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Inflammation Research

Substance P stimulates release of RANKL via COX-2 expression in human dental pulp cells

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Abstract. *Objective:* Our previous study found that substance P (SP), a sensory neuropeptide, was expressed in the dental pulp of rats during experimental tooth movement. We examined the effects of SP on the production of prostaglandin (PG) E_2 and the receptor activator of nuclear factor- B ligand (RANKL) by human dental pulp fibroblast-like (HDPF) cells.
Materials and methods: SP was added to cultured HDPF cells at concentrations ranging from of 10^{-4} to 10^{-12} mol/L. PGE_2 and soluble RANKL (sRANKL) levels were determined using enzyme-linked immunosorbent assay kits. Gene expression was confirmed by RT-PCR analysis. Pit formation assays using dentin slices were carried out to examine the effect of SP on osteoclastogenesis.
Results: The levels of PGE_2 and sRANKL increased in the presence of SP, though the increases were greater in the experimental groups in both a time- and concentration-dependent manner, and the increase of RANKL was partially mediated by PGE_2. The gene expression of cyclooxygenase (COX)-2 and RANKL was up-regulated, and conditioned medium samples obtained from HDPF cells treated with SP induced bone resorption.
Conclusions: SP stimulated the production of PGE_2 and RANKL, and promoted bone resorption. Therefore, SP may be involved in pulpal inflammation and root resorption during orthodontic tooth movement.

Key words: Human dental pulp fibroblast-like cells – Substance P – RANKL – COX-2 – PGE_2

Introduction

The peripheral sensory nervous system contributes to the development of acute and chronic inflammatory processes through the local release of neuropeptides. Substance P (SP), a sensory neuropeptide released from the peripheral endings of sensory nerves during inflammation, can modify the secretion of pro-inflammation cytokines from immunocompetent cells.

A number of different neuropeptides, including SP, are known to be present in the nerve fibers of dental pulp and the periodontal tissues in rats, cats, monkeys, and humans [1–6]. Further, SP-immunopositive nerve fibers have been shown to change their patterns as a result of local pulp trauma possibly indicating that SP-containing fibers take part in the inflammatory process in connection with tissue injury and repair [7]. Recently, it was found that the expression of SP increases in dental pulp in response to buccally directed orthodontic tooth movement (OTM) of the upper first molar in rats [8]. Norevall et al. also suggested that this neuropeptide might be involved in inflammation of the dental pulp during OTM [9].

Prostaglandin (PG) plays important roles in regulating diverse cellular functions in both physiological and pathological conditions [10]. Sundqvist et al. reported that human dental pulp (HDP) fibroblasts (HDPF) to produce PGE_2 following treatment with bradykinin [11], and previous studies have shown that PGE_2 is involved in the pathogenesis of pulpal inflammation [12–14]. PGE_2 has also been reported to be a potent stimulator of bone resorption [12]. PGE_2 production begins when phospholipases liberate arachidonic acid (AA) stored in membrane phospholipids. AA is then converted into PGG_2 by cyclooxygenase (COX), also known as PG endoperoxide synthase, and then PGG_2 is converted into PGH_2 by the peroxidase activity of PG hydroperoxidase [15, 16]. PGH_2 is then transformed into PGE_2 by PGE syntheses.

COX-1 and COX-2, have been characterized, and are known to be encoded by different genes [17, 18, 19, 20]. COX-1 is expressed in a constitutive manner in various tissues [21], while COX-2 is induced following the activation of cells by a variety of proinflammatory agents, such as cytokines [22–24]. Thus, COX-2 may have a role distinct from that of COX-1 in inflammation or bone resorption [25–27].

Osteoclastogenesis is supported by a member of the tumor necrosis factor (TNF) family of proteins that has been shown

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to be identical to the receptor activator of nuclear factor-*k*B ligand (RANKL) [28], TNF-related activation-induced cytokine (TRANCE) [29], and osteoclast differentiation factor (ODF) [30]. Binding of RANKL to receptor activator of nuclear factor-κB (RANK) is known to cause differentiation of osteoclast precursor cells into osteoclasts: RANKL thus promotes osteoclastogenesis and the activity of osteoclasts in bone resorption. RANKL is produced in bone marrow cells, spleen cells, and peripheral blood monocytes which support osteoclastogenesis in the presence of macrophage colony-stimulating factor [31], and RANKL has been shown to stimulate bone resorption by mature osteoclasts *in vitro* [32]. Further, Kanematu et al. showed that PGE_2 induces RANKL expression in marrow stromal cells [33]. However, the relationships between PGE_2 and RANKL in HDPF cells during orthodontic tooth movement have not been elucidated. In the present study, we investigated that the effect of SP stimulation on the production of PGE_2 and soluble RANKL (sRANKL) in HDPF cells *in vitro*: the gene expression of COX-1, COX-2, and RANKL in HDPF cells, and osteoclastogenesis supporting activity by SP using pit formation assays with dentin slices.

Material and methods

Cell cultures

HDPF cells were prepared according to a modified method of Hosoya et al. [34]. Briefly, HDP tissue samples from the roots of pre-molars extracted from 6 healthy young volunteers (3 males, 3 females; 15–25 years old) during the course of orthodontic treatment were obtained, after receiving informed consent from the donors. This study was conducted according to a protocol reviewed by the Board of Nihon University School of Dentistry at Matsudo. The HDP tissue specimens were placed in 35-mm tissue culture dishes, which were covered with sterilized glass coverslips. The medium used was alpha minimum essential medium (α-MEM) (Gibco, Grand Island, NY, USA.), supplemented with 100 µg/ml of penicillin-G (Sigma Chemical Co., St. Louis, MO, USA.), 50 µg/ml of gentamicin sulphate (Sigma), 0.3 µg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA.), and 10 % fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, OH, USA). The culture media were kept at 37 °C in a humidified incubator (Forma CO_2 Incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95 % air and 5 % CO_2. When the cells growing from explants had reached confluence, they were detached with 0.05 % trypsin (Gibco, Grand Island, NY, USA) in phosphate-buffer salines (PBS) for 10 minutes, then subcultured in culture flasks. For the experiments, HDPF cells were used at passages 6 to 9.

Assay methods

To examine the effects of SP (Cayman Chemical, ML, USA) on the production of PGE_2 and sRANKL, approximately 5 x 10^4 HDP cells were transferred to a 24-well plate. Confluent-stage cells were incubated for 24 h in fresh medium containing 2 % FCS in the presence of various concentrations of SP (10^{-12} to 10^{-4} M), and the levels of PGE_2 and sRANKL present in the media were determined using ELISA kit (PGE_2; R&D Systems, MN, USA sRANKL; Biomedica Medizinprodukte GmbH & Co KG, Wien Austria).

Next, the effect of NS-398 (Cayman Chemical, ML, USA), a selective inhibitor of COX-2, on the production of sRANKL was measured by stimulating the cells with SP (10^{-6} M) in the presence or absence of NS-398. SP/NS-398 cultures were pretreated with 10 µM NS-398 for 1 h before adding 10^{-6} M SP.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We extracted the RNA from the HDP cells with an RNeasy mini kit (Qiagen, Co, Tokyo, Japan) following the manufacturer's protocol. The RNA was amplified with an RT-PCR kit, and we obtained 40 µl of purified total RNA. Total RNA was converted to cDNA by ReverTra Ace (Toyobo, Co, Osaka, Japan). PCR amplification was performed using KOD Dash (Toyobo, Co, Osaka, Japan) in a thermal cycler (PTC-0200 DNA Engine, MJ Research, INC. USA). After a hot start, the samples were denatured at 98 °C for 20 s, primer annealed at 55 °C to 60 °C for 2 s, and extened at 74 °C for 30 s for 25 to 30 cycles. PCR primers for COX-1,COX-2,RANKL and β-actin were purchased from Sigma Genosys Co. (Sigma Genosys, Co , Hokkaido, Japan), and were designed referring to the sequences of cDNA that had reported COX-1, COX-2, RANKL, and β-actin [35]. The primers were designed as follows: COX-1: 5'-GGCCTTGGGCCATGGGGTAG-3' and 5'-AGCT GCTCATCGCCCCAGGT-3', COX-2: 5'-AACCCACTCCAAA CACAG-3' and 5'-CTGGCCCTCGCTTATGATCT-3', RANKL: 5'-AG-CAGAGAAAG CGATGGT-3' and 5'-GGGTATGAGAACTTGGGATT-3', β-actin 5'-ATG AGGATCCTCACCGAGCGCGGCTCAGC-3' and 5'-ACACCACTGTGT TGGCGTACAGGTCTTTGC-3'. The PCR products were separated by electrophoresis in a 1.5 % agarose gel and visualized by ethidium bromide staining with UV light illumination. The differences between the PCR products were quantitated by luminescence values. The relative intensities were measured by an image analyzer (Atto densitograph, Atto corp., Tokyo, Japan).

Pit formation on dentin slices by the addition of conditioned medium

The resorptive activity of mature osteoclasts formed *in vitro* was evaluated by examining the ability of the osteoclasts to form resorption pits on dentin slices obtained from mammoth ivory (diameter, 6 mm; thickness, 0.15 mm). Briefly, human osteoclast precursor cells (Hokudo, Tokyo, Japan) were cultured in commercial medium (Hokudo, Tokyo, Japan) at 37 °C for 8 days. After the osteoclasts matured, the commercial medium was changed to conditioned medium obtained from HDP cells after incubated with or without 10^{-6} M SP for 12 h. Following incubation for 48 h, the cells were washed 3 times with PBS. The dentin slices were sonicated in 1 M ammonia solution to remove the cells, then washed and dried. After drying, the slices were mounted onto stubs and sputter coated with platinum for scanning electron microscopy (HCP-2 Hitachi, Tokyo, Japan). We measured the area of the resorption pits per area of dentin slices of four dentin slices and statistical analyzed it in Student's *t*- test.

Statistical methods

Values are shown as the mean±standard deviation (S.D.). Statistical significance was determined using Student's t-test (Figs. 1, 2, 4-B, and Table 1). Data were subjected to one-way analysis of variance (ANOVA) (Figs. 1 and 2). A P value of less than 0.05 was considered statistically significant.

Results

Our recent study suggested that concentrations of SP ranging from 10^{-12} M to 10^{-4} M affect on the production of interleukin (IL)-1β, IL-6, and TNF-α in HDPF cells [36]. The production of these cytokines in relation to osteoclast activity reached a plateau after 12 h of stimulation with 10^{-6} M SP: therefore 10^{-6} M of SP was used to examine the time course

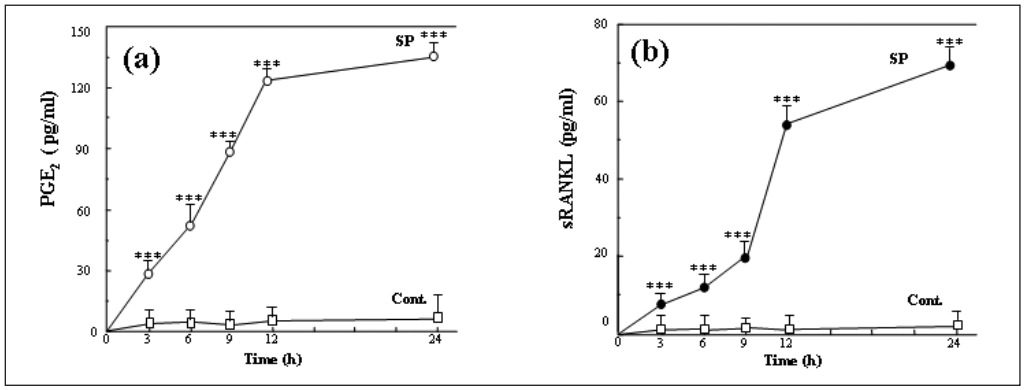

Fig. 1. Time-dependent effects of incubation with substance P (SP) on production of prostaglandin E_2 (PGE_2) and soluble the receptor activator of nuclear factor-B ligand (sRANKL) by human dental pulp fibroblast-like (HDPF) cells in conditioned media. HDPF cells were incubated with or without the indicated concentrations of 10^{-6} M SP for 24 h. Data are expressed as the mean ±S.D. of 6 cultures. PGE_2 and sRANKL were increased by the addition of SP in a time-dependent manner [$p < 0.001$, by one-way analysis of variance (ANOVA)]. Statistically significant from the corresponding control (with out SP) at each concentration. ***$p < 0.001$; □: control; ○: PGE_2 with 10^{-6} M of SP; ●: sRANKL with 10^{-6} M of SP

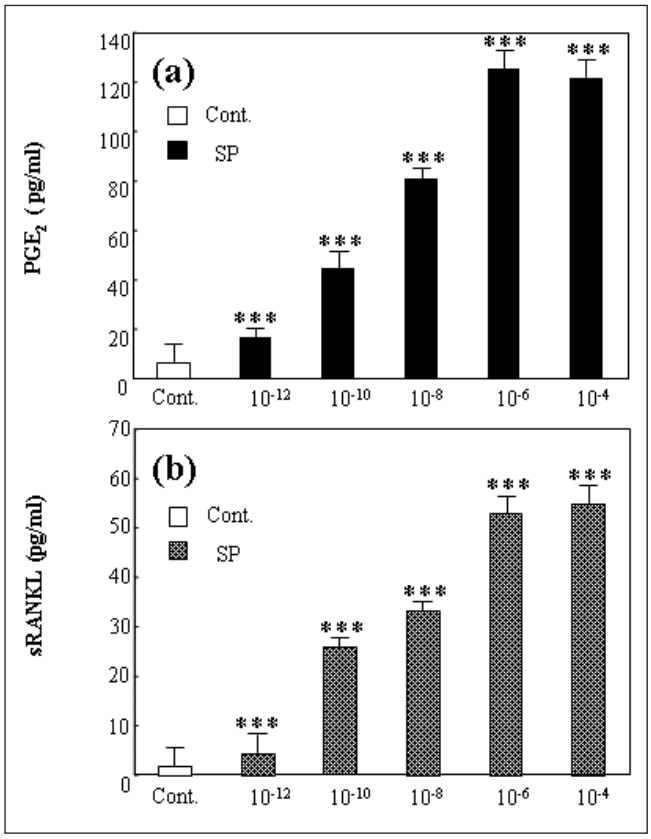

Fig. 2. Dose-dependent effects of SP on PGE_2 and sRANKL production by HDPF cells. SP and PGE_2 production were assayed as described in the Materials and Methods. Data are expressed as the mean ± S.D. of 6 cultures. PGE_2 and sRANKL production was increased following incubation with 10^{-4}–10^{-12}M SP for 24 h in a dose-dependent manner ($p < 0.001$, by one-way ANOVA). The values in the SP-treated groups were significantly increased, compared with the control value (Cont.): ***$p < 0.001$ vs. Control at the same time.

of PGE_2 and sRANKL production. PGE_2 and sRANKL production observed that significantly increased from 3 to 12 h following the addition of SP (Fig. 1 a, b).

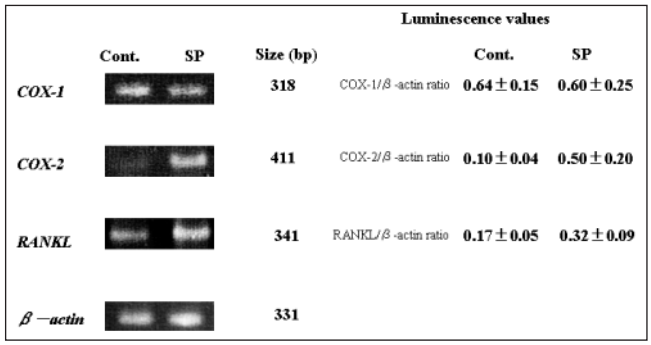

Fig. 3. The expression of cyclooxygenases (COX)-1, COX-2, and RANKL mRNA in HDPF cells subjected to 10^{-6} M SP for 12 h determined with reverse transcription-polymerase chain reaction (RT-PCR). Data are expressed as the mean ± S.D. of 5 cultures. The luminescence value (average gray scale value multiplied by band area) of the DNA band are measured by an image analyzer (Atto densitograph, Atto corp., Tokyo, Japan).

Since the production of PGE_2 and sRANKL plateaued after 12 h of incubation following the addition of SP, this incubation time was chosen for the following experiments. When HDPF cells were incubated in the presence of the indicated amounts of SP for 12 h, production of PGE_2 and sRANKL increased with increases in SP concentration up to 10^{-6} M (Fig. 2 a, b).

To elucidate the molecular mechanisms involved in the alteration of RANKL production via PGE_2, we examined the levels of COX-1, COX-2, and RANKL mRNA in SP-stimulated HDPF cells using an RT-PCR analysis. As shown in Figure 3, the levels of the PCR products corresponding to β-actin were the same in the two experimental groups, thus it was therefore considered acceptable to assume that the amount of PCR product as reflected the level of each mRNA. Bands for COX-2 and RANKL mRNA from the HDPF cells were visible after 30 cycles, and those of the SP-stimulated HDPF cells were more intense than those for the corresponding controls. In contrast, SP did not affect the expression of COX-1 mRNA (Fig. 3).

Table 1. Inhibitiory effect of NS-398 on production of PGE_2 and sRANKL.

	Control	SP	SP + NS398
PGE_2 (pg/ml)	46 ± 12	118 ± 26***	18 ± 3$^{\#\#}$
sRANKL (pg/ml)	16 ± 10	46 ± 106***	20 ± 11$^{\#\#}$

HDP cells were incubated with or without the indicated concentrations of SP (10^{-6} M) or NS398 (10 μM) for 12 hours. Data are expressed as the mean (pg/ml of productions) values ± S.D. of 6 cultures. PGE_2 production was suppressed completely, while that of sRANKL was inhibited by approximately 90% in all of the experiments. Statistically significant (*** $p < 0.001$, compared with control. $^{\#\#}$ $p < 0.001$, compared with SP by one-way ANOVA).

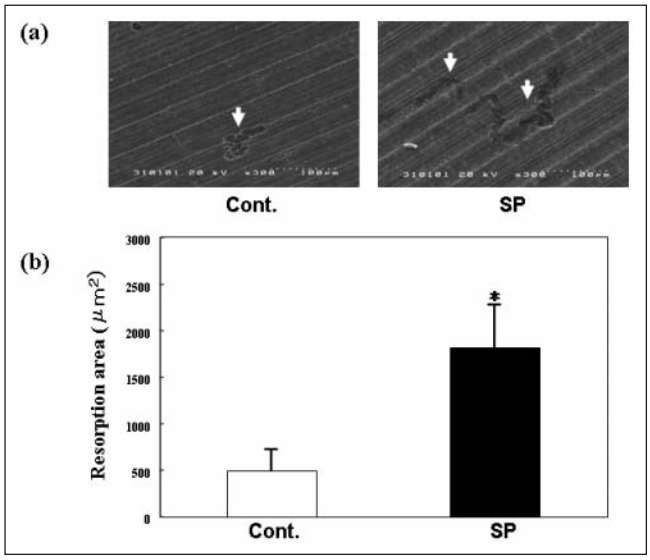

Fig. 4. Scanning electron micrograph images of resorption pits on the surface of dentin slices. (a) Osteoclast precursor cells were cultured on dentin slices for 8 days in basic culture medium. Then we added medium conditioned by HDPF cells cultured with or without 10^{-6} M SP for 12 h, and both the control and SP experimental groups were incubated for 48 h. Resorption pits were observed by scanning electron microscopy (open arrow). Cont, control (without SP): SP, stimulated with SP (x 300). (b) We measured resorption pits per an area of four dentin slices. Data are expressed as the mean ±S.D. of 4 cultures. Significantly different from control (*$p < 0.05$. by Student's *t*-test).

We also observed that the production of PGE_2 increased earlier than that of sRANKL in HDPF cells, showing high levels from 3 to 9 h (Fig. 1). Since PGE_2 has been implicated as a potential inducer of sRANKL, we examined whether the PGE_2 stimulated by SP might affect the production of sRANKL. To clarify this possibility, HDPF cells were incubated with or without NS-398, a selective COX-2 inhibitor, and sRANKL production was measured. As shown in Table 1, when HDPF cells were incubated with 10^{-6} M SP in the presence of 10 μM NS-398, PGE_2 production was suppressed nearly completely, and sRANKL production was inhibited by approximately 90%.

Resorption of mineralized tissue was investigated by measuring the capacity of *in vitro*-formed osteoclasts to resorb into dentin slices. After the osteoclasts matured, the cells were cultured for 48 h in medium obtained from HDPF cells conditioned with or without 10^{-6} M SP. Resorption pits were approximately 4-fold more abundant in the slices treated with SP than in the control group (without SP) (Fig. 4 a, b).

Discussion

Orthodontic forces are known to produce mechanical damage and inflammatory reactions in the periodontium [37], as well as cell damage, inflammatory changes, and circulatory disturbances in the dental pulp [38]. Recently, it was observed that the expression of calcitonin gene-related peptide (CGRP) and SP increase in dental pulp in response to buccally directed OTM in rat upper first molars [8]. Norevall et al. [9] also suggested that these neuropeptides might be involved in inflammation of the dental pulp during OTM. In addition, SP in the dental pulp of cats and rats has been implicated as a mediator of pulpal inflammation [38–40]. In an experimental study by Nicolay et al. [41], SP appeared to increase markedly following the application of orthodontic force in cats: the increase occurred rapidly (3 hours) in the dental pulp and later in the periodontal ligament (PDL) (24 h to 14 days). Therefore, SP may be involved in the initial pulpal inflammation that occurs following application of OTM. In the present study, we determined the levels of PGE_2 in HDPF cells stimulated by SP and found that the neuropeptide significantly increased the production of PGE_2 in both a time- and dose-dependent manner (Figs. 1 and 2). RT-PCR results also showed that the expression of COX-2 and RANKL mRNA was up-regulated, but the expression of COX-1 remained unchanged (Fig. 3). A recent study reported that the expression of SP may be related to PGE_2 in human gingival crevicular fluid during periodontal inflammation and the host response [42], and Gecse et al. [43] found that SP also significantly increases the activity of the arachidonate cascade. Those findings are supported by our present results. In contrast, some studies have indicated that PGE_2 induces SP release from cultured adult rat dorsal root ganglion cells [44] and renal sensory nerves [45]. Thus, additional experiments are necessary to confirm the relationship of SP with PGE_2.

With regard to the relationship between other prostanoids and bone resorption, Yamasaki et al. reported local injection of PGE_1 and PGE_2 induces an increased number of osteoclasts and rapid tooth movement *in vivo* [46]. Katz et al. [47] suggested $PGF_2\alpha$ also stimulates bone resorption *in vitro* and a recent study reported that PGI_2 affects bone resorption in rat experimental tooth movement *in vivo* [48]. However, PGE_1 and PGE_2 were related to acceleration of bone resorption *in vitro* in other studies, but it was suggested that $PGF_2\alpha$ isn't related [49]. There have also been many investigations of the relationship between PGE_2 and RANKL in various cells during bone resorption [50–52]. We therefore chose PGE_2 among the prostanoids for this study, and investigated the effect of SP stimulation on the relationship of PGE_2 and RANKL stimulated by SP in HDPFcells.

The production of PGE_2 occurred earlier than that of RANKL in HDPF cells (Fig. 1). PGE_2 is known to be a bone ab-

sorbing agent that acts on osteoblasts to facilitate osteo-clastogenesis by increasing the secretion of RANKL [53, 54]. Furthermore, PGE_2 expression has been shown to be induced by or increased in the presence of RANKL [55]. To clarify whether the PGE_2 stimulated by SP affects the production of sRANKL, HDPF cells were incubated with or without NS-398, and sRANKL production was measured. As shown in Table 1, the COX-2 inhibitor suppressed PGE_2 production nearly completely, and inhibited sRANKL production by approximately 90%. This is in agreement with the finding of Kanzaki et al. [35] who reported that RANKL up-regulation in mechanically stressed PDL cells was dependent on PGE_2. Okada et al. [54] reported that PGE_2 can stimulate the differentiation of both osteoblasts and osteoclasts *in vitro*. The present results clarify that PGE_2 is an intermediate in the increase of RANKL caused by SP stimulation in HDPF cells.

Conditioned medium obtained from HDPF cells treated with SP stimulated osteoclastic resorption in our dentin pit formation assay (Fig. 4). A recent study found that PGE_2 enhanced RANKL-stimulated formation of osteoclasts in spleen cell cultures; this study supports the notion of a direct effect by prostaglandins on the hematopoietic precursors of osteoclasts [56]. In addition, Takami et al. [57] suggested that RANKL may have a role in the activation of mature osteoclasts and Yasuda et al. [58] demonstrated that RANKL induced pit-forming activity by cultured osteoclasts. Thus, RANKL plays an important role in enhancing the bone resorption activity of osteoclasts. We concluded that resorbed pit enhancement was induced when PGE_2 and sRANKL were included in the conditioned medium obtained from HDPF cell stimulated by SP.

Many pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, activate bone resorption [59–62] and are able to induce osteoclastic activation via a mechanism independent of RANKL [63]. Our laboratory previously reported that SP and CGRP stimulated the production of IL-1β, IL-6, and TNF-α in HDP cells [36]. Therefore, SP-stimulated RANKL and pit formation may be increased by not only PGE_2, but also by those cytokines. Kwan et al. [64] also noted that IL-6, RANKL, and TNF-α/IL-1 also interact within the framework of bone resorption pathophysiology, such as tumor associated osteolysis.

Mori et al. [65] reported that SP stimulated bone remodeling by osteoclasts, and Berggreen indicated that SP-immunoreactive fibers are frequently observed in the resorptive lacunae in dental pulp from replanted teeth. Further, Haug et al. [66] detected SP in root resorptive areas of PDL and dental pulp samples following OTM. Therefore, SP in dental pulp may be involved with root resorption during OTM or following periodontal injury.

In conclusion, SP significantly stimulated the production of PGE_2 and RANKL by HDPF cells, and the increase of RANKL caused by SP stimulation in HDPF cells was partially mediated by PGE_2. In addition, conditioned medium obtained from HDPF cells treated with SP induced bone resorption. Therefore, SP may be involved in pulpal inflammation and root resorption during OTM.

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