

THE INFLUENCE OF SERIAL PASSAGE ON CYCLIC TENSILE STRAIN-INDUCED OSTEOPROTEGERIN SYNTHESIS FROM NORMAL HUMAN OSTEOBLASTS *IN VITRO*

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Abstract The application of mechanical stress is known to be the unique method of analyses on bone metabolism and remodeling in vitro and in vivo. We have investigated on the effects of the application of cyclic tensile strain (CTS) on the syntheses of bone metabolic factors from the normal human osteoblasts in vitro. In this study, we found that different passage numbers affected synthesis of CTS-induced factors from osteoblasts in vitro. Osteoblasts were stimulated with the application of CTS for 3 days, 4 hrs a day. Then, it was found that the application of CTS increased nitric oxide (NO) production, the expression of cyclooxygenase 2 (Cox-2) mRNA, and synthesis of osteopontin (OPN) from osteoblasts during serial passage. However, CTS-induced osteoprotegerin (OPG) synthesis from osteoblasts was shown to change at different passage numbers. In short, increasing at the 3rd passage, CTS-induced OPG synthesis from osteoblasts decreased at the 5th passage. While, though soluble RANKL (sRANKL) release by the application of CTS decreased at 1st passage and did not change at the 5th passage, the expression of receptor activator of nuclear factor- κ B ligand (RANKL) mRNA increased in osteoblasts at the 5th passage, decreasing in those at the 3rd passage. The increase on OPN synthesis the expression of Cox-2 mRNA, and NO production from osteoblasts by the application of CTS was not affected during serial passage. Analyzing on the activity of the p38 mitogen-activated protein kinase (p38 MAPK) in osteoblasts with or without the application of CTS, the p38 MAPK activity in osteoblasts at the 3rd passage inhibited in compared with that at the 3rd passage. From these results, we should use osteoblasts at the 1st passage when analyzing on CTS-induced OPG synthesis with normal human osteoblasts. It is suggested that the regulation of CTS-induced OPG synthesis might be affected by aging of osteoblasts.

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Introduction

Mechanical stress, as well as a number of biochemical factors, is thought to regulate bone metabolism, and the absence of mechanical stress causes reduction of bone matrix protein production, mineral content and bone formation¹⁻³⁾. Although many investigators have reported the relation between bone and mechanical stress including stretch⁴⁾, ultrasound⁵⁾, and gravity⁶⁾, bone remodeling with the application of mechanical stress is not fully understood.

Concerning bone remodeling, many

investigators have studied the interaction between osteoblasts and osteoclasts⁷⁾. Recently, osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) have been shown to play very important roles in bone remodeling^{7,8)}. OPG is a member of the tumor necrosis factor (TNF) receptor family and a soluble decoy receptor against RANKL and soluble RANKL (sRANKL)⁹⁾. OPG, which is produced by osteoblasts and other cells, has been found to be a key factor in the inhibition of differentiation and activation of osteoclasts⁹⁾. On the other hand, RANKL, which is a member of the TNF

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family, activates mature osteoclasts in growth and osteoclastogenesis via their RANK, and sRANKL cleaved from RANKL behaves similar to RANKL as a soluble factor^{8,10}. The increase of RANKL expression and sRANKL release leads to bone resorption and loss⁹. The regulation of OPG synthesis and RANKL expression causes either activation or inactivation of osteoclasts, which deeply affects bone remodeling⁸. Therefore, it has been hypothesized that imbalances of the OPG/RANKL system are related to the pathogenesis of Paget's disease, benign and malignant bone tumors, postmenopausal osteoporosis, rheumatoid arthritis, bone metastases, and hypercalcemia¹¹.

Mechanical stress is known to synthesize other bone metabolic factors, including osteopontin (OPN)¹², nitric oxide (NO)¹³, and cyclooxygenase-2 (Cox-2). OPN, which is secreted by osteoblasts and deposited in the bone matrix, was reported to play a pivotal role in bone formation under tensile mechanical stress because bone formation at the edge of the parietal bone in contact with the expanded suture gap was reduced in OPN deficiency mice¹². While, NO and Cox-2 are inflammatory factors. NO, which synthesized from NO synthase (NOS) encoding three isoforms, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), was reported to induce recovery from bone atrophy by treatment with tail suspension in iNOS deficiency mice injected NOS donor, nitroglycerine¹⁴. It was reported that PGE₂ induced by Cox-2 enhance bone formation because of induction of collagen-I production from human osteoblasts under the application ultrasound¹⁵. Furthermore, it has been reported that NO directly enhanced Cox-2 activity, which increased the production of proinflammatory prostaglandins (PGs) by macrophages in mice¹⁶.

Recent studies have shown that some intracellular signaling pathways, which mediate the biological effects, are induced by physical stimuli. The mitogen-activated protein kinase (MAPK) family has been found to mediate the

signal transduction of external stimulation into intracellular signals, which regulate cell growth and differentiation¹⁷. The extracellular signal-regulated kinase (ERK) pathway is primarily responsive to growth factors and mitogens, but is also activated by hypoxia or osmotic stress and appears to be involved predominantly in cell growth, division, and differentiation^{18,19}. Though weakly activated by growth factors, c-Jun NH₂-terminal kinase (JNK) shows a strong response to cellular stresses such as UV irradiation, protein synthesis inhibitors, and reperfusion following ischemia^{20,21}. On the other hand, p38 MAPK is activated in cellular responses by various environmental stresses, such as hyperosmolarity²². With regard to bone remodeling, some investigators have reported that ERK1/2 activation in osteoblast-like cells was induced by mechanical stress^{23,24}. ROS 17/2.8 cells, an osteoblast-like cell line, have been reported to increase ERK1/2 activation with the application of mechanical cyclic strain²³. Gravity loading has also been reported to induce ERK1/2 activation in MC3T3-E1, an osteoblast cell line, but not p38 MAPK or JNK activation²⁴. These MAPK cascades allow considerable potential for signal transduction as well as the possibility for cross talk among signal transduction pathways, because the substrate specificities of some of their components and upstream signaling molecules overlap.

Some investigators have reported the relation of mechanical stress, bone, and/or MAPK pathways^{25,26}. Tensile stress-induced OPG has been reported to be produced in periodontal ligament cells²⁵ and bone morphogenetic protein-4 (BMP-4)-induced OPG has also been reported to synthesize in bone-marrow stromal cells, ST2 cells, via the p38 MAPK pathway²⁶. However, there have been very few reports indicating that the application of mechanical stress is significantly related to biological responses of human normal osteoblasts via the intracellular signaling

pathway in bone.

Recently, we have demonstrated that in addition to the new regulation of bone remodeling with mechanical stress, the synthesis of osteoprotegerin (OPG) and soluble receptor activator of nuclear factor- κ B ligand (sRANKL) in normal human osteoblasts by the application of cyclic tensile strain^{27,28)}. To analyze on bone metabolism with the application of mechanical stress, we have used normal human osteoblasts though many investigators have used osteoblastic cell lines instead of osteoblasts. However, there are different evidences, which were kind of MAPKs activation and synthesis of bone metabolic factors induced by the application of mechanical stress, between other reports and ours.

In this study, we analyzed passage-dependent characterizations on cultured normal human osteoblasts with the application of CTS on synthesis of OPG and RANKL, including other factors. Furthermore, the activation of the p38 MAPK in osteoblasts was also examined. In this context, we investigated the relation between bone remodeling and the MAPK cascade in each passage numbers of osteoblasts with the application of CTS.

Materials and methods

Normal human osteoblast culture

Clonetics[®] Normal Human Osteoblasts (NHOst) (Sanko Junyaku Co., Ltd, Tokyo, Japan) were normal human osteoblasts isolated from normal human tissues. Since NHOst were cryopreserved in second passage at Clonetics[®] Cell Culture Facility. NHOst passaged in our laboratory for the first time were the 3rd passage in this study. Purchased NHOst were isolated from bones of a one-day-old female normal human neonate and cultured in Osteoblast Growth Medium[™] (OGM[™]) (Sanko Junyaku Co., Ltd) containing 10% fetal bovine serum (CC-4102) (Sanko Junyaku Co., Ltd), ascorbic acid (CC-4398) (Sanko Junyaku

Co., Ltd) , and gentamicin/amphotericin-B (CC-4381) (Sanko Junyaku Co., Ltd) at 37°C in an atmosphere of 5% CO₂ in air. Tripan blue exclusion confirmed viability of >99% of cells in culture^{27, 28)}.

Morphological examination

Osteoblasts at various stages of passage were monitored during cultured and phasecontrast pictures were taken during microscopic examination with a microscope IX70 (Olympus Corporation, Tokyo, Japan)²⁹⁾.

CTS methods

Previously described CTS methods were followed²⁷⁾. After removal of cultured osteoblasts from a dish containing 0.05% trypsin-0.53 mM EDTA, they were transferred onto a 4 cm² fibronectin-coated silicone chamber at a density of 5×10^4 cells/cm² and cultured overnight after the addition of 1 ml medium. The silicone chamber was attached to a Cultured Cell Stretch System NS-300 (Strex Inc., Osaka, Japan) that was driven by a computer controlled stepping motor. Strain magnitudes were 2%, 7%, and 14%, with the stretch rate fixed at 0.2 Hz, 0.25 Hz, and 0.3 Hz, respectively. In this study, the application of uniaxial sinusoidal stretch of 7% and 0.25 Hz CTS was used to induce maximum OPG production (data not shown). After osteoblasts had been allowed to attach to the chamber bottom for 12 hrs, CTS was applied at 37°C and 5% CO₂ once a day for 4 hrs for three successive days. The relative elongation of the silicone membrane was uniform across the whole membrane area^{27,28)}.

Analysis of nitrite accumulation

Nitrite anion (NO₂⁻) accumulation by cultured osteoblasts was determined as previously reported³⁰⁾. The culture supernatants were obtained from 2×10^5 cells/ml osteoblasts stimulated with or without CTS. To measure

the amount of nitrite in the supernatants, a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chem. Company, Ann Arbor, MI, USA) was used. NO production was assessed in the supernatants of osteoblasts as total nitrite by Griess reaction. A total of 100 μ l supernatants was combined with an equal volume of Griess reagent and these samples were incubated at room temperature before quantifying the absorbance at 540 nm. Using a standard curve, the μ mol of nitrite produced was determined and normalized to total cell number in each sample.

Analyses of OPG, sRANKL, and OPN production by ELISA

Cell-free supernatants were obtained from osteoblasts cultured at a density of 5×10^4 cells/cm² immediately in the absence or presence of the application of CTS once a day for 4 hrs for three successive days. The concentrations of OPG, sRANKL, and OPN in these supernatants were assayed by using OPG, sRANKL, and OPN ELISA kits (Cosmo Bio Co., Ltd, Tokyo, Japan). The samples were incubated at room temperature before quantifying the absorbance at 450 nm. Using a standard curve, the concentrations of OPG, sRANKL, and OPN were determined and normalized to total cell number in each sample²⁷⁾.

Reverse transcription

Total RNA isolated from osteoblasts right after the application of CTS for 4 hrs a day for three successive days were isolated with Trizol[®] Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions³¹⁻³⁵⁾. To reverse-transcribe to cDNA, 2.5 μ g of total RNA were added to 10 μ l of reverse transcription mixture containing 1 \times First Strand Buffer (Invitrogen Corp.), 25 μ g/ml Oligo (dT)₁₂₋₁₈ (Invitrogen Corp.), 20 mM DTT, 1 mM of each dNTP, 1.25 $\times 10^3$ units/ml Recombinant RNasin[®] (Promega

Corporation, Madison, WI, USA) , and 1×10^4 units/ml Superscript[™] II (Invitrogen Corp.) and incubated for 45 min at 42°C , followed by 5 min at 99°C and then for 5 min at 5°C.

External standard for real-time RT-PCR

Five kinds of the external standards were constructed by cloning OPG-, RANKL-, OPN-, Cox-2-, or porphobilinogen deaminase (PBGD) -PCR products with recombinant plasmids as described previously^{27,36)}. OPG-, RANKL-, OPN-, Cox-2- and PBGD-cDNA fragments from osteoblasts were amplified by PCR and gel-purified. OPG-, RANKL-, OPN-, Cox-2-, or PBGD-PCR products were ligated into the pCR[®]2.1 vectors (Invitrogen Corp) with the TA Cloning[®] Kit (Invitrogen Corp), respectively. After the plasmids had been introduced into E. coli strain INVaF' (Invitrogen Corp), dilutions of INVaF' carrying the plasmids were plated onto LB agar medium supplemented with 100 μ g/ml ampicillin and 100 μ g/ml X-gal. The plates were incubated overnight at 37°C. On these vectors of OPG-, RANKL-, OPN-, Cox-2- and PBGD-external standards, automated DNA sequencing was performed on double-stranded DNA templates by the dideoxynucleotide chain termination method by using ABI PRIZM 310 (Applied Biosystems Ltd, Foster City, CA, USA).

Real-time RT-PCR for measurement of OPG, RANKL, OPN, Cox-2, and PBGD mRNA expression

To quantify OPG, RANKL, OPN, Cox-2, and PBGD mRNA expression in osteoblasts, a real-time RT-PCR analysis was examined using the LightCycler[™] (Roche Diagnostics, Mannheim, Germany) technology^{27,36,37)}. For amplification, 20 μ l PCR mix reaction buffer including cDNA reverse-transcribed from 50 ng total RNA , 0.5 μ M upstream primer, 0.5 μ M downstream primer, and 1 \times LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) was added to each capillary (Roche Diagnostics).

Used primers of OPG (upstream, 5'-GGC AAC ACA GCT CAC AAG AA-3'; downstream, 5'-CTG GGT TTG CAT GCC TTT AT-3'), RANKL (upstream, 5'-TCC CAT CTG GTT CCC ATA AA-3'; downstream, 5'-CTT GGG ATT TTG ATG CTG GT-3'), OPN (upstream, 5'-TAG AAC GAG TCA GCT GGA TG-3'; downstream, 5'-TGA AAT TCA TGG CTG TGG AA-3'), Cox-2 (upstream, 5'-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3'; downstream, 5'-AGA TCA TCT CTG CCT GAG TAT CTT-3'), and PBGD (upstream, 5'-TCT GGT AAC GGC AAT GCG GC-3'; downstream, 5'-CCA GGG CAT GTT CAA GCT CC-3')³⁸⁾ were prepared. Primer design of OPG, RANKL, OPN, Cox-2, and PBGD was facilitated by using extensive Primer3 and information obtained from the GenomeNet Database Service of the network service at Kyoto University, Japan²⁷⁾. The expression of PBGD mRNA was used as standard³⁸⁾. After heating at 95°C for 10 min, cDNA was amplified for 45 cycles, each cycle consisting of 95°C for 10 sec, 55°C for 5 sec, and 72°C for 10 sec. After PCR has been completed, the LightCycler™ software (Roche Diagnostics) converted the raw data into molecules of target molecules²⁷⁾. Using a standard curve, the number of molecules for OPG, RANKL, OPN, Cox-2 and PBGD mRNA were determined and normalized in each sample. The mean showed the numbers of OPG, RANKL, OPN, and Cox-2 mRNA molecules per 50 pg total RNA. The levels of expression for PBGD mRNA in osteoblasts stimulated either with or without CTS were about the equal number of molecules (data not shown)^{27, 36)}.

p38 MAPK inhibition studies

Preliminary experiments showed that the optimal concentration for inhibition of p38 MAPK without cytotoxicity was 10 μ M SB203580 (Cosmo Bio Co., Ltd)³⁹⁾. To assess the effects of the inhibitor on p38 MAPK activation, SB203580 was added to the culture medium 1 hr prior to

the application of CTS. Osteoblasts at the 3rd passage were then subjected to cyclic strain for 20 min and lysate samples of osteoblasts were collected for Western immunoblotting analysis (data not shown)^{39,40)}. To examine the effects of the exposure of SB203580 on OPG production, sRANKL release, and the expression of OPG and RANKL mRNA in osteoblasts, 0.1, 1, or 10 μ M SB203580 was added to 5×10^4 cells/cm² osteoblasts in the culture medium 1 hr prior to the application of CTS on day 1, and osteoblasts were subjected to CTS once a day for 4 hrs for three successive days. Cell-free supernatants obtained and total RNA isolated from osteoblasts right after the application of the last CTS, the concentrations of OPG and sRANKL in the supernatants were measured and the levels of OPG and RANKL mRNA expression in the cells were analyzed by a real-time RT-PCR method, respectively.

Western immunoblotting analyses

Western immunoblotting analyses were performed as previously described^{41,42)}. Osteoblasts were stimulated either with or without CTS for 15, 30, or 45 min. After being washed three times with ice-cold PBS at the end of a cyclic stretch, osteoblast samples in the absence and presence of the application of CTS were lysed with 50 μ l Blue Loading Buffer (New England BioLabs, Inc., Beverly, MA, USA) including 0.5 μ l Protease Inhibitor Cocktail (Sigma-Aldrich, Inc., Milwaukee, MI, USA). After lysed osteoblast samples were then transferred to microtubes and treated with heat at 95°C for 5 min, 10 μ l of each sample was loaded onto SDS-PAGE gel. After run, proteins were electrophoretically transferred onto PVDF membrane (Millipore Corp. Bedford, MA, USA). After blocking with SuperBlocking™ Blocking Buffer (Perbio Science, Helsingborg, Sweden), including 5% nonfat dry milk, the membrane was incubated with each primary antibody (Ab) in SuperBlocking™ Blocking Buffer

at room temperature for 1 hr. After washing, the membrane was incubated with HRP-conjugated secondary anti-rabbit Ab in SuperBlocking™ Blocking Buffer. Anti-phospho-p38 MAPKinase rabbit polyclonal Ab and anti-p38 MAPKinase rabbit polyclonal Ab were used as the primary Abs. These primary and secondary Ab were purchased from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan). Immunoactivity was determined by using the SuperSignal® West Pico Chemiluminescent Substrate (Perbio Science) and assessed by exposing film to the blots.

Statistical analysis

Data are presented as mean±S.E. for the number of independent experiments as indicated in figures 2, 3, 4, 5, 6, and 7. Data are expressed as the mean±S.D. of values from triplicate experiments in figures 8 and 9. The statistical significance of the differences was assessed by the Wilcoxon signed rank test. The results were analyzed by using the StatView II statistical program (Abacus Concepts Inc., Berkeley, CA, USA) for Macintosh computers.

Results

Morphological examination at various stages of passage

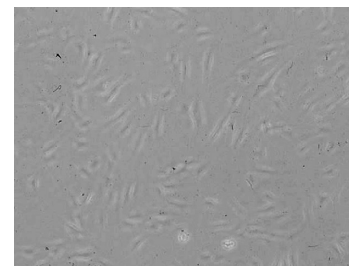
Cultured osteoblasts at passage numbers 3, 5, and 7 were shown in Figure 1.

While osteoblasts at the 3rd passage exhibited the characteristic spindle feature, osteoblasts started to become flattened and enlarged. Osteoblasts at the 7th passage had enlarged cytoplasmic volume and diminished extent of characteristic spindle feature. As osteoblasts took 7 days to reach confluence during early passages of culture, cells older than 8 passages scarcely reached confluence for 14 days.

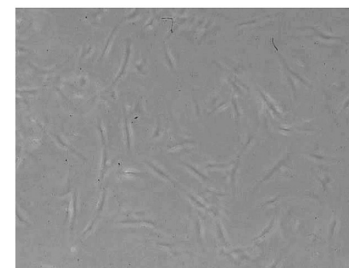
CTS-induced OPG synthesis from osteoblasts during serial passage

We investigated that the application of CTS

Passage 1



Passage 3



Passage 5

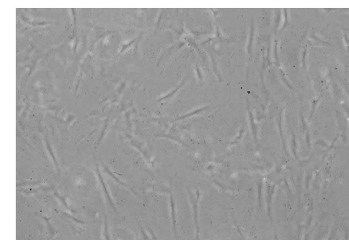


Figure 1 Morphological examination of cultured normal human osteoblasts at various stages of passage. Light microscopic observations of normal human osteoblasts cultured to passage numbers 3, 5, and 7.

affected normal human osteoblasts on OPG and RANKL synthesis²⁷. In this study, it was examined whether passage numbers of cultured osteoblasts affect OPG synthesis with the application of CTS. It was examined that CTS-induced OPG production from osteoblasts at passage numbers 3, 4, 5, 6, and 7. It was found that OPG production from osteoblasts with the application of CTS was lower than that without the application of CTS at the 5th passage, though being higher than that without the application of CTS at the 1st passage (Figure 2). From these results, it was found that CTS-induced OPG production from osteoblasts was dependently inhibited through passage numbers. There were not significant differences at passage numbers 4, 5, and 6.

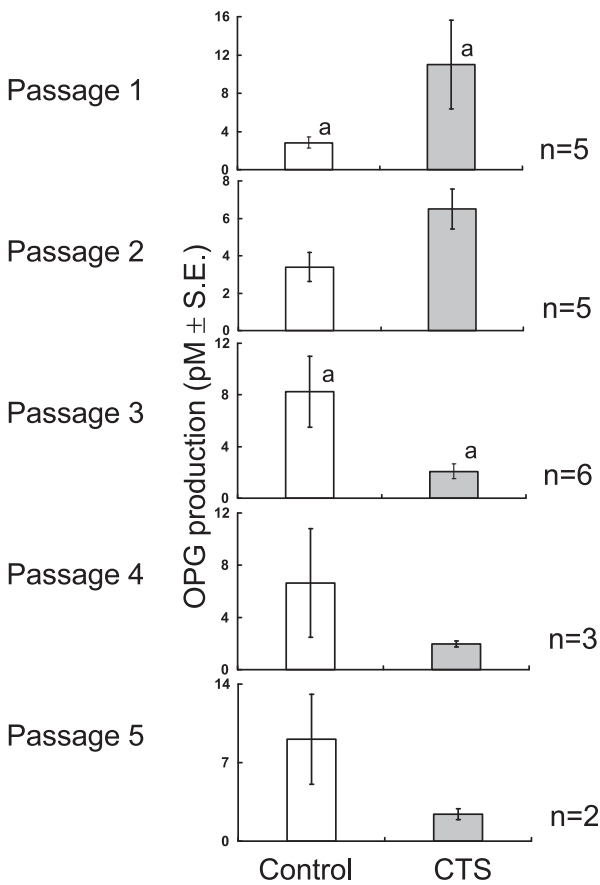


Figure 2 CTS-induced OPG synthesis from osteoblasts of passage numbers 3, 4, 5, 6, and 7. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained and total RNA were isolated from osteoblasts soon after the application of the last CTS, and OPG production and the expression of OPG mRNA were measured by ELISA and a real-time RT-PCR analysis, respectively. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from five independent experiments.
^a Differences are statistically significant ($p < 0.05$).

CTS-induced OPG synthesis from osteoblasts at the 3rd and 5th passage

Production of CTS-induced OPG protein was shown to be affected by differences of passage numbers in Figure 2. It was attempted whether the expression of CTS-induced OPG mRNA was affected by differences of passage numbers. Though the levels of the expression of OPG

mRNA in osteoblasts with the application of CTS were higher than those without the application of CTS at the 3rd passage, the levels of the expression of OPG mRNA in osteoblasts with the application of CTS were lower than those without the application of CTS at the 5th passage (Figure 3). From this result, synthesis of CTS-induced OPG was inhibited by differences of passage numbers.

sRANKL release and the expression of RANKL mRNA in osteoblasts stimulated with the application of CTS at the 3rd and 5th passage

We reported that the application of CTS reduced sRANKL release and the expression of RANKL mRNA from osteoblasts at the 3rd passage²⁷. To examine whether passage numbers were affected on sRANKL release and the expression of RANKL mRNA from osteoblasts with the application of CTS, it was attempted on sRANKL release and the expression of RANKL mRNA from osteoblasts with or without the application of CTS at the 3rd and 5th passage. Though sRANKL release was reduced from osteoblasts at the 3rd passage by the application of CTS, there was not a statistically significant difference between the application of CTS and control on sRANKL release from osteoblasts at the 5th passage. However, though the expression of RANKL mRNA was reduced in osteoblasts at the 3rd passage by the application of CTS, the expression of RANKL mRNA was induced in osteoblasts at the 5th passage by the application of CTS (Figure 4).

CTS-induced OPN synthesis from osteoblasts at the 3rd and 5th passage

It is known that the application of mechanical stress induce enhancement of synthesis of OPN, Cox-2, and NO from cells. In order to analyze whether synthesis of CTS-induced OPG, Cox-2, and NO was affected by differences of passage numbers, it was attempted on synthesis of

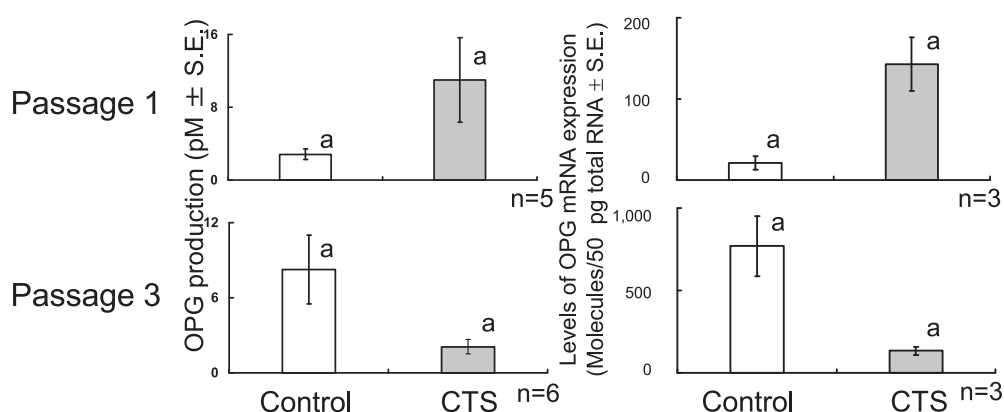


Figure 3 CTS-induced OPG synthesis from osteoblasts at the 3rd and 5th passage. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained and total RNA were isolated from osteoblasts soon after the application of the last CTS, and OPG production and the expression of OPG mRNA were measured by ELISA and a real-time RT-PCR analysis, respectively. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from five independent experiments.
^a Differences are statistically significant ($p < 0.05$).

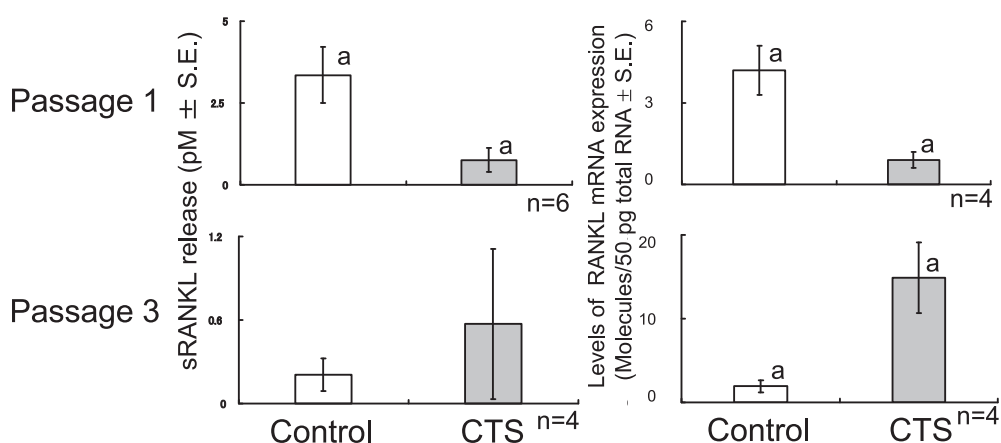


Figure 4 sRANKL release and expression of RANKL mRNA from osteoblasts with the application of CTS at the 3rd and 5th passage. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained and total RNA were isolated from osteoblasts soon after the application of the last CTS, and sRANKL release and the expression of RANKL mRNA were measured by ELISA and a real-time RT-PCR analysis, respectively. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from five independent experiments.
^a Differences are statistically significant ($p < 0.05$).

OPN, the expression of Cox-2 mRNA, and NO production from osteoblasts with or without the application of CTS at the 3rd and 5th passage. It was shown that synthesis of OPN protein and mRNA (Figure 5), the expression of Cox-2 mRNA (Figure 6), and NO production

(Figure 7) from osteoblasts with the application of CTS at the 3rd and 5th passage were not affected. Synthesis of these factors induced by the application of CTS was higher than that of control after the 4th passage (data not shown).

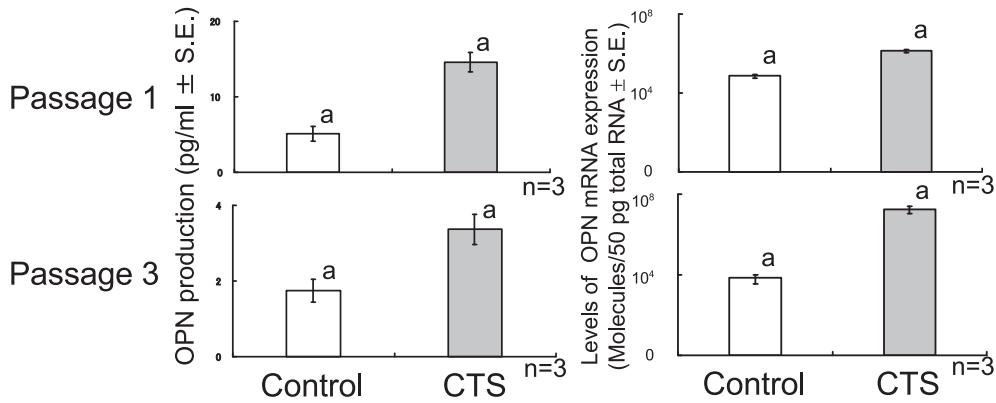


Figure 5 CTS-induced OPN synthesis from osteoblasts at the 3rd and 5th passage. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained and total RNA were isolated from osteoblasts soon after the application of the last CTS, and OPN production and the expression of OPN mRNA were measured by ELISA and a real-time RT-PCR analysis, respectively. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from three independent experiments.

^a Differences are statistically significant ($p < 0.05$).

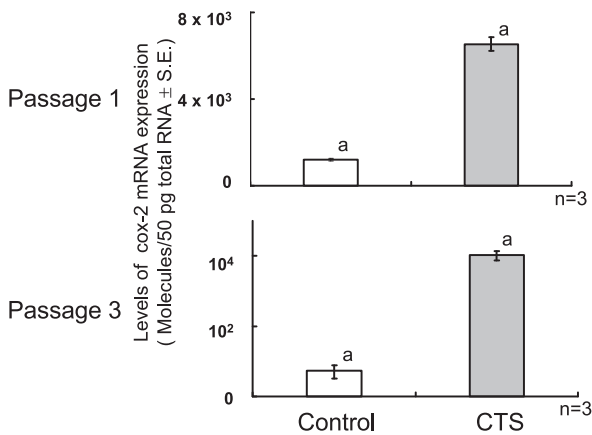


Figure 6 The expression of Cox-2 mRNA from osteoblasts with the application of CTS at the 3rd and 5th passage. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained and total RNA were isolated from osteoblasts soon after the application of the last CTS, the expression of Cox-2 mRNA were measured by a real-time RT-PCR analysis. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from three independent experiments.

^a Differences are statistically significant ($p < 0.01$).

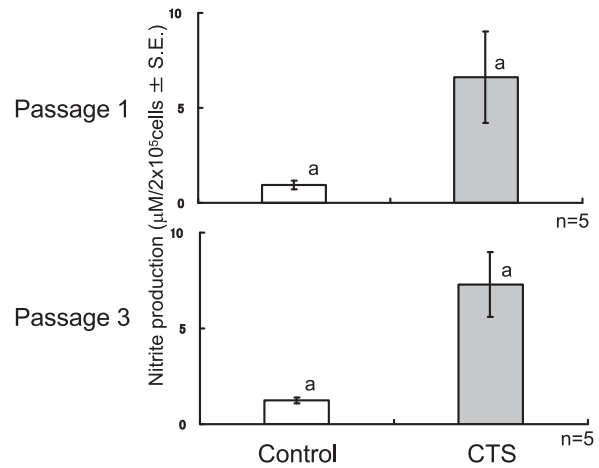


Figure 7 CTS-induced NO production from osteoblasts at the 3rd and 5th passage. Osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained soon after the application of the last CTS, nitrite production was measured by Griess reaction. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean ± S.E. of values from three independent experiments.

^a Differences are statistically significant ($p < 0.01$).

Effects of p38 MAPK inhibitor on OPG production and sRANKL release from osteoblasts at the 3rd passage under the application of CTS

To examine whether p38 MAPK activation

was related to OPG synthesis and sRANKL release from osteoblasts, p38 MAPK inhibitor, SB203580, was employed in the experiment³⁹. With pretreatment of 0.1, 1, or 10 μM SB203580

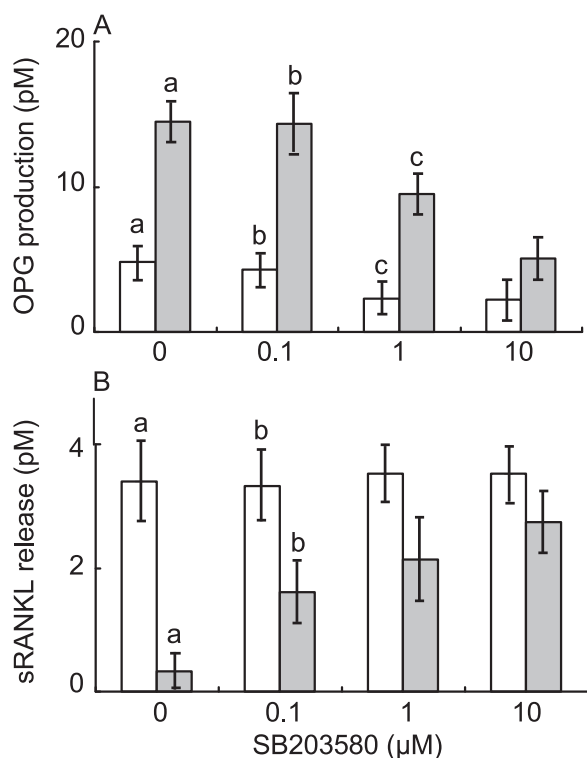


Figure 8 Effects of p38 MAPK inhibitor on OPG production and sRANKL release from osteoblasts with the application of CTS at the 3rd passage. After 0.1, 1, or 10 mM SB203580 were added to osteoblasts in the culture medium 1 hr prior to the application of CTS on day 1, osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Cell-free supernatants were obtained 2 days after the application of the last CTS, and OPG production (A) and sRANKL release (B) were measured by ELISA. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean±S.D. of values from triplicate experiments.

^{a,b} Differences are statistically significant ($p<0.01$).

^c Differences are statistically significant ($p<0.05$).

for 1 hr before the application of CTS, it was found that CTS-induced OPG production was dose-dependently suppressed by SB203580 (Figure 8A) and inhibition of sRANKL release by the application of CTS was simultaneously abrogated by 1 and 10 μ M SB203580 (Figure 8B). From these results, it was thought that the increase of CTS-induced OPG production and abrogation of CTS-reduced sRANKL release

from osteoblasts were induced via the p38 MAPK pathway.

Effects of p38 MAPK inhibitor on the expression of OPG and RANKL mRNA in osteoblasts at the 3rd passage under the application of CTS

Furthermore, a real-time RT-PCR analysis was attempted whether SB203580 affected the expression of OPG and RANKL mRNA in osteoblasts stimulated with the application of CTS. With pretreatment of SB203580 for 1 hr before the application of CTS, it was also found that the expression of CTS-induced OPG mRNA was dose-dependently suppressed (Figure 9A) and inhibition of RANKL mRNA expression by the application of CTS was simultaneously abrogated by pretreatment with SB203580 (Figure 9B). From these results, it was shown that the application of CTS induced the increase of OPG synthesis and abrogation of CTS-reduced sRANKL release and RANKL mRNA expression in osteoblasts via the p38 MAPK pathway and it was speculated that p38 MAPK activation in osteoblasts might regulate bone remodeling.

The time course of p38 MAPK activation in osteoblasts stimulated with the application of CTS at the 3rd and 5th passage

We reported that activation of p38 MAPK was an important role for regulating synthesis of OPG and RANKL in osteoblasts with the application of CTS²⁷. To examine how p38 MAPK in osteoblasts at the 3rd or 5th passage were activated by the application of CTS, the time courses of p38 MAPK activation in osteoblasts with the application of CTS are shown in figure 10. p38 MAPK activation in osteoblasts at the 3rd passage peaked after 15-30 min of the application of CTS and then declined. However, p38 MAPK activation in osteoblasts at the 5th passage was minimum and scarcely increase after 15, 30, and 45 min

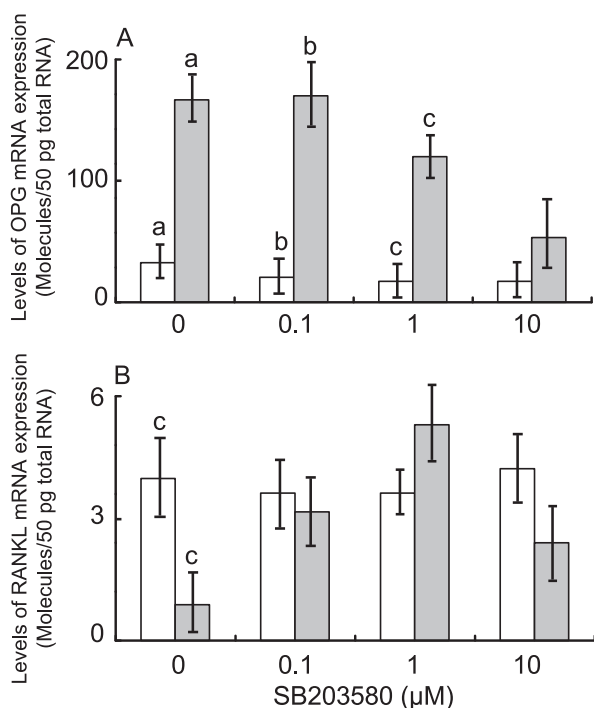


Figure 9 Effects of p38 MAPK inhibitor on the expression of OPG and RANKL mRNA in osteoblasts with the application of CTS at the 3rd passage. After 0.1, 1, or 10 μM SB203580 were added to osteoblasts in the culture medium 1 hr prior to the application of CTS on day 1, osteoblasts were cyclic-strained once a day for 4 hrs for three successive days (■). Total RNA isolated immediately after the application of the last CTS, the levels of OPG (A) and RANKL (B) mRNA expression were measured by a real-time RT-PCR analysis. After real-time PCR has been completed, the LightCycler™ software converted the raw data into molecules of target molecules. Using a standard curve, the numbers of molecules of OPG and RANKL mRNA were determined and normalized in each sample. The mean showed the numbers of OPG and RANKL mRNA molecules per 50 pg total RNA. The control sample (□) was osteoblasts in the absence of the application of CTS. Data are expressed as the mean±S.D. of values from triplicate experiments.

^{a,b} Differences are statistically significant ($p<0.01$).

^c Differences are statistically significant ($p<0.05$).

of the application of CTS. From these results, it was shown that p38 MAPK activation by the application of CTS was affected by differences of passage numbers and the application of CTS resulted in a selective activation of p38 MAPK

in osteoblasts at the 3rd passage (Figure 10).

Discussion

Clinically and generally, mechanical stress and training are thought to contribute to the development of solid bone. Until now, we have reported that normal human osteoblasts stimulated with the application of 7%, 0.25 Hz CTS regulates synthesis of OPG and RANKL via p38 MAPK pathway²⁷.

In this study, furthermore, to analyze characterization of normal human osteoblasts stimulated with the application of CTS, we investigated on passage-dependent changes of synthesis of OPG and RANKL and activation of p38 MAPK in cultured normal human osteoblasts stimulated with the application of CTS. It was found that synthesis of OPG in osteoblasts with the application of CTS was lower than that of control at the 5th passage, though being higher than that without the application of CTS at the 3rd passage (Figures 2 and 3). On the other hand, It was found that the levels of the expression of RANKL mRNA in osteoblasts with the application of CTS was higher than that of control at the 5th passage, though being lower than that without the application of CTS at the 3rd passage (Figure 4). Synthesis of OPN, the levels of the expression of Cox-2 mRNA, and NO production, which are known to be induced by the application of mechanical stress, in osteoblasts stimulated with the application of CTS were higher than those of control at every passage numbers (Figures 5-7). Furthermore, we were shown that pretreatment with p38 MAPK inhibitor, SB203580, against osteoblasts stimulated with the application of CTS inhibited synthesis of CTS-induced OPG and abrogated suppression of CTS-reduced RANKL synthesis at the 3rd passage (Figures 8, 9). Then, it was shown that p38 MAPK in osteoblasts was scarcely activated by the application of CTS at the 5th passage, activated by the application

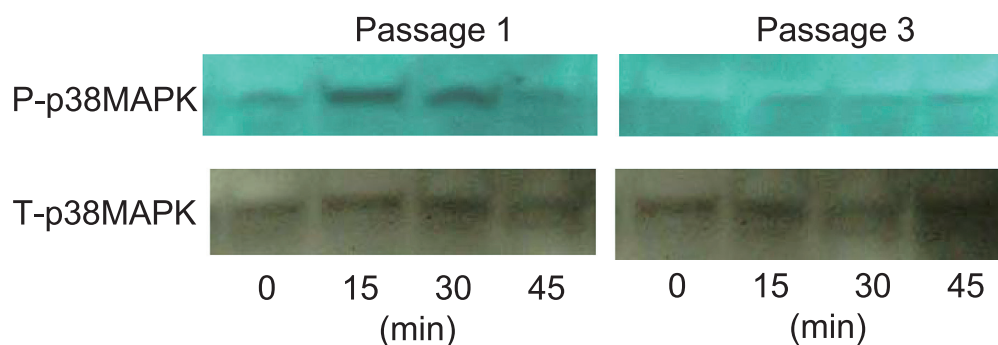


Figure 10 The time courses of the activation of p38MAPK and ERK1/2 in osteoblasts with the application of CTS at the 3rd and 5th passage.

CTS at the 3rd passage (Figure 10). From these results, it was shown that the biological reactions, under the application of CTS on synthesis of OPG and RANKL changed though the culture duration of passage on normal human osteoblasts.

A number of osteoblastic cell lines are available, including mouse MC3T3-E1 cell line, mouse ST-2 cell line, rat ROS 17/2.8 cell line, human MG-63 cells, and so on⁴³⁻⁴⁷. To analyze bone metabolism under the application of mechanical stress, other investigators have used these osteoblastic cell lines⁴³⁻⁴⁶. The application of 16% cyclic tensile stretch has been reported to result in induction of OPG mRNA and protein and reduction of RANKL mRNA and sRANKL release in MC3T3-E1⁴³. As with the present report, this cited study indicates that a stretch induced OPG synthesis and reduced RANKL synthesis as our reports. A marked increase of OPG production has been observed in stretched MG-63 with the application of physical strain, though RANKL production was not affected by the application of physical strain⁴⁶. This cited report also indicated that stretched cells were apt to increase OPG synthesis. On the other hand, uniform equibiaxial mechanical strain of 0.25% has been reported to reduce RANKL mRNA expression in murine bone marrow cells⁴⁸. In the present study, in which the

expression of RANKL protein in osteoblasts with the application of CTS was not examined, it was suggested that the application of strain might reduce not only sRANKL release and RANKL mRNA expression but also RANKL expression from bone-related cells. These cited reports would seem to support our experimental results of OPG synthesis by the application of CTS, although they contained no direct discussion of osteoblasts.

Some investigators have reported findings on the relation between mechanical stress and MAPKs activation in osteoblastic cells^{39,45}. It has been reported that the application of cyclic strain (peak $3400 \mu\epsilon = 0.34\%$, 1 Hz) for 10 min induced ERK1/2 activation in ROS 17/2.8, rat osteosarcoma cells^{49,50}. Nitric oxide and prostaglandins induced by this cited cyclic strain has been reported to induce proliferation and differentiation of osteoblasts via ERK1/2 pathway⁴⁹. It has also been reported that the application of g-loading with a centrifugal force of 12-27g (= 0.012-0.027%) caused induction of ERK1/2 activation but not p38 MAPK or JNK in mouse MC3T3-E1 osteoblasts²⁴. In this study, however, the application of 7%, 0.25 Hz CTS induced p38 MAPK activation but not ERK1/2 activation in osteoblasts at the 3rd passage. The findings of these reports were interestingly the opposite of our results on p38

MAPK and ERK1/2 activation. The reason for such differences might be the difference in the elongation of stretch in these experiments. Seven percent elongation in our study was much greater than that in the two cited reports. Other reasons might be the difference between murine cell lines and human normal osteoblasts, and/or differences in strain methods.

Although an increase in p38 MAPK activation was observed beginning 15 min after the application of CTS, p38 MAPK activation declined after 45 min of the application of CTS (Figure 10). CTS-induced p38 MAPK activation in osteoblasts was thought to be associated with the regulation of OPG synthesis, sRANKL release, and RANKL mRNA expression.

In Figures 8-A and 9-A, CTS-induced OPG synthesis is shown to be inhibited by SB203580. p38 MAPK activation in cells has been reported to enhance OPG synthesis²⁵⁾. Bone morphogenetic protein-4 (BMP-4), which induces differentiation of osteoblasts, has been reported to increase OPG synthesis in a mouse bone-marrow-derived stromal cell line, ST2, via the p38 MAPK pathway, but not in the ERK1/2 pathway because OPG synthesis in ST2 stimulated with BMP-4 was reduced by p38 MAPK inhibitor, SB203580 but not by ERK1/2 inhibitor, PD98059²⁵⁾. This cited report shows that p38 MAPK activation resulted in OPG production in bone and supports our findings, although the stimulators and cells were different from those in our study.

As shown in Figure 8-B and 9-B, inhibition of sRANKL release and RANKL mRNA expression by the application of CTS was abrogated by the pretreatment of SB203580. This experiment indicated that the p38 MAPK pathway was associated with sRANKL release. However, in Figure 9-B, not dose-dependently abrogated by 10 μ M SB203580, CTS-reduced RANKL mRNA expression was thought to be regulated by the differences of the levels of p38

MAPK activation⁵¹⁾. Furthermore, the analyses of the relationship between the p38 MAPK activation and the synthesis of enzymes cleaving into sRANKL, for example TNF- α converting enzyme (TACE), were thought to need^{52,53)}.

From the present results, the application of 7%, 0.25 Hz CTS, which is thought to place considerable strain on osteoblasts, induced an increase of OPG synthesis and a decrease of sRANKL release and RANKL mRNA expression in osteoblasts. Furthermore, it simultaneously caused induction of p38 MAPK activation and reduction of ERK1/2 activation in osteoblasts. Regarding intracellular signaling, synthesis of OPG and sRANKL was considered to be regulated in osteoblasts activated by the application of CTS via the p38 MAPK pathway. Consequently, it was suggested that CTS-induced p38 MAPK activation in osteoblasts could play an important role in cellular responses of bone remodeling. This study yielded important evidence on bone remodeling by CTS, and its findings strongly suggest that human osteoblasts subjected to mechanical stress might modulate and regulate bone metabolism.

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