Abstract: High-magnitude mechanical strain inhibits bone nodule formation by reducing expression of bone morphogenetic protein-2 (BMP-2), Runt-related transcription factor 2 (Runx2), and muscle segment homeobox 2 (Msx2). Mechanical strain also induces production of proinflammatory factor prostaglandin E2 (PGE2) by osteoblasts. We measured the effect of mechanical strain-induced PGE2 production on bone nodule formation and expression levels of bone formation-related factors. Osteoblast-like cells isolated from fetal rat calvariae were loaded with 18% cyclic tension force (TF) for 48 h in the presence or absence of NS-398, a selective inhibitor of cyclooxygenase-2. To investigate the effect of TF-induced PGE2 on bone formation, bone nodule area on day 21 was measured by von Kossa staining. BMP-2, Runx2, and Msx2 expression levels were examined at 1 day after TF loading. Bone nodule formation was significantly inhibited by TF but was restored to control level by PGE2 inhibition. Furthermore, TF loading-induced reductions in expressions of these factors were restored to control level by PGE2 suppression. These results indicate that PGE2 production induced by high-magnitude mechanical strain inhibits bone nodule formation by reducing expression levels of bone formation-related factors.

Keywords: mechanical strain; bone-nodule; PGE2; Runx2; osteoblast.

Introduction

Tooth movement occurs through remodeling of alveolar bone by application of orthodontic force, a process that is strongly associated with activity of osteoblast and osteoclasts in alveolar bone. To induce physiological bone remodeling without damaging teeth or periodontal tissue, the optimal level of force must be applied. Mitsui et al. (1) reported that a lead weight producing 1.0 g/cm2 of continuous compressive force induced in vitro mineralization by increasing expression of bone morphogenetic protein-2 (BMP-2), Runt-related transcription factor 2 (Runx2), and Osterix. In contrast, a continuous compressive force of 3.0 g/cm2 reduced mineralization and BMP-2 expression in the human osteoblastic osteosarcoma cell line Saos-2. Koyama et al. (2) reported that a compressive force of 3.0 g/cm2 induced production of the proinflammatory cytokines interleukin (IL)-1β, -6, and -8 and tumor necrosis factor (TNF)-α, which promote osteoclast formation.

Using methods similar to those of the present study, Jacobs et al. (3) found that a cyclic tension force of 5% elongation at 60 cycles/min induced bone formation in vitro by increasing alkaline phosphatase, collagen I, and osteoprotegerin expression in periodontal ligament cells. Agarwal et al. (4) reported that a cyclic tension force of 12.5-18.0% elongation at 0.3 cycles/min enhanced synthesis of proinflammatory factors in human periodontal ligament cells. Furthermore, we recently reported that a high-magnitude cyclic tension force...
(18% elongations at 6 cycles/min) significantly inhibited bone nodule formation and reduced BMP-2, Runx2, and muscle segment homeobox 2 (Msx2) expression in rat calvarial cells (5). These findings suggest that optimal force enhances, while excessive force inhibits, bone formation. However, whether factors related to bone formation are directly affected by mechanical stress is unclear.

Prostaglandin (PG) E injection around the tooth accelerates orthodontic tooth movement (6-8). PGE\(_2\) has a potent osteoclast-inducing effect that is mediated by cyclooxygenase-2 (COX-2). Mirsaidi et al. (9) reported that a high PGE\(_2\) concentration inhibits mineralization by human osteoblasts. Moreover, PGE\(_2\) production by osteoblasts is induced by application of mechanical stress, and production is positively related to the magnitude of mechanical stress applied (10).

We examined the effect of PGE\(_2\) production induced by high-magnitude mechanical strain on bone nodule formation and the expression of the bone formation-related factors BMP-2, Runx2, and Msx2 in rat calvarial cells. Using the experimental model of Bellows et al. (11,12), we investigated the effects of high-magnitude mechanical strain and PGE\(_2\) on bone nodule formation and expression levels of bone formation-related factors.

### Materials and Methods

#### Rat calvarial cell culture procedures

The procedures used for osteogenic cell isolation and culture were previously described by Fushiki et al. (5) and Bellows et al. (11).

#### Mechanical strain loading

Calvarial cells were exposed to mechanical strain with or without \(10^{-6}\) M NS-398 (Merck KGaA, Darmstadt, Germany), a selective inhibitor of COX-2, as described by Fushiki et al. (5). Control cells (Control) and control cells supplemented with NS-398 (Control+NS) were loaded with 0% elongation and cultured on plates of the same type.

#### Measurement of bone nodule area

At 18 days after TF loading, bone nodule area was assayed using the von Kossa technique as previously described (5). The area of von Kossa-stained bone nodules in each well was measured with ImageJ software (ver. 1.51s, win 64-bit; National Institutes of Health, Bethesda, MD, USA). Briefly, a binarized image at a threshold value of 60 was generated from a photograph of a 32-mm-diameter area of each 34-mm well, and the area with a threshold value of 0-60 was measured. The average of three wells per group was calculated.

### PGE\(_2\) assay

The amount of PGE\(_2\) in culture medium after TF loading for 48 h was measured by using an enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (KGE004B; R&D Systems Inc., Minneapolis, MN, USA) in accordance with the manufacturer’s instructions. The average PGE\(_2\) concentration of three wells per group was calculated.

### Reverse transcription-polymerase chain reaction

Total RNA was extracted 1 day after TF loading for 48 h (day 1), as previously described by Fushiki et al. (5). The gene-specific primers used are shown in Table 1.

### Statistical analysis

Values are shown as means ± standard deviations (SD). SPSS software version 16.0 (SPSS Japan, Tokyo, Japan) was used to perform one-way analysis of variance with the Tukey test. A \(P\) value of <0.05 was considered to indicate statistical significance.

### Results

#### PGE\(_2\) levels

PGE\(_2\) level was significantly lower in the Control+NS group than in the Control group and significantly higher in the TF-for-48-h group (18% TF) than in the Control and Control+NS groups. However, the PGE\(_2\) level in the TF with NS-398 (18% TF+NS) group was similar to that of the control (Fig. 1).

#### Effect of TF on bone nodule formation

Fewer bone nodules were observed in the 18% TF group than in the Control and Control+NS groups. However,
addition of NS-398 increased the number of bone nodules in the 18% TF group (Fig. 2A). Bone nodule area did not significantly differ between the Control and Control+NS groups and was significantly smaller in the 18% TF group than in the Control and Control+NS groups (Fig. 2B).

**BMP-2, Runx2, and Msx2 expression**

On day 1, mRNA expression levels of BMP-2, Runx2, and Msx2 were determined (Fig. 3A-C). BMP-2 expression level in the 18% TF group was 42% that of the Control group but recovered in the 18% TF+NS group, although the increase was not significant (P = 0.075). The bars show the means ± SD of three experiments; *P < 0.05, significant decrease vs. the Control and Control+NS groups.

Runx2 expression level was significantly lower in the 18% TF group, 72% that of the Control group, but significantly increased, to the control level, in the 18% TF+NS group. The bars show the means ± SD of three experiments; **P < 0.01, significant decrease vs. the Control and Control+NS groups. †P < 0.05, significant increase vs. the 18% TF group.

Msx2 expression level was significantly lower in the 18% TF group, approximately half that of the Control group, but was equivalent to that of the control level in the 18% TF+NS group. The bars show the means ± SD of three experiments; **P < 0.01, significant decrease vs. the Control and Control+NS groups. †P < 0.05, significant increase vs. the 18% TF group.

The bone nodule area in the 18% TF+NS group was similar to those in the Control and Control+NS groups.
and Msx2 did not significantly differ between the Control and Control+NS groups. The BMP-2 expression level in the 18%TF group was 42% that of the Control group (Fig. 3A; \( P < 0.05 \)) but recovered in the 18%TF+NS group, although the increase was not significant (\( P = 0.075 \)). The Runx2 expression level in the 18%TF group was significantly lower—72% that of the Control group—but significantly recovered, to that of the control, in the 18%TF+NS group (Fig. 3B; \( P < 0.05 \)). The Msx2 expression level in the 18%TF group was significantly lower, approximately half that of the Control group, but significantly recovered, to that of the control, in the 18%TF+NS group (Fig. 3C; \( P < 0.05 \)).

**Discussion**

In orthodontic treatment, sequential bone remodeling is induced by therapeutic mechanical strain (13,14). We previously reported (5) that high-magnitude mechanical strain inhibits bone nodule formation and reduces Runx2, BMP-2, and Msx2 expression levels in rat calvarial cells. Mechanical stress applied to osteoblasts may induce production of not only bone formation-related factors (such as BMP-2, Runx2, and Msx2), but also proinflammatory factors such as IL-1, IL-6, TNF-α, and PGs. PGE2, a potent proinflammatory factor, is produced by osteoblasts in response to mechanical stress, and the magnitude of the increase is dependent on the strength of the compressive force (10). Mirdadi et al. (9) reported that PGE2 inhibits mineralization of bone nodules by human osteoblasts. Therefore, we investigated the relationship of mechanical stress-induced PGE2 production with bone nodule formation and BMP-2, Runx2, and Msx2 expression.

PGE2 production in calvarial cells was significantly increased by 18% TF loading and reduced by the addition of NS-398. In contrast, bone nodule area was reduced to 40% of control by 18% TF loading and recovered to the control level after addition of NS-398. According to reports of Bellows et al. (11) and Ozawa et al. (15), we hypothesized that the number of bone nodules can be used as a measure of bone nodule area. We noted that PGE2 production induced by high-magnitude TF inhibited bone nodule formation.

We investigated the effects of TF loading on PGE2 production and BMP-2, Runx2, and Msx2 expression. BMPs, transforming growth factor superfamily member, induces not only bone formation but also osteoblast differentiation of mesenchymal cells. BMP-2 is a potent osteoblastic differentiation factor that is important in regulating bone formation and remodeling (16) and enhancing proliferation and differentiation of human osteoblasts (17,18). Runx2 is a transcription factor essential for osteoblast differentiation and bone nodule formation (19,20), and its activity is induced by BMP-2 through Smad1/Smad5 signaling (21,22). Msx2 regulates osteoblast proliferation and differentiation via a Runx2-independent mechanism (23,24).

We previously reported (5) that Runx2 and Msx2 expression levels at 1 day after TF loading for 48 h were higher than those at 4 and 7 days; therefore, expression levels of these factors at 1 day after TF loading were examined in the present study. BMP-2, Runx2, and Msx2 expression levels were significantly inhibited by 18% TF loading but recovered to control levels with the addition of NS-398. These results suggest that the inhibition of bone nodule formation by 18% TF loading was due to reduced expression of the bone formation-related factors BMP-2, Runx2, and Msx2 and by increased production of PGE2. However, a previous study reported that proinflammatory factors other than PGs, such as IL-1β and TNF-α, were induced by excessive mechanical stress (2). These factors likely affect bone nodule formation via inhibition of BMP-2 expression.

In conclusion, mechanical strain-induced PGE2 production inhibited bone nodule formation by reducing expression levels of bone formation-related factors.

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**Conflict of interest**

The authors have no conflict of interest to declare.

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