**,** in the PDL when periodontal tissue suffers inflammatory or mechanical injuries [10]. PDL cells also constitutively produce both TIMP-1 and -2 proteins, which inhibit the activity of MMPs. In infections such as periodontitis, lipopolysaccharide (LPS) from gram-negative microbacterial endotoxin induces the production of various inflammatory cytokines such as interleukin (IL) -1α, IL-1β and TNF-α in PDL cells to promote active bone resorption [11,12]. However, the effects of intermittent tensile stress in regulating the expression of these factors in cultured cells derived from adult human periodontal tissue and their modulation in the

presence of LPS have not been investigated.

In this study, we investigated how intermittent tensile stress regulates the mRNA expression of OPG, RANKL, TIMPs and MMPs, and the OPG protein content of conditioned medium**,** using a Flexercell Strain Unit in cultures of PDL cells. We assessed the modulation of LPS on induced mRNA expression in order to elucidate the role of tensile stress in the progression of inflammation and alveolar bone resorption in periodontal and periapical diseases. Our studies show that expression of OPG is regulated by tensile stress and these changes in expression are modulated by the presence of endotoxin in PDL cells. Furthermore, diverse signaling pathways exist to transduce intracellular signaling of tensile stress in PDL cells, leading to the induction of expression of specific mRNAs.

Materials and Methods

Materials

Materials used in this work were obtained from the following sources: Fetal bovine serum (FBS), alpha modified Eagle's minimum essential medium (α-MEM), HEPES buffer, trypsin/EDTA solution, *Salmonella abortus equi* LPS, cycloheximide, indomethacin, genistein, PD098059, cytochalasin B and Y-27632 were from Sigma (St. Louis, MO, USA); penicillin-G was from Banyu (Tokyo, Japan), and streptomycin from Meiji (Tokyo, Japan).

Cell cultures

The protocol for this experiment was reviewed and approved by the Hokkaido University Graduate School of Dental Medicine Ethics Committee, and informed consent was obtained from all volunteers. Pieces of human PDL were obtained from premolars extracted for orthodontic reasons from healthy young donors. Explants were taken exclusively from the middle of tooth roots, to exclude intermixing of gingivae and dental

pulp, according to the methods described by Ragnarsson *et al.* [13]. The resected tissue was immediately rinsed several times in phosphate buffered saline and transferred to a 24-well culture plate (Costar, NY, USA). Tissues were cultured in α-MEM containing 100 units/ml penicillin-G, 0.1 mg/ml streptomycin and 25 mM HEPES buffer, supplemented with 20% FBS, at 37 °C in a humidified atmosphere of 5% CO_2 in air; the medium was changed every 3 days throughout the experiment. Outgrowth cells were subcultured and maintained in α-MEM supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C and used for experiments at passage four.

Mechanical strain devices

PDL cells were seeded at a density of 1.0×10^5 cells/well on flexible-bottomed culture plates coated with type I collagen (Flex I; Flexcell International Co. McKeesport, PA, USA), and cultured in α-MEM supplemented with 2% FBS. A Flexercell Strain Unit (Flexcell International Co.) was used to generate tensile stress in PDL cells. This unit was placed in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, and a vacuum was

applied across the plate surface according to instructions provided by the manufacturer. During the application of tensile stress, the cells were maintained in α-MEM supplemented with 2% FBS.

Inhibitors

Protein synthesis and signal transduction pathways that mediated cellular responses to tensile stress were investigated using specific inhibitors: Cycloheximide (10 μM) for inhibition of *de novo* protein synthesis, indomethacin (10 μM) for inhibition of cyclooxygenase (COX), genistein (20 μM) for inhibition of tyrosine kinase, PD098059 (10 μM) for specific inhibition of extracellular signal-related kinase (ERK), cytochalasin B (10 μM) for inhibition of actin de-polymerization, and Y-27632 (10 μM) for a protein inhibitor of RhoK. With regard to the concentration of each inhibitor, we used the effective doses previously reported [14,15]. After PDL cells were pre-incubated in the presence of each inhibitor for 30 min to permit these compounds to penetrate into the cells and block their respective pathways, intermittent stretching with 20% elongation, 10

cycles/min was applied to PDL cells in culture for 48 hr.

Total RNA isolation

Total cellular RNA was isolated from each culture using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to protocols provided by the manufacturer. In order to remove contaminated genomic DNA, total RNA samples were treated with RNase-Free DNase I (Qiagen) at 37°C for 30 min.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Two μg of total RNA was used as a template to synthesize first-strand complementary DNA (cDNA) with oligo (dT) primer and reverse transcriptase, using the Omniscript RT Kit (Qiagen). Then, l μl of the cDNA mixture was subjected to polymerase chain reaction (PCR) amplification using specific primers. The primer sequences, amplification cycles and annealing temperatures used in this study are shown in Table 1. Each PCR reaction was carried out in a 50 μl mixture containing 1 μl of cDNA, 5 μl of 10× Qiagen PCR

buffer, 10 μl of 5× Q-Solution, 1 μl of 10 mM each deoxynucleotide triphosphate mix, 0.1 μM each of sense and antisense primers, and 0.5 μl of Taq DNA polymerase (Qiagen). Each reaction consisted of initial denaturation at 94°C for 3 min, followed by 3-step cycling: Denaturation at 94°C for 30s, annealing at a temperature optimized for each primer pair (Table 1) for 30s and extension at 72°C for 1 min. After the requisite number of cycles, reactions underwent a final extension at 72°C for 10 min. Amplification products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. The relative intensity of the gel bands was measured using NIH Image software and results were normalized to the mRNA level of GAPDH, a housekeeping enzyme. We performed these experiments using samples from at least five different cell preparations and quantification of mRNA was confirmed using the same cell sample at least in triplicate.

Quantitative real time PCR

Quantification of OPG mRNA was performed using an ABI Prism 7000 sequence

detection system (Applied Biosystems, Foster City, CA, USA). One μg of each RNA sample was subjected to reverse transcription using the omniscript RT kit (Qiagen) and RNase-free DNase Set (Qiagen) in a total volume of 20 μl. Then, 2.5 μl of the reaction mixture were incubated with the double-stranded DNA dye SYBR Green I (Qiagen) in a total volume of 25 μl. The primers used for detection were as described in Table 1. All reactions were run in with a hot start pre-incubation step of 10 min at 95°C, following by cycles of 15 sec at 95°C, 1min at 60°C. We performed these experiments using samples from at least three different cell preparations and quantification of mRNA was confirmed using the same cell sample at least in triplicate. The amount of template was quantified using the comparative cycle threshold method as outlined in the manufacturer's technical bulletin. Measured OPG mRNA levels were normalized to the mRNA level of β-actin.

Enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of OPG concentration

Conditioned media harvested from cultured cells were used as samples. The

concentration of OPG was determined by enzyme-linked immunosorbent assay (ELISA) using an OPG ELISA Kit (Biomedica, Vienna, Austria). Measurements were performed in triplicate in samples diluted 1:10 according to the manufacturer's instructions in triplicate. In brief, 100 µl of assay buffer, 50 µl of sample and 50 µl of detection antibody were added to a well. After incubation for 16 hr at 4°C, the wells were washed with wash buffer. Two hundred µl of streptavidin-horseradish peroxidase conjugate were added to all wells and then incubated for 60 min at room temperature. After wells were washed, substrate was added to all wells and incubated for 20 min, followed by stop solution. The absorption was determined with a microplate reader at 450 nm against 620 nm as a reference.

Enzyme immunoassay (EIA) for the quantitative determination of bicyclo prostaglandin E2 (PGE2)

Conditioned media harvested from cultured cells were used as samples. The concentration of bicyclo prostaglandin (PG) E2 of the conditioned medium was

determined using a bicyclo PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Bicyclo PGE2 is a stable breakdown product of PGE2 and 13,14-dihydro-15-keto PGE2. Due to the inherent instability of 13,14-dihydro-15-keto PGE2, we quantified it as bicyclo PGE2 to estimate PGE2 biosynthesis in cultures. The assay is based on the competition between free bicyclo PGE2 and a bicyclo PGE2 tracer-acetylcholinesterase conjugate (bicyclo PGE2 tracer) for a limited number of bicyclo PGE2-specific rabbit antiserum binding sites. In brief, for derivatization of standard and samples, 300 µl of carbonate buffer was added and incubated at 37 °C overnight. Four hundred µl of phosphate buffer was then added, followed by 300 µl of EIA buffer. After sample, bicyclo PGE2 tracer and bicyclo PGE2 antiserum were added to wells, the plate was incubated for 18 hr at room temperature, and then washed with wash buffer. For development, 200 µl of Ellman’s reagent was added to all wells and the plate was incubated for 60 min in the dark. The resultant color reaction was measured with a microplate reader at 405 nm, the color development being inversely proportional to the concentration of free bicyclo PGE2 measured.

Statistical analysis

Values were calculated as the mean ± standard deviation (SD). Some data were subjected to multiple measurement analyses of variance (ANOVA) and Student's *t*-test was used for analysis of the difference between the cultures tested. A *P* value of less than 0.05 was accepted as significant.

Results

Initially, PDL cells were exposed to stretching at a frequency of 10 cycles/min; each cycle consisting of 3s of strain and 3s of relaxation with 20% elongation for 2 days. No damaged, dead or apoptotic cells were observed microscopically in stretch-loaded cultures (Fig. 1). The distribution of orientation of cells grown on stretched culture dishes was different from the uniform random distribution observed in non-stretched cultures (Fig. 1). Cells from stretched and non-stretched cultures were harvested and extracted to quantify the level of mRNA expression by RT-PCR. Fig. 2A shows the amplification products of OPG cDNA separated on a 2% agarose gel. Although no significant change in the OPG mRNA expression level was observed with strain from 0 hr through to 24 hr of culture, the expression level of OPG mRNA was up-regulated by exposure to mechanical strain for 48 hr in comparison with non-stretched cultures at the corresponding time (Fig. 2A). This induction of OPG mRNA was also observed at 72 or 120 hrs of culture. Analysis of the OPG mRNA/GAPDH mRNA ratio in the stretched culture is shown in Fig. 2B. To further confirm the specific induction of expression of the

transcript by stretch, we performed quantitative real-time RT-PCR analysis. The OPG mRNA copies were increased to about 3-fold within 48 h compared to no stretch controls (Fig. 2C). We then asked whether different magnitudes of strain affect the OPG mRNA expression level in PDL cells. The expression of OPG mRNA was significantly increased by mechanical strain with 20% or 25% elongation in comparison with the level of 0% elongation cultures, while no significant difference was seen with 5% elongation (Fig. 3).

Next, we examined whether stretch-induced OPG mRNA expression was modulated by the presence of LPS in PDL cells, by incubating PDL cell cultures with 1 μg/ml of LPS and applying tensile stress. In the presence of LPS, OPG mRNA expression did not increase with 20% elongation, 10 cycles/min, for 2 days (Fig. 4), indicating that LPS suppressed expression of tensile stress-induced OPG mRNA in PDL cells.

The elevated expression of OPG mRNA in stretched cells was further confirmed by ELISA for quantification of OPG protein using conditioned medium from these cells. The amount of OPG protein in stretched cultures increased 3-fold compared

with non-stretched cultures at 48 hr (Table 2). In cultures treated with tensile stress and LPS in combination, the OPG protein level did not alter compared to the non-stretched cultures (Table 2) confirming the changes in mRNA expression shown by RT-PCR analysis (Fig. 4).

Mechanisms translating mechanical stress into a signal that activates increased OPG gene expression are as yet unidentified. In order to investigate whether up-regulation of OPG mRNA expression by stretching was dependent on *de novo* protein synthesis, we added cycloheximide to the PDL cell cultures. Cycloheximide did not reduce the level of OPG mRNA expression induced by stretching (Fig. 5). In conrast, indomethacin suppressed strain-induced OPG mRNA expression, suggesting that OPG mRNA induction by tensile stress in PDL cells was mediated by cyclooxygenase and/or PG synthesis (Fig. 5). The concentration of PGE2 in the conditioned medium also increased in stretched cultures (Table. 3). Genistein also significantly suppressed stretch-induced OPG mRNA expression, suggesting that strain-induced OPG mRNA expression was also dependent on protein tyrosine phosphorylation by certain tyrosine

kinases (Fig. 5). In contrast, PD098059, cytochalasin B and Y-27632 showed no inhibitory effects on the up-regulation of OPG mRNA (Fig. 5). These results indicate that strain-induced OPG mRNA expression in PDL cells is not mediated by signaling molecules related to ERK, RhoK or actin de-polymerization.

Previously, PDL cells were shown to produce RANKL which activates mature osteoclasts and causes the differentiation of preosteoclastic cells to osteoclasts. To elucidate the effect of tensile stress on RANKL mRNA expression in PDL cells, we performed RT-PCR analysis using primers specific for RANKL cDNA. Although PDL cells expressed RANKL mRNA, its expression level did not change when the cells were loaded by intermittent stretching with 20% elongation at 10 cycles/min for 2 days (Fig. 6).

MMPs are a family of metalloenzymes, which, once activated, can digest various protein components of the ECM. To address whether mechanical strain affects matrix degradation, we measured the levels of TIMP-1, -2, MMP-1 and -2 mRNA expression in PDL cells. RT-PCR analysis of TIMP-1 and -2 cDNAs revealed that tensile

stress increased TIMP-1 and -2 mRNA expression by PDL cells in comparison to non-strained cells (Fig. 7A and B). In contrast, expression levels of MMP-1 and -2 mRNA in PDL cells did not change with exposure to stretching with 20% elongation at 10 cycles/min for 2 days (Fig. 7C and D). We further examined whether the aforesaid inhibitors were able to inhibit the expression of TIMP-1 mRNA induced by mechanical strain. Stretch-induced TIMP-1 mRNA expression was inhibited by the addition of cycloheximide but not indomethacin, genistein, PD098059, cytochalasin B or Y-27632, suggesting that stretch-induced TIMP-1 mRNA expression depended on *de novo* protein synthesis (Fig. 8).

Discussion

The PDL is a soft connective tissue, which serves to anchor the tooth to the alveolus and functions as a cushion between two mineralized tissues. It has been shown that certain mechanical stresses appear to regulate cellular function in PDL cells. In addition, mechanical strain is known to be one of the stimuli for alveolar bone remodeling. Previously, many devices have been used to simulate the loading of mechanical forces on cells *in vitro*. Since PDL cells are repeatedly stretched and relaxed by mechanical forces under normal conditions *in vivo*, we used the Flexercell system to apply intermittent tensile stress to PDL cells in culture. We estimated that an orthodontic force of 1 N on the average human premolar equates to a strain level of approximately 1% in the PDL cell cultures [23]. In most clinical studies, 500–3,000 g of bite force have been used [24]. Therefore, we subjected PDL cells in culture to cyclic stretching with 20% elongation at a frequency of 10 cycles/min, each cycle consisting of 3s of strain and 3s of relaxation; thus we propose that our experiment is a valid reproduction of the physiologic mechanical forces of mastication.

There have been several studies regarding the regulation of mRNA expression and protein synthesis in PDL cells under stretched conditions. They include studies on mRNA expression of type I collagen, fibronectin, tropoelastin, COX-2, IL-1ß, protein synthesis of transforming growth factor-β1, macrophage colony stimulating factor and others [25-27]. It is thought that an increased or decreased expression of specific mRNAs and production of proteins by PDL cells is a significant part of their adaptive response to several types of mechanical stress. We found an up-regulation of OPG mRNA and protein and stable expression of RANKL in PDL cells exposed to tensile stress, suggesting that the soluble protein may modulate the differentiation of osteoclasts rather than a direct interaction between PDL cells and preosteoclasts/osteoclasts, at least under physiologic loading of stress in PDL cells. Using an *in vivo* experimental model of tooth movement in rat, Kobayashi *et al.* [28] demonstrated that preexisting osteoclasts disappeared from the bone surface through apoptosis during a force-induced rapid shift from bone resorption to bone formation. They also showed that a marked increase in OPG mRNA expression was observed in the stretched cells on the tensioned distal bone

surface, simultaneously with the loss of osteoclasts. Since osteoclast formation is dependent upon RANKL expression, strain-induced reductions in this factor may contribute to the accompanying reduction in osteoclastogenesis [5]. Therefore, the conditions of cyclic stretching used in our experiments may represent the tensioned distal alveolar bone surface and appropriate tensile stress may reduce the differentiation and/or function of surrounding osteoclasts in PDL tissue. The degradation of ECM proteins is a critical aspect of tissue damage due to mechanical stress and inflammation. Under our experimental conditions of stretched cells, TIMP-1 and -2 mRNA increased, but MMP-1 and -2 mRNA did not, suggesting that certain appropriate stress may act as an inhibitor of the degradation of ECM in the PDL. In inflamed periodontal tissues, PDL cells are infected by various bacterial endotoxins, which are capable of producing MMPs and various inflammatory cytokines such as IL-1 and -6. These cytokines promote bone resorption, which may in turn initiate or augment periodontal disease [29]. In our study, the combination of the presence of LPS and loading with tensile stress reduced expression of OPG mRNA in PDL cells.

What is the mechanism by which tensile stress controls expression of OPG in PDL cells? It is possible that stretching with 20% elongation enhanced the activity of COX and thus increased production of PGE2. There are two COX isoforms, COX-1 and COX-2, which are variably expressed in different cell types. We were not able to determine which isoform of COX is up-regulated by cyclic stretching in PDL cells. Several previous reports have indicated that COX-2 is a key molecule in the response of PDL cells to various mechanical stresses [30]. We could adequately explain the cellular response to tensile stress in terms of regulation of intracellular COX and PGE2 release alone. It is proposed that induction of OPG mRNA in PDL cells by stretching could partially involve an intracellular tyrosine kinase cascade, since genistein partially blocked the induction of OPG mRNA in the stretched cells. Furthermore, protein synthesis inhibitor affected the decrease in TIMP-1 mRNA expression induced by tensile stress, suggesting that complex pathways are involved in stress-induced mRNA expression. In yeast, upon a shift to high osmolarity, cells rapidly activate a mitogen-activated protein kinase cascade, the high-osmolarity glycerol pathway, which orchestrates part of the

transcriptional response [31]. Possibly, the function of the related mammalian pathway may be to mediate activation of the response of PDL cells to tensile stress.

In the present study, we demonstrated that when tension was applied to PDL cells with the addition of LPS, there was a significant difference in the expression levels of OPG between cells subjected to tension forces and untensioned cells. It is already established that toll-like receptor (TLR) 4 is a cell surface receptor of LPS and that MD2 acts as a cell surface co-receptor [32]. PDL cells express TLR4 and the several functions of LPS in PDL cells may be mediated by TLR4 [33]. Activation of NF-kappa B, p38 MAP kinase and c-Jun N-terminal kinase is known to be involved in both the intracellular signaling via TLR [32] and the various responses to mechanical stress. Therefore, it is possible that LPS modulates activation of the signaling molecules activated by tensile stress in PDL cells. It has been reported that LPS enhances the production of several inflammatory cytokines including IL-1β, -6, and -8 in PDL cells [29]. Therefore, the soluble cytokines in conditioned medium could modulate the response of PDL cells to tensile stress. Further investigation is needed to determine the molecular mechanisms

involved in the effects of tensile stress and the presence of LPS on PDL cells.

In summary, we have provided evidence that expression of OPG and TIMP-1 and -2 is specifically up-regulated by the application of tensile stress at levels similar to those to which PDL cells are subjected under physiologic conditions. Because OPG is involved in the regulation of osteoclastogenesis and bone resorption, it is tempting to speculate that a change in its expression is an immediate adaptive response of PDL tissue to the altered tensile stress. Moreover, loading by cyclic stretching is likely to increase the up-regulation of TIMP in PDL cells. Appropriate tensile stress may act to conserve physiologic function in the PDL via OPG and TIMP production. In turn, endotoxins may augment tissue and bone resorption via a reduction of OPG production in the PDL.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Scientific Research in Priority Areas (C) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1. Verna C, Zaffe D, Siciliani G (1999) Histomorphometric study of bone reactions during orthodontic tooth movement in rats. Bone 24:371-379

2. Banes AJ, Tsuzaki M, Yamamoto J, Fischer T, Brigman B, Brown T, Miller L (1995) Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals. Biochem Cell Biol 73:349-365

3. Lambert Ch A, Nusgens BV, Lapiere Ch M (1998) Mechano-sensing and mechano-reaction of soft connective tissue cells. Adv Space Res 21:1081-1091

4. Petrov AG, Usherwood PN (1994) Mechanosensitivity of cell membranes. Ion channels, lipid matrix and cytoskeleton. Eur Biophys J 23:1-19

5. Khosla S (2001) the OPG/RANKL/RANK system. Endocrinology 142:5050-5055

6. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ (1997) Osteoprotegerin: a novel secreted protein

involved in the regulation of bone density. Cell 89:309-319

7. Kanzaki H, Chiba M, Shimizu Y, Mitani H (2002) Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappa B ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 17:210-220

8. Cawston T (1998) Matrix metalloproteinases and TIMPs: properties and implications for the rheumatic diseases. Mol Med Today 4:130-137

9. Hayakawa T (2002) Multiple functions of tissue inhibitors of metalloproteinases (TIMPs): a new aspect involving osteoclastic bone resorption. J Bone Miner Metab 20:1-13

10. Hou LT, Yaeger JA (1993) Cloning and characterization of human gingival and periodontal ligament fibroblasts. J Periodontol 64:1209-1218

11. Wilson M (1995) Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogenesis of chronic periodontitis. Sci Prog 78:19-34

12. Wiebe SH, Hafezi M, Sandhu HS, Sims SM, Dixon SJ (1996) Osteoclast activation in

inflammatory periodontal diseases. Oral Dis 2:167-180

13. Ragnarsson B, Carr G, Daniel JC (1985) Isolation and growth of human periodontal igament cells in vitro. J Dent Res 64:1026-1030

14. Onodera S, Nishihira J, Iwabuchi K, Koyama Y, Yoshida K, Tanaka S, Minami A (2002) Macrophage migration inhibitory factor up-regulates matrix metalloproteinase-9 and -13 in rat osteoblasts. Relevance to intracellular signaling pathways. J Biol Chem 277:7865-7874

15. Kletsas D, Basdra EK, Papavassiliou AG (2002) Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. J Cell Physiol 190:313-321

16. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175-179

17. Gasson JC, Golde DW, Kaufman SE, Westbrook CA, Hewick RM, Kaufman RJ,

Wong GG, Temple PA, Leary AC, Brown EL (1985) Molecular characterization and expression of the gene encoding human erythroid-potentiating activity. Nature 315:768-771

18. Stetler-Stevenson WG, Brown PD, Onisto M, Levy AT, Liotta LA (1990) Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumor cell lines and human tumor tissues. J Biol Chem 265:13933-13938

19. Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ (1986) Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein. J Biol Chem 261:6600-6605

20. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA, Goldberg GI (1988) H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 263:6579-6587

21. Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S (1987) Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in

human lung cancers. Cancer Res 47:5616-5619

22. Nakajima-Iijima S, Hamada H, Reddy P, Kakunaga T (1985) Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the introns. Proc Natl Sci Acad USA 82:6133-6137

23. Andersen KL, Norton LA (1991) A device for the application of known simulated orthodontic forces to human cells in vitro. J Biomech 24:649-654

24. Coffey JP, Williams WN, Turner GE, Mahan PE, Lapointe LL, Cornell CE (1989) Human bite force discrimination using specific maxillary and mandibular teeth. J Oral Rehabil 16:529-536

25. Pavlin D, Gluhak-Heinrich J (2001) Effect of mechanical loading on periodontal cells. Crit Rev Oral Biol Med 12:414-424

26. Shimizu N, Goseki T, Yamaguchi M, Iwasawa T, Takiguchi H, Abiko Y (1997) In vitro cellular aging stimulates interleukin-1 beta production in stretched human periodontal-ligament-derived cells. J Dent Res 76:1367-1375

27. Kimoto S, Matsuzawa M, Matsubara S, Komatsu T, Uchimura N, Kawase T, Saito S

(1999) Cytokine secretion of periodontal ligament fibroblasts derived from human deciduous teeth: effect of mechanical stress on the secretion of transforming growth factor-beta 1 and macrophage colony stimulating factor. J Periodontal Res 34:235-243

28. Kobayashi Y, Hashimoto F, Miyamoto H, Kanaoka K, Miyazaki-Kawashita Y, Nakashima T, Shibata M, Kobayashi K, Kato Y, Sakai H (2000) Force-induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor beta and osteoprotegerin expression. J Bone Miner Res 15:1924-1934

29. Wilson M, Reddi K, Henderson B (1996) Cytokine-inducing components of periodontopathogenic bacteria. J Periodontal Res 31:393-407

30. Yamaguchi M, Shimizu N, Goseki T, Shibata Y, Takiguchi H, Iwasawa T, Abiko Y (1994) Effect of different magnitudes of tension force on prostaglandin E2 production by human periodontal ligament cells. Arch Oral Biol 39:877-884

31. Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66:300-372

32. Daipke A,Heeg K (2002) Signaling integration following Toll-like receptor triggering.

Crit Rev Immunol 22:217-250

33. Hatakeyama J, Tamai R, Sugiyama A, Akashi S, Sugawara S, Takada H (2003) Contrasting responses of human gingival and periodontal ligament fibroblasts to bacterial cell-surface components through the CD14/Toll-like receptor system. Oral Microbiol Immunol 18:14-23

Figure legends

Figure 1 PDL cells of stretched and non-stretched cultures. PDL cells were seeded at a density of 1.0×10^5 cells/well on Flex I culture plates and cultured in α-MEM supplemented with 10% FBS for 3 days. Then, cells were loaded with (+) or without (-) tensile stress with 20% elongation at 10 cycles/min for 2 days using a Flexercell strain unit. During application of the mechanical stress, the cells were incubated in α-MEM supplemented with 2% FBS. Original magnification ×100.

Figure 2 Osteoprotegerin (OPG) mRNA expression with the application of tensile stress in human periodontal ligament (PDL) cells. PDL cells were seeded at a density of 1.0×10^5 cells/well on Flex I culture plates, and cultured in α-MEM supplemented with 10% FBS for 3 days. Then, cells were loaded with (+) or without (-) tensile stress for the indicated hours with 20% elongation at 10 cycles/min using a Flexercell strain unit. Total RNA was extracted from the cells and the expression levels of OPG mRNA were determined by RT-PCR (A and B) and quantitative real time PCR (C) as described in

Materials and Methods. A: Agarose gel electrophoresis demonstrating amplification products of OPG and GAPDH cDNAs. B: Relative density of OPG mRNA to GAPDH mRNA. C: Ratio of OPG mRNA to β-actin mRNA copies measured by quantitative real time PCR. The results shown are the mean ± SD of five independent experiments (B) and three independent experiments (C). * Significant difference from stress (-) culture (P<0.05): ANOVA, Student's *t*–test.

Figure 3 Effects of percentage of elongation on OPG mRNA expression with the application of tensile stress in human periodontal ligament (PDL) cells. PDL cells were seeded at a density of 1.0×10^5 cells/well on Flex I culture plates, and cultured in α-MEM supplemented with 10% FBS for 3 days. Then, cells were loaded with (+) or without (-) tensile stress for the indicated percentage of elongation at 10 cycles/min using a Flexercell strain unit for 48 hours. Total RNA was extracted from the cells, the expression levels of OPG mRNA were determined by RT-PCR as described in Materials and Methods. A: Agarose gel electrophoresis demonstrating amplification products of

OPG and GAPDH cDNA. B: Relative density of OPG mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments. * Significant difference from stress (-) culture (P<0.05): ANOVA, Student's *t*–test.

Figure 4 Effects of tensile stress and *Salmonella abortus equi* lipopolysaccharide (LPS) in combination on OPG mRNA expression of PDL cells. PDL cells were seeded at a density of 1.0×10^5 cells/well on Flex I culture plates, and cultured in α-MEM supplemented with 10% FBS for 3 days. Then, cells were incubated with LPS (1 μg/ml) (+) or vehicle (-) and loaded with (+) or without (-) tensile stress with 20% elongation at 10 cycles/min for 48 hours. Total RNA was extracted from the cells, and expression levels of OPG mRNA were determined by RT-PCR as described in Materials and Methods. A: Agarose gel electrophoresis demonstrating amplification products of OPG and GAPDH cDNA. B: Relative density of OPG mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments. * Significant difference from stress (-) culture (P<0.05): ANOVA, Student's *t*–test.

Figure 5 Effects of inhibitors on OPG mRNA expression induced by tensile stress in PDL cells. PDL cells were seeded at a density of 1.0×10^5 cells/well on Flex I culture plates, and cultured in α-MEM supplemented with 10% FBS for 3 days. Then, cells were cultured in a medium containing cycloheximide (10 μM), indomethacin (10 μM), genistein (20 μM), PD098059 (10 μM), cytochalasin B (10 μM), Y-27632 (10 μM) or vehicle (control) for 30 min. The cells were cultured with (+) or without (-) loading with tensile stress with 20% elongation at 10 cycles/min for 48 hours. Total RNA was extracted from the cells, and expression levels of OPG mRNA were determined by RT-PCR as described in Materials and Methods. A: Agarose gel electrophoresis of the products of PCR using specific primers for OPG or GAPDH. B: Relative density of the OPG mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments. * Significant difference from stress (-) culture ($P<0.05$): Student's *t*–test.

Figure 6 Receptor activator of NF-kappa B ligand (RANKL) mRNA expression with

the application of tensile stress in PDL cells assessed by RT-PCR. PDL cells were seeded at a density of 1.0×10^5 cells/well, and cultured for three days. The cells were exposed to LPS (1 μg/ml) (+) or vehicle (-) and were loaded with (+) or without (-) tensile stress with a rate of 20% elongation at 10 cycles/min for 48 hours. Total RNA was extracted from the cells, and expression levels of RANKL mRNA were determined by RT-PCR. A: Agarose gel electrophoresis of the products of PCR using specific primers for RANKL or GAPDH. B: Relative density of RANKL mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments.

Figure 7 Tissue inhibitors of matrix metalloprotease (TIMPs) or matrix metalloproteases (MMPs) mRNA expressions with the application of tensile stress with or without LPS in PDL cells. PDL cells were seeded at a density of 1.0×10^5 cells/well, and cultured for 3 days. Then, cells were incubated with LPS (1 μg/ml) (+) or vehicle (-) and loaded with (+) or without (-) tensile stress with 20% elongation at 10 cycles/min for 48 hours. Total RNA was extracted, and then the expression levels of TIMP-1, -2 (A and

B), MMP-1 or -2 (C and D) mRNA were determined by RT-PCR. A: Agarose gel electrophoresis of the products of PCR using specific primers for TIMP-1, -2 or GAPDH. B: Relative density of expression levels of TIMP-1 or -2 mRNA to GAPDH mRNA. C: Agarose gel electrophoresis of the products of PCR using specific primers for MMP-1, -2 or GAPDH. D: Relative density of MMP-1 or -2 mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments. * Significant difference from stress (-) culture ($P<0.05$): Student's *t*–test.

Figure 8 Effects of inhibitors on TIMP-1 mRNA expression induced by tensile stress in PDL cells. PDL cells were seeded at a density of 1.0×10^5 cells/well, and cultured for 3 days. Then, cells were cultured in medium containing cycloheximide (10 μM), indomethacin (10 μM), genistein (20 μM), PD098059 (10 μM), cytochalasin B (10 μM), Y-27632 (10 μM) or vehicle (control). The cells were cultured with (+) or without (-) loading with tensile stress with 20% elongation at 10 cycles/min for 48 hours. Total RNA was extracted from the cells, the expression levels of TIMP-1 mRNA were determined by

RT-PCR. A: Agarose gel electrophoresis of the products of PCR using specific primers for TIMP-1 or GAPDH. B: Relative density of TIMP-1 mRNA to GAPDH mRNA. The results shown are the mean ± SD of five independent experiments. * Significant difference from stress (-) culture ($P<0.05$): Student's *t*–test.

Fig. 1

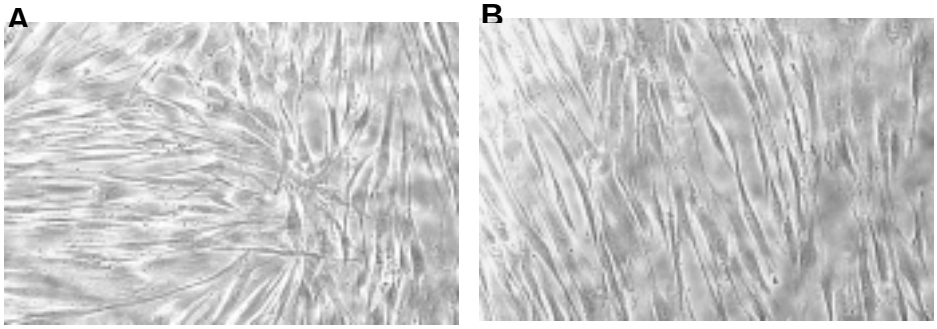


Fig. 2

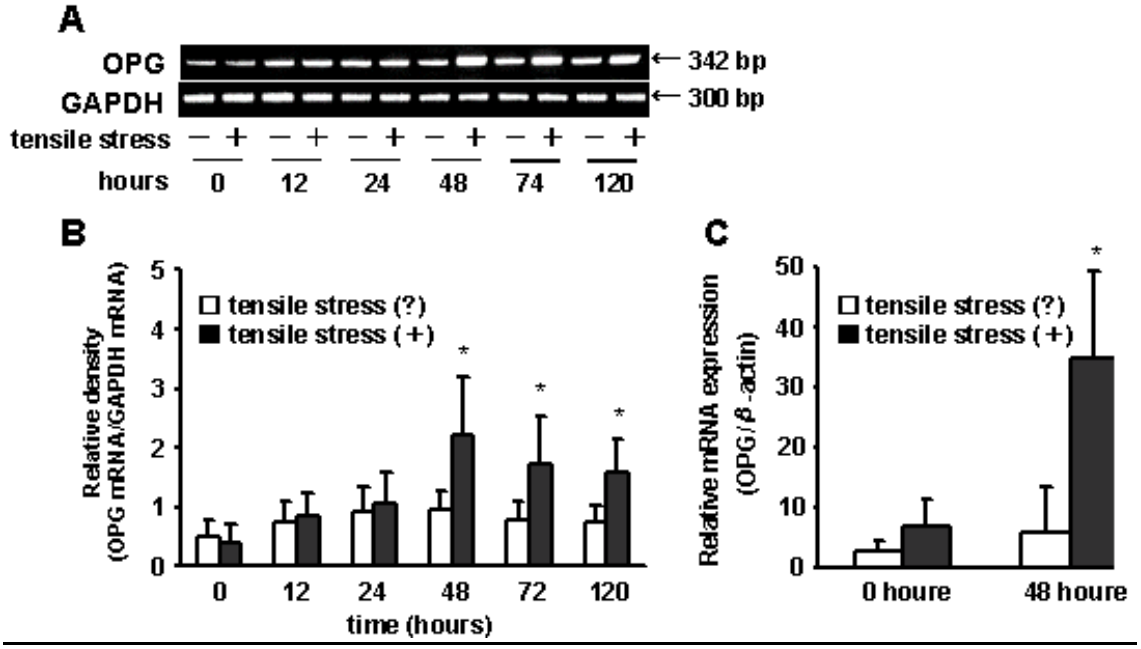

A
OPG
GAPDH
342 bp
300 bp
tensile stress
hours
0
12
24
48
74
120
B
Relative density
(OPG mRNA/GAPDH mRNA)
tensile stress (?)
tensile stress (+)
time (hours)
C
Relative mRNA expression
(OPG/β-actin)
0 houre
48 houre

Fig. 3

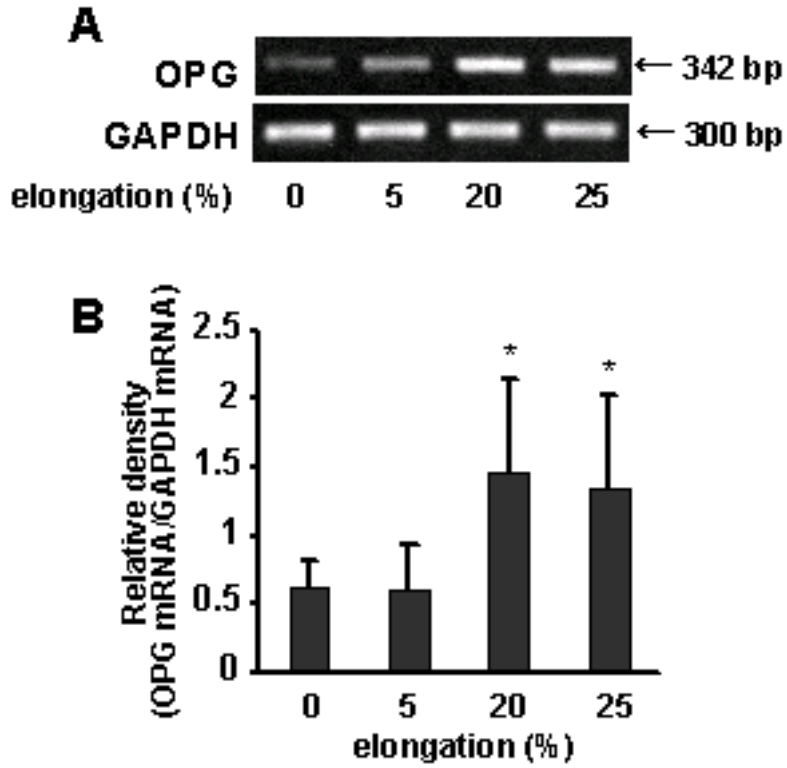

A
OPG
GAPDH
342 bp
300 bp
elongation (%)
0
5
20
25
B
Relative density
(OPG mRNA/GAPDH mRNA)
2.5
2
1.5
1
0.5
0
*
*
0
5
20
25
elongation (%)

Fig. 4

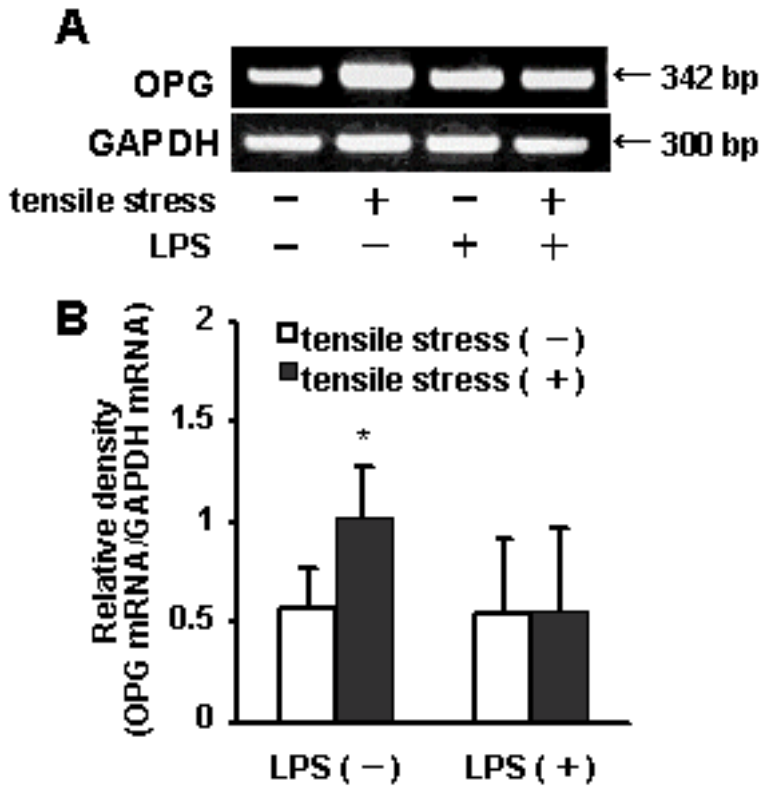

A
OPG
GAPDH
342 bp
300 bp
tensile stress
LPS
B
Relative density
(OPG mRNA/GAPDH mRNA)
tensile stress (−)
tensile stress (+)
LPS (−)
LPS (+)

Fig. 5

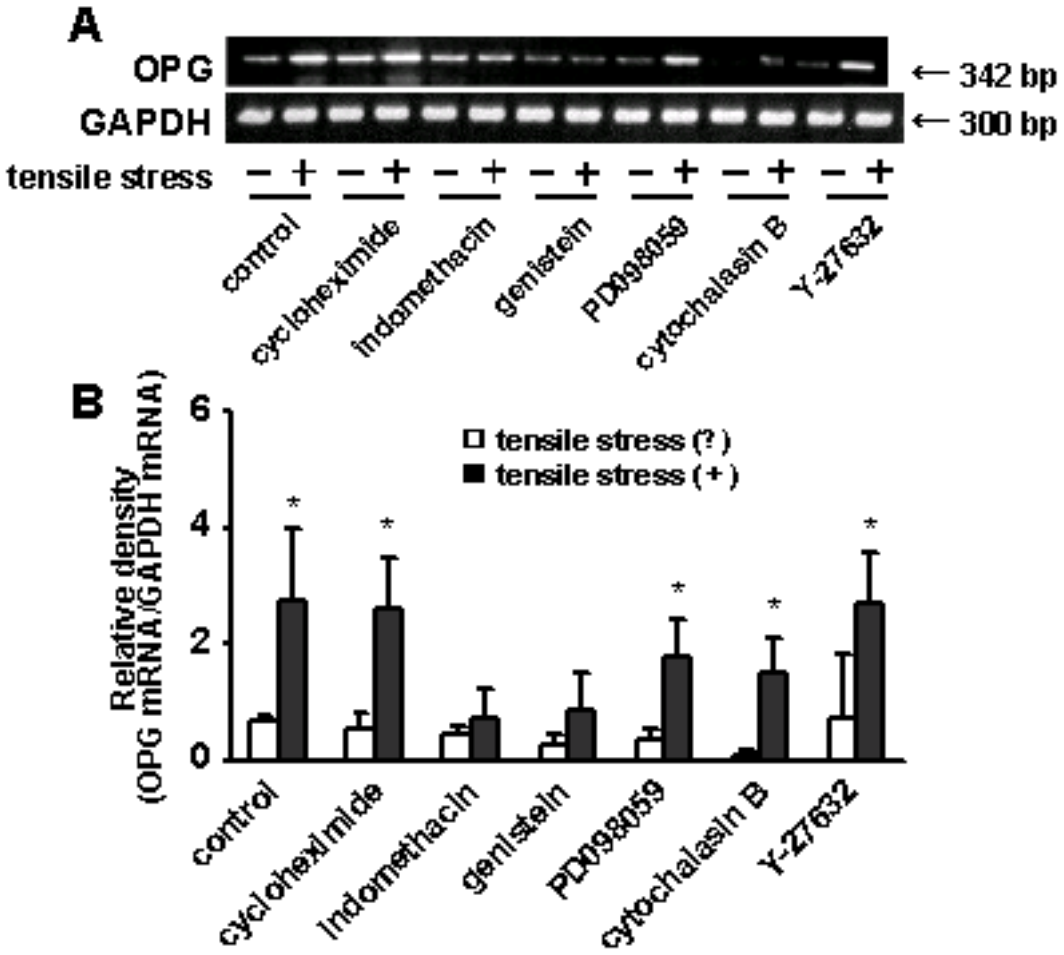

A
OPG
GAPDH
← 342 bp
← 300 bp
tensile stress
control
cycloheximide
indomethacin
genistein
PD098059
cytochalasin B
Y-27632
B
Relative density (OPG mRNA/GAPDH mRNA)
tensile stress (?)
tensile stress (+)
0
2
4
6
*

Fig. 6

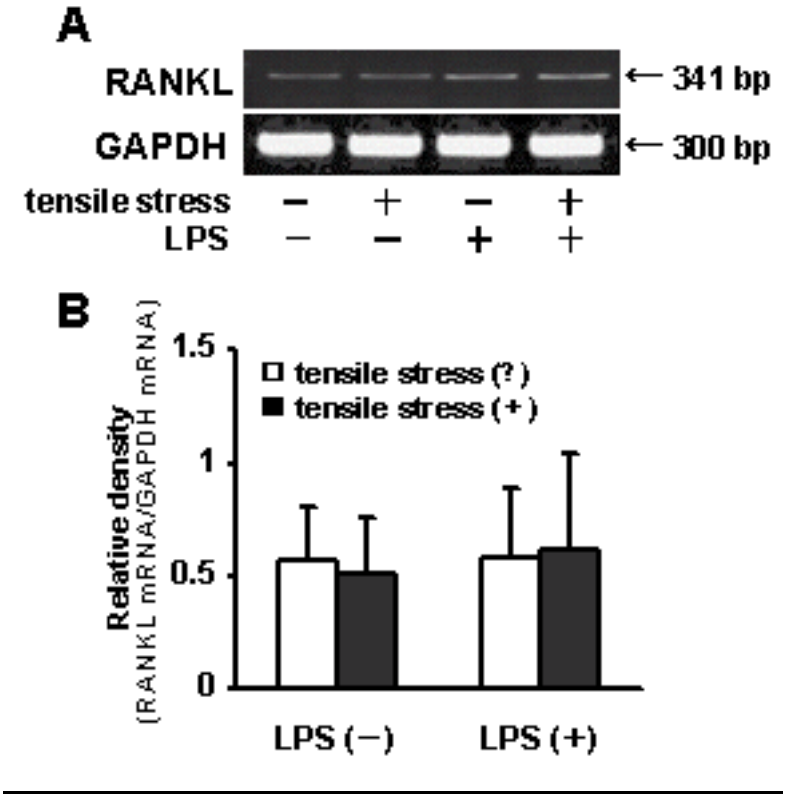

A
RANKL
GAPDH
← 341 bp
← 300 bp
tensile stress − + − +
LPS − − + +
B
Relative density
(RANKL mRNA/GAPDH mRNA)
1.5
1
0.5
0
tensile stress (?)
tensile stress (+)
LPS (−)
LPS (+)

Fig. 7

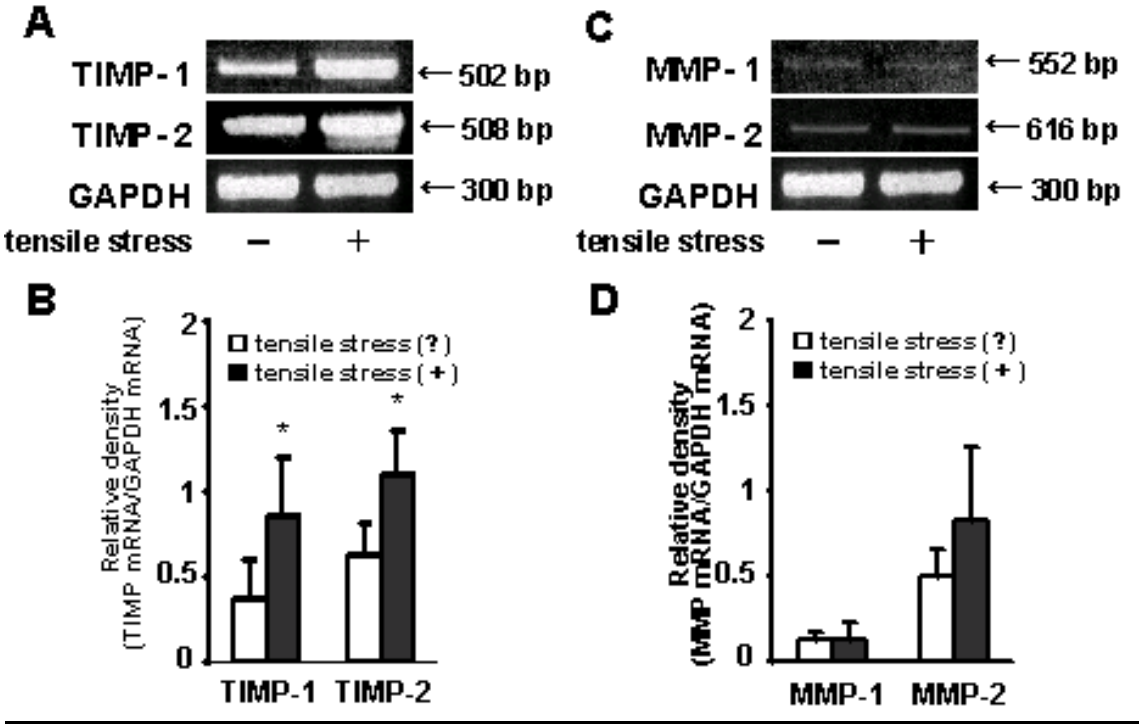
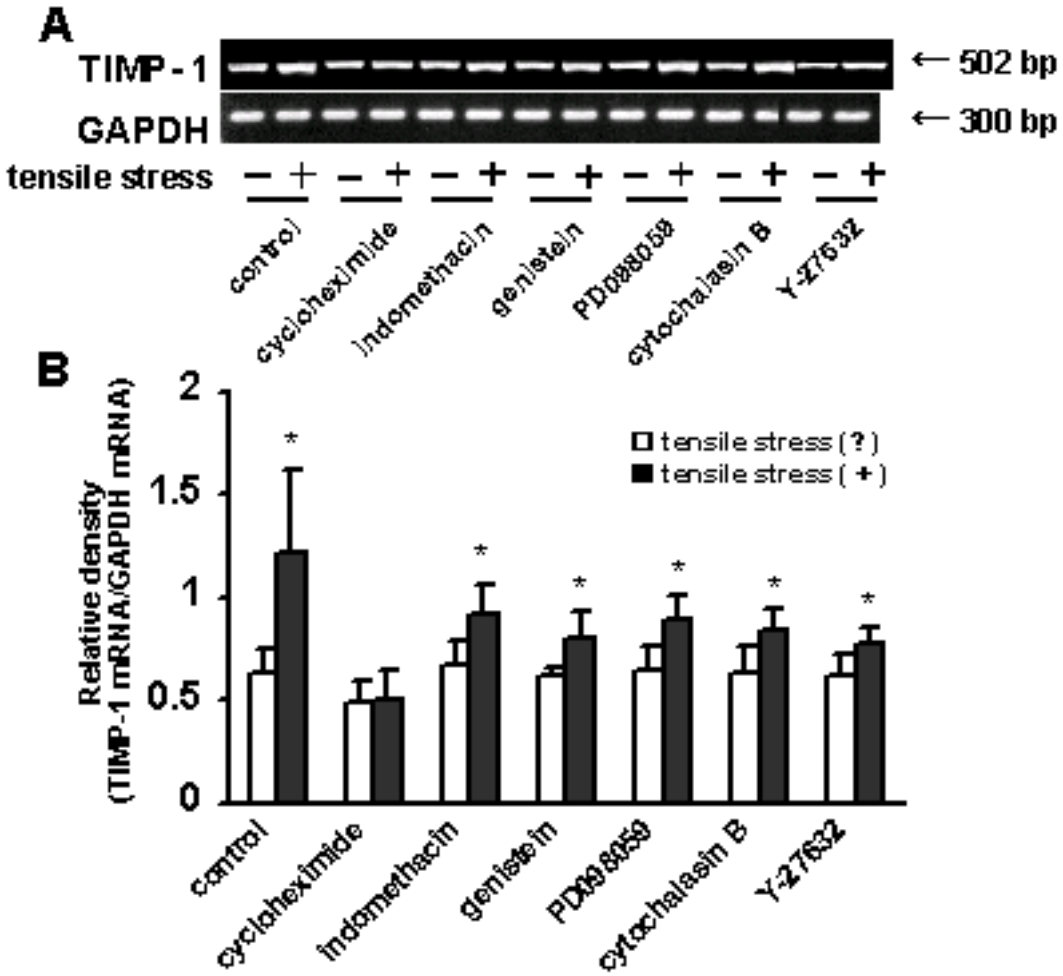

A
TIMP-1
TIMP-2
GAPDH
502 bp
508 bp
300 bp
tensile stress – +
C
MMP-1
MMP-2
GAPDH
552 bp
616 bp
300 bp
tensile stress – +
B
Relative density (TIMP mRNA/GAPDH mRNA)
tensile stress (?)
tensile stress (+)
TIMP-1 TIMP-2
D
Relative density (MMP mRNA/GAPDH mRNA)
tensile stress (?)
tensile stress (+)
MMP-1 MMP-2

Fig. 8


A
TIMP-1
GAPDH
← 502 bp
← 300 bp
tensile stress
control
cycloheximide
indomethacin
genistein
PD098059
cytochalasin B
Y-27632
B
Relative density
(TIMP-1 mRNA/GAPDH mRNA)
tensile stress (?)
tensile stress (+)
0
0.5
1
1.5
2
*