Original

Conditioned medium from rat dental pulp reduces the number of osteoclasts via attenuation of adhesiveness in osteoclast precursors

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Abstract: Dental pulp is known to play crucial roles in homeostasis of teeth and periodontal tissue. Although resorption of bone around the roots of nonvital teeth is occasionally observed in clinical practice, little is known about the role of dental pulp in osteoclastogenesis. Here we evaluated the effects of conditioned medium (CM) from rat dental pulp on osteoclastogenesis. It was found that the CM reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts, but did not alter the mRNA levels of nuclear factor of activated T-cells, cytoplasmic 1 and TRAP. To further understand the mechanism behind these results, we evaluated the effects of CM on osteoclast precursors and found that the CM removed cell processes, resulting in a significant reduction in the number of

attached cells and an increase in the number of freely floating cells. Furthermore, the CM suppressed the mRNA levels of focal adhesion kinase and paxillin, which are involved in cell adhesiveness and spreading. Collectively, the present results show that CM from dental pulp serves as an inhibitor of osteoclastogenesis by reducing the number and adhesiveness of osteoclast precursors, suggesting novel therapeutic applicability for osteoporosis.

Keywords: rat dental pulp; osteoclast precursors; osteoclasts; adhesiveness.

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Introduction

Dental pulp is surrounded and sealed by dentine, enamel, and cementum, and this airtightness contributes to protecting dental pulp from bacteria in the oral environment (1,2). Once hard dental tissues are destroyed by caries, bacteria may invade into the dental pulp, resulting in its inflammation (3). If this inflammatory situation continues, the dental pulp may become nonvital, followed by bone resorption around its root (4).

In dental pulp, genes such as calcitonin gene-related peptide, transient receptor potential vanilloid-1 (TRPV1),

matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor receptors are known to be expressed (5-9). Several studies have shown that these genes are involved in development of periapical lesions (7-9). For instance, ablation of TRPV1-expressing neurons in dental pulp results in destruction of periapical bone (8). It is also reported that periapical lesions in MMP-9-knockout mice are larger than those in wild-type mice (7). Furthermore, a blocking antibody against VEGFR-2 in mice with periapical lesions induced by the pulp exposure promotes the extent of the lesions (9).

Dental pulp is composed of various cells, extracellular matrix, nerves, and capillaries. Most of the cells are fibroblasts (1), but immune cells such as macrophages, dendritic cells, and lymphocytes are also present. Macrophages can differentiate into multinucleated osteoclasts in the presence of receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). However, little is known about the role of the dental pulp in osteoclastogenesis.

In this study, we prepared conditioned medium (CM) from dental pulp and examined its effects on osteoclastogenesis. Osteoclast precursors were cultured with RANKL in the presence and absence of the CM, and tartrate-resistant acid phosphatase (TRAP) staining was conducted to identify multinucleated osteoclasts. To evaluate the effects of the CM on osteoclast precursors, the cells were cultured in the presence and absence of the CM without RANKL, and the numbers of attached and freely floating cells were determined. To assess the adhesiveness of osteoclast precursors in the CM, the mRNA levels of focal adhesion kinase (FAK) and paxillin were determined using quantitative real-time PCR.

Materials and Methods

Animals

For harvesting of bone marrow cells and isolation of dental pulp, Wistar rats were purchased from Chubu Kagaku Shizai (Nagoya, Japan). Thirty-one male rats were used in this study. The protocols for animal experiments were approved by the Aichi Gakuin University Animal Research Committee (approval number: AGUD320).

Cell culture

Male Wistar rat bone marrow cells, isolated from long bones (femur and tibia), were cultured in α MEM containing 10% fetal bovine serum and antibiotics (Wako, Osaka, Japan). Cells were maintained at 37°C with 5% CO₂ in a humidified incubator. They were then stained with trypan blue and the numbers of live and dead cells were counted using a hemacytometer.

Preparation of osteoclast precursors from bone marrow cells and osteoclastogenesis

Primary osteoclast precursors were prepared as described previously (10). In brief, bone marrow cells were cultured with 10 ng/ml M-CSF (PeproTech, Rocky Hills, NC, USA) for 3 days, and the surface-attached cells were used as osteoclast precursors. For osteoclastogenesis, these cells were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL (PeproTech).

Preparation of CM from rat dental pulp and tail tissue

The rats were killed by cervical dislocation, and their maxillary incisors were extracted for isolation of the dental pulp. The tails excluding the skin were cut at the same length as the dental pulps. These tissues were then cultured in α MEM containing 10% fetal bovine serum and antibiotics for 3 days. The culture medium was collected and centrifuged at 1,000 rpm for 5 min, and the supernatants were collected and passed through a 0.22- μ m pore filter (Millipore, Darmstadt, Germany). The filtrate was used as CM in the various experiments.

TRAP staining

After culture of osteoclast precursors from bone marrow cells for 72 h with RANKL in the presence and absence of CM, the cells were fixed in 10% formalin neutral buffer solution and incubated in sodium acetate buffer (0.1 M, pH 5) containing naphthol AS-MX phosphate, Fast Red Violet LB Salt, and MnCl₂ in the presence of sodium tartrate at 37°C for 60 min for TRAP staining. The number of TRAP-positive cells containing three or more nuclei was determined.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plus mini kit and an RNeasy Micro kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using TaKaRa Thermal Cycler Dice Real Time System III with THUNDERBIRD SYBR qPCR mix kits (TOYOBO, Osaka, Japan). The PCR cycling conditions were 95°C for 10 min for pre-denaturation, 40 cycles at 95°C/15 s for denaturation, and 60°C/1 min for extension. We evaluated the mRNA levels of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), TRAP, FAK, and paxillin using the PCR primers listed in Table 1. GAPDH was used as an internal control.

Table 1 Real-time PCR primers for mRNAs

Gene	Forward primer	Reverse primer
FAK	5'- CGTGAAGCCTTTTCAAGGAG -3'	5'- TCCATCCTCATCCGTTCTTC -3'
paxillin	5'- CCAGCACCAAAAATTCCAGT -3'	5'- GGTCCAGCTCAGAGAGGTTG -3'
NFATc1	5'- ACGATGTGGAGGTGGAAGAC -3'	5'- GGACGCCTCAGAGTTACAGC -3'
TRAP	5'- GAGAACGGTGTGGGCTATGT -3'	5'- GTGAAGCCACCCAGAGAGTC -3'
GAPDH	5'- ATGACTCTACCCACGGCAAG -3'	5'- TACTCAGCACCAGCATCACC -3'

Fig. 1 Inhibitory effects of dental pulp CM on osteoclastogenesis. We cultured osteoclast precursors with 50 ng/mL RANKL and dental pulp CM for 72 h. (A) Suppression of differentiation of TRAP-positive multinucleated osteoclasts from osteoclast precursors by dental pulp CM ($n = 6$). Note that the RANKL administration group was also treated with 10% FBS, and that the RANKL plus CM group was also treated with 2% FBS and the remaining FBS in the CM. (B) Suppression of differentiation of TRAP-positive multinucleated osteoclasts from osteoclast precursors by dental pulp CM ($n = 6$). Note that the RANKL administration group was also treated with 10% FBS, and that the RANKL plus CM group was also treated with 10% FBS and the remaining FBS in the CM. Data are expressed as mean $\pm$ S.D. The double asterisks indicate $P < 0.01$.

Statistical analysis

Three to six independent experiments were conducted, and data were expressed as mean $\pm$ S.D. Statistical significance was evaluated using Student's t -test at $P < 0.05$. The single and double asterisks indicate $P < 0.05$ and $P < 0.01$, respectively.

Results

Inhibitory effects of dental pulp CM on osteoclastogenesis

The primary aim of this study was to evaluate the effects of dental pulp CM on osteoclastogenesis. We employed TRAP staining for the RANKL group (10% fetal bovine serum - FBS) and the RANKL & dental pulp CM group

Fig. 2 Lack of apparent effects of CM derived from tail tissue on osteoclastogenesis. We cultured osteoclast precursors with 50 ng/ml RANKL and dental pulp or tail CM for 72 h. The CM from the tail did not reduce the number of RANKL-induced TRAP-positive multinucleated osteoclasts ($n = 5$). Data are expressed as mean $\pm$ S.D. The double asterisks indicate $P < 0.01$. NS indicates no significance.

Fig. 3 Dose-dependent reduction in the number of TRAP-positive multinucleated osteoclasts by culture with dental pulp CM for 72 h (concentration of CM: 1-fold to 1/32-fold) ($n = 3$). Data are expressed as mean $\pm$ S.D. The double asterisks indicate $P < 0.01$.

(2% or 10% FBS plus remaining FBS in the CM). The results showed that the CM reduced the number of TRAP-positive multinucleated osteoclasts (Fig. 1). In subsequent experiments, we used the RANKL group (10% FBS) and the RANKL & CM group (10% FBS and remaining FBS in the CM). To evaluate whether the observed inhibitory effect of the dental pulp CM is

specific, we also employed CM derived from the tail tissue. The number of TRAP-positive multinucleated osteoclasts was significantly reduced by the dental pulp CM, but not by the tail CM (Fig. 2). The number of multinucleated osteoclasts was significantly reduced by the dental pulp CM at various doses (1-fold to 1/8-fold) in a dose-dependent manner (Fig. 3).

Fig. 4 Lack of apparent effects of dental pulp CM on the mRNA levels of NFATc1 and TRAP. (A) mRNA levels of NFATc1 and TRAP at 48 h after RANKL administration ($n = 4$). (B) mRNA levels of NFATc1 and TRAP at 72 h after RANKL administration ($n = 4$). Data are expressed as mean $\pm$ S.D.

Lack of detectable effects of dental pulp CM on NFATc1 and TRAP

Although the dental pulp CM reduced the number of TRAP-positive multinucleated osteoclasts, it did not alter the levels of mRNA for NFATc1 and TRAP induced by RANKL (Fig. 4).

Reduction in the number of osteoclast precursors and attenuation of their adhesiveness by dental pulp CM

To clarify the mechanism responsible for the reduction in the number of TRAP-positive multinucleated osteoclasts without any alteration in the expression of mRNAs for NFATc1 and TRAP, we evaluated the effects of dental pulp CM in the absence of RANKL. Dental pulp CM without RANKL significantly reduced the number of cells with processes (Fig. 5A, B). It also increased the number of freely floating cells and decreased the number of cells attached to the surface (Fig. 5C). However, there was no significant difference in the number of dead cells (Fig. 5D). Furthermore, the CM attenuated the levels of mRNA for FAK and paxillin, which are known to regulate cell adhesion and spreading (Fig. 5E).

Discussion

We demonstrated in this study that CM derived from dental pulp reduced the number of TRAP-positive multinucleated osteoclasts. In spite of the reduction in TRAP-positive multinucleated osteoclasts by dental pulp

CM, the CM did not alter the mRNA levels of NFATc1 and TRAP. These results suggest that the CM did not affect osteoclast differentiation directly, but reduced the number of osteoclast precursors and attenuated their adhesiveness, resulting in the reduced number of multinucleated cells. Our results strongly indicate that the observed reduction in the number of multinucleated cells in response to dental pulp CM is not attributable to factors such as the serum concentration in the medium, which are not directly linked to dental pulp. To eliminate any possibility of serum-driven effects on osteoclastogenesis, we prepared two CM groups: 2% FBS plus remaining FBS in the CM, and 10% FBS plus remaining FBS in the CM (Fig. 1A, B, respectively). In both groups, the CM-driven reduction in the number of TRAP-positive multinucleated osteoclasts was observed regardless of the FBS concentration.

The CM derived from dental pulp reduced the expression of mRNAs for FAK and paxillin in osteoclast precursors. FAK is known to be involved in cell adhesion, proliferation and cytoskeletal rearrangement in various cells (11-15). For instance, it is reported that knockdown of FAK in osteoblastic MC3T3-E1 cells caused them to adopt a round morphology and attenuated their proliferation, adhesion, migration, and differentiation (15). Myeloid-specific FAK-knockout mice revealed loss of FAK, resulting in reduction of bone resorption by osteoclasts (14). Furthermore, Owen et al. reported that

Fig. 5 Reduction in the number of osteoclast precursors and attenuation of their adhesiveness by dental pulp CM. We cultured osteoclast precursors with 10 ng/mL M-CSF and dental pulp CM (1/2-fold) for 24–72 h. (A) Disappearance of cell processes in osteoclast precursors upon culture with dental pulp CM. (B) Ratio of the number of the cells with processes after culture with dental pulp CM for 72 h ($n = 3$). (C) Numbers of viable cells, both floating freely and attached, after culture with dental pulp CM for 72 h ($n = 3$). (D) Numbers of dead cells, both floating freely and attached, after culture with dental pulp CM for 72 h ($n = 3$). (E) Effects of culture with dental pulp CM for 24 and 48 h on expression of FAK and paxillin mRNAs ($n = 4$). Data are expressed as mean $\pm$ S.D. The single and double asterisks indicate $P < 0.05$ and $P < 0.01$, respectively.

FAK regulates lamellipodium formation, adhesion, and motility in macrophages (12). Paxillin is also known to regulate cell migration, adhesion, and spreading (16–20). It is reported that paxillin regulates actin reorganization (16), and enhances cell spreading and stretching in macrophages (20). It is also reported that paxillin is required for organization of podosome rings in osteoclasts (18). Collectively, these studies suggest that dental pulp CM may reduce cell adhesion via downregulation of FAK and paxillin in osteoclast precursors.

Several studies have reported interactions of macrophages with dental pulp cells and stem cells (DPSCs) (21–24). However, it is still unclear whether co-cultured macrophages and dental pulp cells/DPSCs can produce an inflammatory response. Yonehiro et al. reported that co-culture of immortalized dental pulp cells and

macrophages upregulated the production of inflammatory cytokines such as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in response to lipopolysaccharide (LPS) stimulation (22). In contrast, Lee et al. reported that co-culture of DPSCs and macrophages markedly attenuated the secretion of tumor necrosis factor α (TNF- α) in response to LPS (23). It has also been reported that CM from DPSCs increased the M2 polarization of M1 macrophages that had been activated by LPS, resulting in an increase of anti-inflammatory cytokines (24). These reports suggest that the immune responses of macrophages in response to LPS may depend on the presence of other cells. Furthermore, it has been reported that RANKL is expressed in dental pulp cells, and that co-culture of CD14 $^{+}$ monocytes derived from human peripheral blood and dental pulp cells promotes

the number of osteoclasts via RANKL-RANK signaling (21). The present results suggest that the direct and indirect interactions of dental pulp cells with macrophages have differential effects on osteoclastogenesis.

Many studies have reported that the CM from DPSCs and human exfoliated deciduous teeth provides a powerful tool for investigating bone and dental pulp tissue regeneration, focal cerebral ischemia, and diabetes mellitus (25-29). Moreover, the factors secreted from DPSCs have been analyzed (26,29,30). TGF- β 1 and FGF-2 in the CM from DPSCs have been reported to counteract cytotoxicity induced by the base resin TEGDMA (26). Bronckaers et al. have reported that the CM from DPSCs contains various angiogenesis-related molecules such as vascular endothelial growth factor (VEGF), MCP-1, plasminogen activator inhibitor-1 (PAI-1), and endostatin (30). Piva et al. have also reported that the CM from DPSCs contains the angiogenic proteins endothelin, insulin-like growth factor binding protein-3 (IGFBP-3), pentraxin3, serpin E1, serpin F1, thrombospondin 1, tissue inhibitor of metalloproteinase-1, and VEGF (29). IGFBP-3 is reported to inhibit cell adhesion through downregulation of FAK in human umbilical vein endothelial cells (31). Although the present study revealed that dental pulp CM suppressed cell adhesion and reduced the mRNA levels of FAK and paxillin, further analysis will be necessary to identify the specific molecules in CM that are responsible for the down-regulation of FAK and paxillin, and suppression of cellular adhesion.

We also found that dental pulp CM reduced the number of TRAP-positive multinucleated osteoclasts due to attenuation of osteoclast precursor adhesiveness. Therefore, CM from the dental pulp of extracted teeth might be a potentially useful therapeutic option for osteoporosis if administered systemically.

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Conflict of interest

The authors have no conflict of interest to declare.

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