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Dental follicle cell-conditioned medium enhances the formation of osteoclast-like multinucleated cells

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SUMMARY An influx of mononuclear cells and the subsequent increase of osteoclasts around tooth germs suggests that the dental follicle (DF) regulates or influences bone resorption required for tooth eruption. In order to study the effects of DF cell products on osteoclast formation during tooth eruption, a conditioned medium (CM) was created in which DF cells were added to mouse bone marrow cultures. Tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated cells were formed in the presence of 10 nM 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. The CM, dose-dependently, stimulated the formation of TRAP-positive cells in the presence of 1,25$(OH)_2D_3$ for 14 days culture. The number of these cells decreased due to degradation in the control culture. A semi-solid methylcellulose assay in the presence of CM showed little expression of colony-stimulating activity. These results suggest that the DF cells of a developing tooth produce factor(s) that enhance osteoclast formation and bone resorption necessary for tooth eruption.

Introduction

For a tooth to erupt there must be resorption of bone and primary tooth roots overlying the crown of the erupting tooth. The overlying bone and primary teeth resorb and the tooth moves into the space created by the resorption. In an osteopetrotic toothless rat, the impairment of bone resorption causes delayed or absent tooth eruption (Seifert *et al*., 1988). Such observations suggest the possible existence of a signal for bone resorption (Proffit, 1993). A previous report has asserted that a dental follicle (DF) is required for bone resorption in the enlargement of a bony passageway for pre-erupting tooth movement (Cahill *et al*., 1988). The DF is a loose connective tissue that encapsulates the enamel organ and differentiates to the supporting tissue of the tooth (Ten Cate, 1980). The number of osteoclasts and tartrate resistant acid phosphatase (TRAP)-positive mononuclear cells is known to increase in the bony crypt adjacent to the DF before the onset of tooth eruption (Wise and Fan, 1989). Such an influx of mononuclear cells and the subsequent increase of osteoclasts in the DF and bony crypts suggest that the follicle regulates or influences bone resorption required for tooth eruption.

The DF cells *in vitro* appear to be primary fibroblasts, although some may be derived from undifferentiated mesenchymal cells (Wise *et al*., 1992). Fibroblasts, such as L929 cells, have been well known as a strong source of CSF-1 (M-CSF), which can stimulate the proliferation or differentiation of osteoclast-like cells (Scheven *et al*., 1997). It has been shown that both the CSF-1 protein and its mRNA are present in the DF *in vitro* and *in vivo* (Wise *et al*., 1995). A recent report demonstrates that CSF-1 is secreted by the DF cells (Grier *et al*., 1998). These findings indicate that DF cells may have the potential to release an osteoclast-stimulating factor such as

CSF-1 and directly contribute to the osteoclastogenesis required for tooth eruption. However, the effect of CSF-1 on osteoclast formation is controversial. *In vitro* assays using bone marrow precursor cells showed that CSF-1 is essential for formation of osteoclasts (MacDonald *et al*., 1986; Tanaka *et al*., 1993). In contrast, some studies in murine bone marrow cultures have noted that CSF-1 could inhibit osteoclast-like cell induction by $1,25(OH)_2D_3$ (Takahashi *et al*., 1991; Perkins and Kling, 1995). Thus, there is a possibility that the DF cells perform another role for osteoclast formation required for tooth eruption even if they have a potential to produce CSF-1. In the present study, the role of the DF cell and, in particular, the direct effects on the formation of TRAP-positive osteoclast-like cells during tooth eruption were investigated.

Materials and methods

Dental follicle cell conditioned media

Harlan Sprague–Dawley rats were obtained from Nihon Crea Inc., Osaka, Japan. The first mandibular molars were surgically isolated from 6–7-day-old postnatal rats under a microscope (Stemi 2000, Zeiss, Germany). A recent report has shown that CSF-1 is secreted by dental follilcle cells harvested from this dental developmental stage (Grier *et al*., 1998). The DF cells were separated from the first molars with 1 per cent trypsin/1 mM EDTA and 0.25 per cent trypsin/1 mM EDTA, and cultured in six-well plastic plates (Corning, Corning, NY) as previously described (Wise *et al*., 1992). The DF cells were grown to confluence with Eagle's modified minimum essential medium (MEM; Nissui, Tokyo, Japan) containing 15 per cent foetal calf serum (FCS; GIBCO BRL, ML). After one or two passages of the culture, the cells were plated in 35-mm dishes (Corning; 2.0 ml/dish). The isolated cells from the DF grew to confluence in almost 2 weeks and remained fibroblastic in shape: elongation with processes at each pole through two passages. No contamination of epidermal cells was observed after two passages. Three days after the last medium change, the media were centrifuged at 3000 rpm for 10 minutes and the supernatants were stored at –30°C (conditioned medium, CM).

Bone marrow culture

Bone marrow cells were aseptically isolated and enriched, as previously described (Takahashi *et al.*, 1988), from the long bones of 6–8-week-old male ICR mice (Nihon Crea Inc.). Briefly, these cells were flushed from the bone marrow cavity of femurs and tibiae with cold α-MEM (Sigma, St Louis, MO) supplemented with 10 per cent FCS. The cells were washed twice and seeded in 24-well plates (Corning) at a density of 1.5×10^6 cells/ml. The CM were added and cultured with α-MEM containing 5 per cent FCS in the presence or absence of 10 nM $1,25(OH)_2D_3$ in a total volume of 0.5 ml. The CM was replaced every 3 days with fresh medium and the hormone. The cells were incubated at 37°C in a 5 per cent CO_2 humidified atmosphere. After the 7th and 14th days, the cells were fixed with 10 per cent neutral formalin and ethanol/acetone, and stained for TRAP, which was used as a marker enzyme for osteoclasts. Staining for TRAP was performed according to the modified method of Wijngaert and Burger (1986) by incubating the fixed cells for 20 minutes at room temperature in an acetate buffer containing naphthol AS-BI phosphate (Sigma) as a substrate and hexazonium pararosaniline solution as coupler in the presence of 20 mM tartaric acid. TRAP positive multinucleated giant cells were manually scored using a light microscope.

Colony-forming assay

The mouse bone marrow cells were resuspended (1.5×10^6 cells/ml) in α-MEM containing 20 per cent FCS and 1.5 per cent methycellulose (Wako, Osaka, Japan). Cell suspension (0.1 ml) and 0.3-ml test sample of the CM or 100 units/ml CSF-1 (Genzyme, Cambrige, MA) were cultured in triplicate in six-well plates (1 ml/well). Cultures were incubated at 37°C in a 5 per cent CO_2 humidified atmosphere and scored on day 7 for colony formation. Aggregates of over 50 cells were counted as a colony under microscopic observation.

Statistical analysis

All data were analysed for significance by means of ANOVA. Statistical significance required a P value of <0.05.

Results

Multinucleated TRAP-positive cells were formed from the bone marrow in the culture with α-MEM containing 10 nM $1,25(OH)_2D_3$ within 7 days. No TRAP-positive multinucleated cells were observed in the culture without $1,25(OH)_2D_3$ throughout the experiment. When 50 per cent CM (v/v) harvested from the culture of DF cells was added to the basal medium (α-MEM) containing 5 per cent FCS, there was no significant difference in the number of TRAP-positive multinucleated cells formed in 7 days (Figure 1). CM alone could not cause formation of TRAP-positive multinucleated cells from bone marrow without $1,25(OH)_2D_3$.

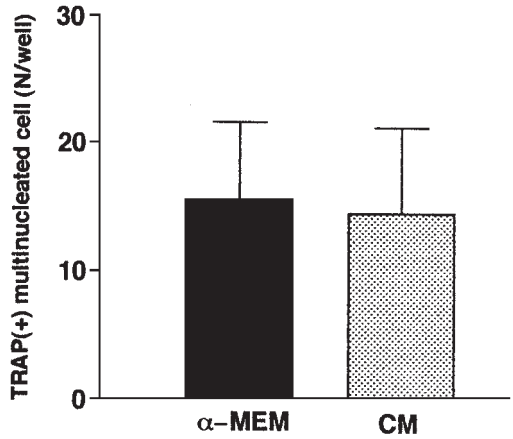

Figure 1 Effect of the conditioned medium on the formation of TRAP-positive multinucleated cells in 7 days. Mouse marrow cells were cultured with or without the 50 per cent (v/v) CM in the presence of $1,25(OH)_2D_3$. After culturing, TRAP-positive multinucleated cells were counted. Data are expressed as the mean ± SEM of three cultures. Statistical significance was determined using ANOVA ($P < 0.05$).

It has been reported that the number of TRAP-positive multinucleated cells decrease after 8–10 days and return to the control level on day 14 in the presence of 10 nM $1,25(OH)_2D_3$ Takahashi *et al*., 1988). As seen in Figure 2, the disappearance of TRAP-positive cells in the control after 14 days seemed to be due to the degradation or death of these cells. In the presence of the CM, on other hand, the formation of TRAP-positive multinucleated cells in bone marrow culture was significantly enhanced (Figure 3). The CM had a strong effect on the formation of TRAP-positive cells at 14 days,

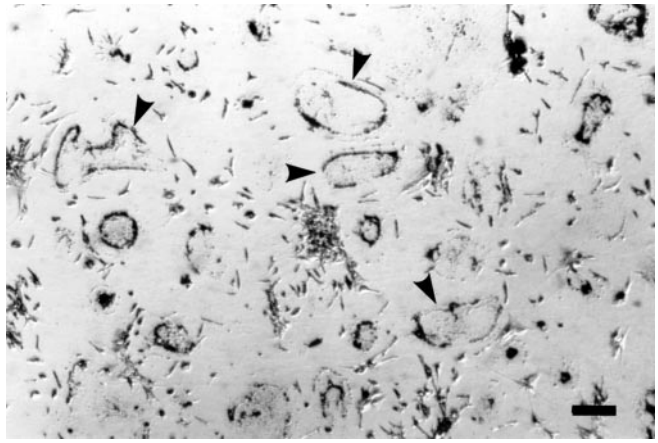

Figure 2 At 14 days, the TRAP-positive multinucleated cells that were induced by $1,25(OH)_2D_3$ had disappeared from the culture without the conditioned medium. The disappearance of the TRAP-positive cells (arrowheads) seemed to be due to the degradation of those cells. Cells were then fixed and stained for TRAP. Bar = 200 μm.

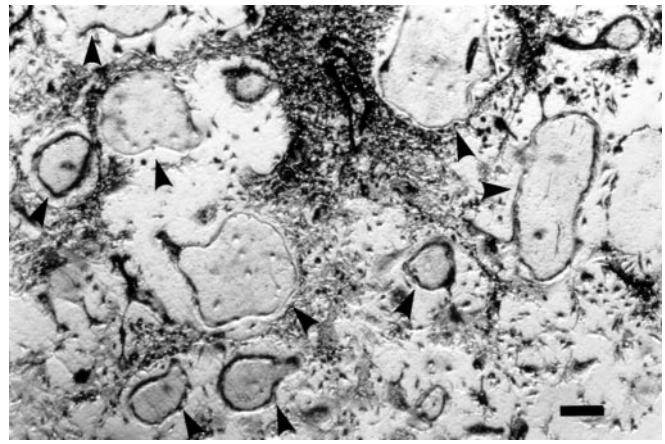

Figure 3 TRAP-positive multinucleated cells (arrowheads) formed in mouse bone marrow cultures. Mouse marrow cells were cultured with 50 per cent CM derived from the DF cell culture in the presence of $1,25(OH)_2D_3$ for 14 days. Bar = 200μm.

showing a typical dose-dependent histogram for CM-stimulated formation of TRAP-positive multinucleated cells (Figure 4). The maximal number in 12 independent cultures treated with 50 per cent CM in the presence of $1,25(OH)_2D_3$ was 83.6 ± 11.6.

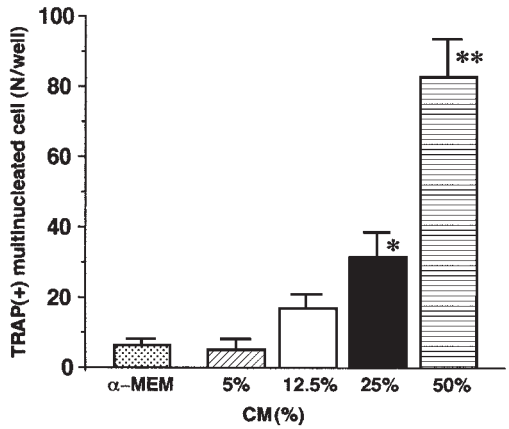

Figure 4 Effect of an increasing concentration of CM on the formation of TRAP-positive multinucleated cells induced by $1,25(OH)_2D_3$. Mouse marrow cells were cultured with increasing concentrations of the conditioned medium. After culture for 14 days, TRAP-positive multinucleated cells were counted. Data are expressed as the mean ± SEM of three cultures. Significantly different from the culture in α-MEM without CM (*$P < 0.05$) and between the culture with 25 and 50 per cent CM (**$P < 0.01$).

The CM was harvested from the confluent culture of DF cells in the presence of 15 per cent FCS for 3 days, because quiescent or growth-arrested fibroblasts in media with low concentrations of FCS has been reported to show little production of CSF-1 (Falkenburg *et al.*, 1990). The serum level (15 per cent) of the CM raises the possibility that high concentration of FCS itself may stimulate TRAP-positive multinucleated cell formation in the bone marrow culture even if no CM is added. To determine whether the induced increased TRAP-positive multinucleated cell formation was due to contained serum factor, the bone marrow cells were cultured for 14 days with basal medium (α-MEM) containing 12.5 per cent FCS (thus containing concentrations equivalent to 50 per cent CM with 5 per cent FCS) and 5 per cent FCS. No significant increase in the number of TRAP-positive cells occurred between the cultures with α-MEM containing 12.5 per cent FCS (6.4 ± 1.7) and 5 per cent FCS (1.2 ± 0.4). In addition, the number of TRAP-positive cells induced by $1,25(OH)_2D_3$ in the basal medium containing 10 per cent FCS (3.0 ± 1.9), as well as 5 or 12.5 per cent at 14 days was less than at 7 days. These results can exclude the effects of serum concentration in the culture with the CM.

Table 1 Effect of CM on colony-stimulating activity by mouse bone marrow cells in semi-solid methylcellulose medium. Bone marrow cells (1.5×10^6 cell/ml) were cultured in the presence of CM [30 per cent (v/v)] or CSF-1 (100U/ml). After 7 days culture, the number of colonies that consisted of 50 or more cells was counted under inverted phase microscopy. Data are expressed as mean ± SEM of six samples and are representative of three performed assays.

Treatment	No. of colonies
Vehicle	3.8 ± 0.9
Conditioned medium	7.5 ± 1.3
CSF-1 (100 U/ml)	87.7 ± 7.9*

*$P < 0.05$ compared with the vehicle.

To test whether DF cells secrete factors that include CSFs, growth factors, or cytokines, the colony-forming activity of the CM using mouse bone marrow cultures on semi-solid methylcellulose medium was examined. The colony formation was not stimulated by 30 per cent CM (Table 1). This result suggests the stimulating activity of TRAP-positive multinucleated cell formation in the CM may not be due to CSFs.

Discussion

The addition of the CM derived from DF cells into the bone marrow culture for 14 days enhanced TRAP-positive multinucleated cell formation by $1,25(OH)_2D_3$. The multinucleated cells exhibited typical features of osteoclasts (Figure 3). In previous studies, these multinucleated cells have been reported to have functional attributes of osteoclasts. TRAP-positive cells respond to osteotropic hormones such as $1,25(OH)_2D_3$ and cause pit formation on dentine slices (Takahashi *et al*., 1988). Osteoclasts are haematopoietic in origin and numerous factors, including marrow environment, regulate osteoclast formation, and bone resorption.

During the osteoclast differentiation process, factors can act at fusion and/or proliferation stages of osteoclast-genesis. The most active hormone, 1,25-dihydroxyvitamin D_3, acts as a potent stimulant of osteoclast formation. In the absence of $1,25(OH)_2D_3$, no TRAP-positive multinucleated cell formation occurred in the culture with or without the CM. Since $1,25(OH)_2D_3$ is one of several systemic hormones, it would be reasonable to assume that another factor influences osteoclast formation around the DF during tooth eruption. CSFs are haematopoietic growth factors that may also act as stimuli for osteoclast formation. CSF-1 appears to stimulate both the proliferation and differentiation of osteoclast precursors in co-culture with bone marrow cells (Tanaka *et al*., 1993). Wise *et al*. (1995) demonstrated the expression of CSF-1 mRNA and CSF-1 protein in the DF. A recent report shows that DF cells secrete CSF-1 protein into culture media (Grier *et al.*, 1998). Although CSF-1 is an essential factor for osteoclast-genesis, the role of CSF-1 in osteoclast development is controversial. TRAP-positive multinucleated cell formation was found to be inhibited in a dose-dependent fashion by CSF-1 when the marrow cells were treated with CSF in the presence of $1,25(OH)_2D_3$ for 8 days (Takahashi *et al*., 1991). The addition of exogenous CSF-1 into stromal cell/murine bone marrow co-culture also causes a dose-dependent decrease in TRAP-positive multinucleated cells (Perkins and Kling, 1995). In the present experiment using the mouse bone marrow, the number of TRAP-positive multinucleated cells at day 7 showed no significant difference between the CM and in α-MEM containing 10 per cent FCS (Figure 1). Semi-solid methylcellulose cultures showed little colony formation induced by any concentration up to 30 per cent (v/v) of CM (Table 1). These results suggest that the CM does not contain a sufficient concentration of CSF-1 to cause colony formation and/or inhibition of TRAP-positive multinucleated cell formation during 7 days.

In mouse marrow cultures, osteoclast-like multinucleated cells first appear on day 5 in the presence of $1,25(OH)_2D_3$, and the number of the cells increase to a peak on days 6–8 and decreases thereafter (Takahashi *et al*., 1988). In the present investigation, TRAP-positive multinucleated cells induced by $1,25(OH)_2D_3$, with the culture as a control, also decreased at day 14 when those cells seemed to be in degradation

(Figure 2). When the CM was added to the culture, the number of TRAP-positive multinucleated cells increased 2.5-fold at day-14 over that of day 7 (Figure 4). It has been shown that long-term exposure of CSF-1 on osteogenetic cells does not cause inhibition on osteoclast formation. Perkins and Kling (1995) reported that addition of exogenous CSF-1 beyond 6 days in co-culture of ST-1 stromal cells and murine bone marrow cells had a decreasing ability to inhibit osteoclast formation. MacDonald *et al.* (1986) demonstrated that the subsequent addition of $1,25(OH)_2D_3$ to bone marrow cultures initially treated with CSF-1, significantly enhanced osteoclast-like cell formation. These reports suggest that CSF-1 is necessary for the early proliferative stage of the osteoclast in long-term bone marrow cultures, but is no longer required for the cellular fusion phase of osteoclast formation (Biskobing *et al.*, 1995). The addition of CSF-1 to bone marrow derived macrophages or osteoclasts causes a down-regulation of the CSF-1 receptor (Guilbert and Stanley, 1986; Amano *et al.*, 1995). CSF-1 existing in the CM may no longer be effective for inhibiting osteoclast formation at day 14 in this culture system due to the down-regulation of the CSF binding site on the osteoclast precursors. The number of TRAP-positive multinucleated cells was not increased in the presence of the CM and $1,25(OH)_2D_3$ at day 7, but was at day 14. It is suggested that the stimulatory effect of TRAP-positive multinucleated cell formation in the CM is not related to CSF-1.

Further osteogenetic factors may stimulate the formation of TRAP-positive multinucleated cells. Parathyroid hormone and interleukin-1 (IL-1) stimulate osteoclast-like cell formation in human or mouse bone marrow culture systems (Takahashi *et al.*, 1988; Thomson *et al.*, 1986). Fibroblasts derived from human periodontal ligament (PDL) have been shown to release PGE_2 in the culture fluid (Saito *et al.*, 1994). These factors themselves have the ability to induce osteoclast-like cells in the absence of $1,25(OH)_2D_3$ (Roodman, 1996). Symons *et al.* (1995) demonstrated that cultured molars of both normal and microphthalmic mice secreted TGF-β, as well as IL-1α. Moreover, TGF-β, a known IL-1 inhibitor, modulates both osteoclastic bone resorption and migration and osteoclast differentiation (Mundy, 1991). Epidermal growth factor (EGF), which is known to cause the premature eruption of rat incisors, binds to the DF in early development of the tooth (Partanen and Thesleff, 1987). EGF has been shown to stimulate cell proliferation in odontogenic cells (Steidler and Reade, 1981). The addition of murine EGF into marrow culture followed by $1,25(OH)_2D_3$ significantly stimulates multinucleated cell formation (Takahashi *et al.*, 1986). Since these factors have a role in modulating cell proliferation and differentiation, there is a potentially important interaction between a locally produced factor and a systemic osteogenetic hormone [$1,25(OH)_2D_3$] in the formation of TRAP-positive multinucleated cells.

The DF cells have a potential to differentiate to PDL cells. Once the follicle cells have differentiated into paradental tissue, their behaviour is irreversibly altered. The results of Saito *et al.* (1994) indicate that the conditioned media derived from human PDL cells does not contain IL-1α. The influx of osteoclasts in the early postnatal stage is diminished, followed by tooth growth and drift (Wise and Fan, 1989). The stimulating activity of TRAP-positive multinucleated cell formation may be one of the characteristics of DF cells in the early stage of tooth eruption.

Conclusions

The present data show that CM derived from the culture of DF cells enhances the formation of osteoclasts from the bone marrow cells in the presence of $1,25(OH)_2D_3$. Therefore, the DF cells of a developing tooth produce factors that can enhance and maintain osteoclast formation. During the period when the crown of a tooth is being formed and positional adjustment (pre-eruptive drift) occurs in the alveolar bone, there is a possibility that a tooth germ, including the DF, secretes a bone resorbing factor which is needed for a tooth eruption pathway in the alveolar bone. The existence of the DF interposed between the enamel organ and the adjacent bony crypt, releases the factor around a tooth germ,

which contributes to the influx of osteoclasts in this specific location. The results of the present study suggest that there are chemical messengers from DF cells, which play a role as a signal for inducing resorption of the alveolar bone adjacent to the tooth during this phase of eruption.

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