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ORIGINAL ARTICLE

Enamel matrix derivative does not affect osteoclast formation or bone resorption in cultures of mouse bone marrow macrophages or human monocytes

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ABSTRACT

Objective: Enamel matrix derivative (EMD) is widely used under the brand name Emdogain® to promote periodontal regeneration in surgical treatment of periodontitis and peri-implantitis. The molecular mechanisms are unclear, but it has been proposed that EMD has stimulatory effects on the root cementum and periodontal ligament cells. Since dental implants lack these structures, we hypothesized that EMD-induced bone gain involve interactions with osteoclast precursor cells, with consequent inhibitory effect on osteoclast formation and/or activity. The aim was to evaluate this hypothesis.

Material and methods: Primary mouse bone marrow macrophages (BMMs) and human peripheral blood monocytes were cultured in the presence of receptor activator nuclear factor-κB ligand (RANKL) to stimulate osteoclast formation. A purified Emdogain® fraction was added to the cell cultures and the effect on number and size of newly formed osteoclasts were evaluated. In cultures on natural bone slices, bioanalytical methods were used to assay osteoclast number and bone resorption.

Results: EMD had a negative effect on osteoclastogenesis in mouse cultures on plastic surface, whereas addition of EMD to osteoclast precursor cells on bone substrate did not affect osteoclast formation or bone resorption.

Conclusions: The results on natural bone matrix contradict a direct effect of EMD on osteoclast precursor cells.

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KEYWORDS
Enamel matrix derivative; Emdogain; osteoclast formation; bone resorption

Introduction

Periodontitis and peri-implantitis are two challenging inflammatory conditions involving disruption of the normally well-balanced bone remodelling process performed by bone-resorbing osteoclasts and bone-forming osteoblasts. When osteoclast activity exceeds osteoblast activity, the net result is the loss of jawbone that supports a tooth or implant. Successful treatment requires resolution of inflammation and, ideally, regeneration of the jawbone.

Enamel matrix derivative (EMD) is a protein extract of porcine foetal tooth buds, which is widely used in periodontal regenerative surgery as adjunctive therapy to promote wound healing and accelerate new periodontal tissue formation [1,2]. The rationale for EMD uses in periodontal therapy is that it will help to create a regenerative environment mimicking the tooth developmental environment [3]. Numerous clinical studies verify that EMD applied to the root surfaces induces tooth cementum and ligament formation, increases alveolar bone, enhances the probing attachment level and improves pocket depth reduction [2,4]. The osteopromotive potential of EMD has also been demonstrated in various animal models [5–9].

Despite much effort to elucidate the mechanism, no consensus has been reached regarding how EMD affects periodontal tissue regeneration. However, *in vitro* studies demonstrate that EMD improves the behaviours of osteoblasts, endothelial cells, and periodontal ligament cells by affecting the cells' attachment, spreading, proliferation, survival and expression of various transcription and growth factors [2,10,11]. Moreover, EMD upregulates the expression of osteoprotegerin (OPG) and downregulates receptor activator of nuclear factor-κβ ligand (RANKL) [11–13], which are key molecules in regulating the balance between bone resorption and bone formation under normal physiological conditions. RANKL is expressed by osteoblast lineage cells, and binds to its receptor (RANK) on osteoclast precursor cells of the monocyte/macrophage linage, thereby stimulating their differentiation into mature osteoclasts. OPG is produced by many cell types, including osteoblasts, and acts as a soluble decoy receptor for RANKL, preventing RANKL from binding

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to RANK and thereby inhibiting osteoclast formation and activity [11–13]. Overall, these data suggest that EMD may modulate the RANK-RANKL-OPG system towards bone formation.

We hypothesized that EMD contributes to net bone gain in periodontal healing by directly targeting osteoclast precursor cells, thereby inhibiting osteoclast formation and/or activity. This possibility is not supported by two previous studies demonstrating that EMD can enhance osteoclast formation in crude mouse bone marrow cell (BMC) cultures [14,15]. However, BMC cultures contain multiple different cell types, including bone marrow stromal cells, which theoretically could contribute to the increased osteoclast formation observed in response to EMD.

Although RANKL-stimulated mouse BMC and bone marrow macrophage (BMM) cultures are widely used models of osteoclastogenesis, the most relevant and beneficial effects of EMD for regenerative periodontal therapy have been identified from clinical studies in humans [1,2,4,16,17]. The aim of this study was to explore the impact of EMD on osteoclast formation and bone resorption in pure cultures of isolated both mouse and human osteoclast precursor cells and to demonstrate possible differences between species.

Material and methods

Mice

CsA male mice from our own inbred colony were used in the study. The mice were housed under a 12:12 h dark/light cycle in a temperature- and humidity-controlled (22 °C/50%) room, with *ad libitum* access to standard chow (Special Diet Service #801730).

Ethical approval

Animal care and experiments were approved by the Regional Animal Research Ethics Committee at the Court of Appeal of Northern Norrland (permission No. A6-17).

EMD purification

Emdogain® is supplied in 0.3-mL vials at a concentration of 30 mg/mL (Straumann AG, Basel, Switzerland). To remove the acidic propylene glycol alginate (PGA) gel and obtain a neutral pH, the contents of one vial were dialysed against 1× PBS (pH 7.4) and diluted 12-fold to a final concentration of 2.5 mg/mL prior to use in cell experiments.

Mouse bone marrow macrophages (BMMs)

Femurs and tibiae from 5- to 7-week-old male mice were dissected and cleaned of adhering tissues. The marrow cavity was flushed and M-CSF-induced BMMs were prepared as previously described [18,19]. Briefly, after erythrocyte lysis, the cells were cultured in α-MEM supplemented with 10% foetal bovine serum, L-glutamine (Life Technologies Ltd., Europe BV) and antibiotics (streptomycin, penicillin and gentamycin) (Gibco, Grand Island, NY) (hereafter referred to as complete α-MEM) and recombinant mouse M-CSF (30 ng/mL) (R&D Systems/Biotechne, Abingdon, UK) in Corning® non-treated suspension culture dishes (Corning Inc., Corning, NY). In these dishes, BMMs are adherent, but not stromal or lymphoid cells. After 3 d of incubation, non-adherent cells were discarded and the adherent BMMs were harvested and seeded for continued experiments, as described below.

Osteoclast formation among mouse BMM cultures on plastic dishes and bone slices

The protocol used for studies of osteoclast formation in mouse BMM cultures was basically adopted from Conaway et al. [19]. In brief, mouse BMMs were seeded in 5-μL droplets (5×10^3 cells) in 96 multiwell plates (Nunc, Roskilde, Denmark). The cells were left to adhere for 10 min, followed by addition of 200 μL complete α-MEM supplemented with mouse M-CSF (30 ng/mL) and RANKL (4 ng/mL) (R&D Systems/Biotechne), with or without EMD (1, 10 or 100 μg/mL). The cells were incubated at 37 °C under 5% CO_2 for 3 d (72 h). Then the culture media was harvested and stored at −20 °C until analysis of the active isoform 5b of tartrate-resistant acid phosphatase (TRAP 5b) (Immunodiagnostic systems [IDS], Copenhagen, Denmark). Adherent cells were fixed at the bottom of the 96-well plates, and stained for TRAP using the leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instruction. TRAP-positive cells with three or more nuclei were considered osteoclasts. Images were acquired using an Olympus BX41 light microscope.

Mouse BMMs were also seeded on bovine cortical bone slices (IDS) placed at the bottom of 96-well plates (Nunc) (5×10^3 cells/well) in 200 μL complete α-MEM, supplemented with mouse M-CSF (30 ng/mL) and RANKL (4 ng/mL), with or without EMD (100 μg/mL). These cells were incubated at 37 °C under 5% CO_2 for 6 d. Culture media was changed on day 3. On day 6, the media was harvested and stored in aliquots at −20 °C until analysis of TRAP 5b and C-terminal type I collagen (CTX-1) (IDS). The cells grown on bone slices were fixed and stained for TRAP as described above. The number of TRAP-positive cells and area of resorption pits were visualized using light microscopy (Olympus BX41).

Human peripheral blood monocytes

Negatively selected 'untouched' monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) using an indirect magnetic labelling system. Briefly, blood was drawn from healthy volunteers into vacutainer tubes supplemented with EDTA as an anticoagulant. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare), following the manufacturer's instructions. From the PBMC fraction, monocytes were immunomagnetically purified (negative selection) using the Pan

monocyte isolation kit and MACS columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

In vitro osteoclastogenesis in human monocyte cultures on plastic dishes and bone slices

The protocol used for studies of osteoclast formation in human monocyte cultures was basically adopted from Lionikaite et al. [20]. In brief, human monocytes were seeded in 96-well plates (1×10^5 cells/well) in complete α-MEM supplemented with recombinant human M-CSF (30 ng/mL) (R&D Systems) and RANKL (3 ng/mL), with or without EMD (100 μg/mL). Then the cells were incubated at 37 °C under 5% CO_2 for various time periods. Culture media was changed after 3 d (72 h) in all treatment groups. Media was harvested at defined time-points and stored in aliquots at −20 °C until TRAP 5b analysis. Adherent cells were fixed at the bottom of the 96-well plates, and stained for TRAP as described above. TRAP-positive cells with three or more nuclei were considered osteoclasts. Images were acquired using an Olympus BX41 light microscope.

Human monocytes were also seeded on bovine cortical bone slices placed at the bottom of 96-well plates (5×10^3 cells/well) in 200 μL of complete α-MEM, supplemented with human M-CSF (30 ng/mL) and RANKL (3 ng/mL), with or without EMD (100 μg/mL). These cells were incubated at 37 °C under 5% CO_2 for various time periods. Then, the culture media was harvested and stored in aliquots at −20 °C until analysis of TRAP 5b and CTX-1. The cells grown on bone slices were fixed and stained for TRAP, as described above. The number of TRAP-positive cells and area of resorption pits were visualized using light microscopy (Olympus BX41).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7 (San Diego, CA). Student's *t*-test (two-tailed) was used to compare the numbers of osteoclasts and concentrations of TRAP 5b and CTX-1 in culture media. All experiments were repeated at least twice with comparable results. All data are presented as mean ± SD. Significance levels were set to $p < .05$.

Results

Emdogain purification

EMD is commercially available in a PGA formulation under the brand name Emdogain®. We found that addition of Emdogain® (100 μg/mL) to BMM cultures resulted in a drop in pH and that the cells died (data not shown). Hence, for further studies the substance was first dialysed against PBS (pH 7.4) to remove the acidic gel.

EMDs impact on osteoclast formation in mouse BMM cultures on a plastic surface

To investigate the effect of EMD on osteoclastogenesis, we cultured primary BMMs from CsA mice in the presence of M-CSF and RANKL, with or without EMD. After 72 h, a large number of TRAP-positive multinucleated osteoclasts had formed (Figure 1(A)). However, addition of EMD (100 μg/mL) to the culture media yielded significant reductions of both the size and number of osteoclasts formed (Figure 1(A,B)). EMD-supplemented cultures also showed significantly lower amounts of TRAP 5b secreted into the culture media, reflecting the reduced number of mature osteoclasts (Figure 1(C)). Lower EMD concentrations (1 or 10 μg/mL) did not appear to affect osteoclast formation (data not shown). EMD alone (1, 10 or 100 μg/mL), without RANKL, had no visible effect on M-CSF-stimulated mouse BMMs cultured on a plastic surface (data not shown).

EMDs impact on osteoclast formation and bone-resorbing capacity in mouse BMM cultures on bone slices

To explore whether EMD also affected osteoclast formation when cells were cultured on a physiological substrate, and to investigate whether bone-resorbing capacity was affected, we cultured mouse BMMs on bovine cortical bone slices in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL). After 6 d of culture, we observed numerous osteoclasts and clear resorption pits on all bone slices, regardless of EMD supplementation (Figure 2(A)). The culture media was harvested on day 6, and analysed for TRAP 5b and CTX-1 levels. EMD addition did not influence TRAP 5b or

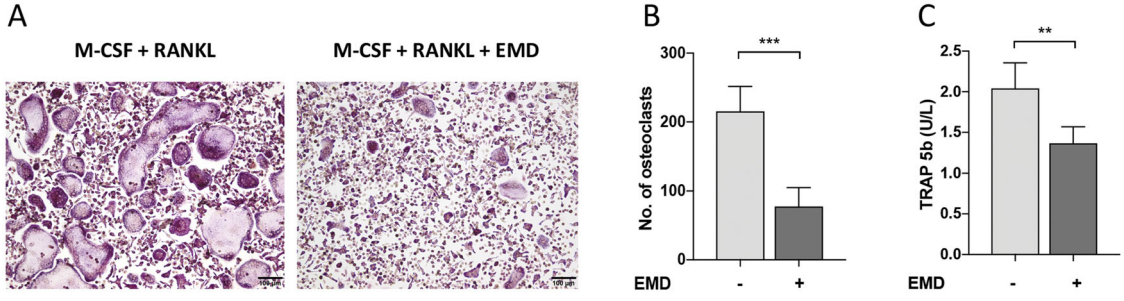

Figure 1. EMD inhibits RANKL-induced osteoclastogenesis in mouse BMM cultures on a plastic surface. (A) Mouse BMMs cultured in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL) for 3 d (72 h), and then stained for TRAP ($n = 6$ wells per treatment group). Representative images are shown. Original magnification ×100. (B) The number of osteoclasts per well and (C) TRAP 5b activity levels accumulated in culture media after 72 h incubation. Data represent the mean ± SD from six observations. $^{**}p < .01$; $^{***}p < .001$.

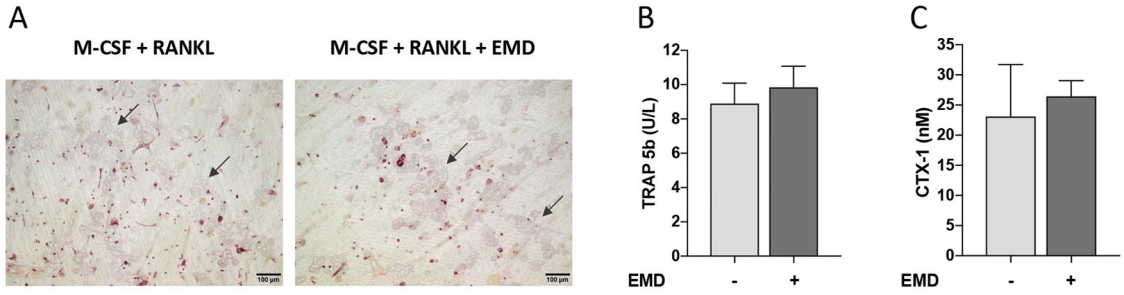

Figure 2. EMD does not affect RANKL-induced osteoclast formation in mouse BMM cultures on bone slices. (A) Mouse BMMs were cultured in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL), for 6 d and then stained for TRAP ($n = 6$ bone slices per treatment group). Representative images are shown. Arrows indicate resorption pits. Original magnification ×100. On day 6, we measured the (B) TRAP 5b and (C) CTX-1 levels in culture media. Since all media was changed on day 3 (after 72h incubation), these values represent the TRAP 5b and CTX-1 levels released between days 3 and 6. Data represent mean ± SD from six observations.

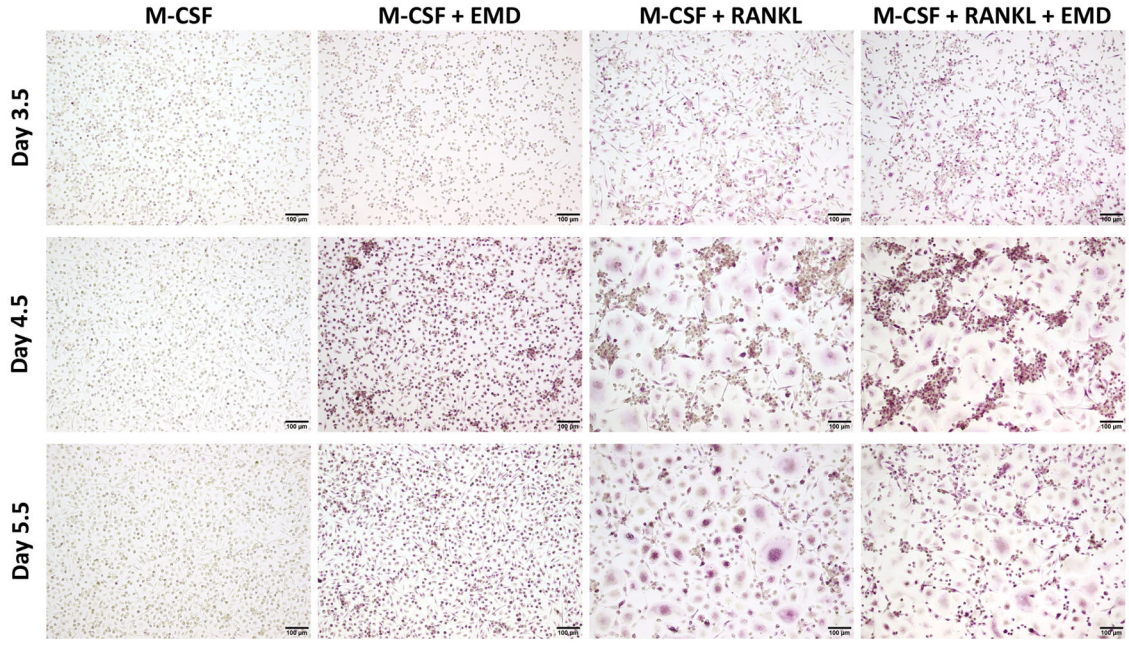

Figure 3. EMD stimulates differentiation of mononucleated TRAP-positive cells but does not affect RANKL-induced osteoclast formation in human monocyte cultures on a plastic surface. Human monocytes were cultured in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL) for 3.5, 4.5 and 5.5 d, and then stained for TRAP ($n = 4$ wells per treatment group and time-point). Representative images are shown. Original magnification ×100.

CTX-1 levels (Figure 2(B,C)). The addition of EMD without RANKL did not affect osteoclastogenesis in mouse BMM cultures on bone slices (data not shown).

EMDs impact on osteoclast formation in human monocyte cultures on a plastic surface

Human monocytes that were isolated from peripheral blood and cultured in the presence of M-CSF and RANKL differentiated into TRAP-positive multinucleated osteoclasts within 4–5 d (Figure 3). Addition of EMD (100 μg/mL) to the culture media did not appear to affect the formation of mature osteoclasts, but resulted in a significantly increased proportion of TRAP-positive mononucleated cells. At around 4.5 d, we observed marked clustering of mononuclear cells, similar to a previous description of the first sign of RANKL-induced osteoclastogenesis in human monocytes [20]. This phenomenon was not affected by EMD. Addition of EMD (100 μg/mL) without RANKL also induced differentiation of monocytes into TRAP-positive mononucleated pre-osteoclasts. However, no mature osteoclasts were formed unless RANKL was added to the culture media.

EMDs impact on osteoclast formation and bone-resorbing capacity in human monocyte cultures on bone slices

Human monocytes were cultured for 10.5 d on bone slices in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL). The monocytes differentiated into multinucleated osteoclasts in the presence of RANKL, and resorption pits were clearly apparent after 10.5 d, regardless of EMD presence (Figure 4(A)). Similar to human monocytes cultured on a plastic surface, the addition of EMD without RANKL induced the differentiation of TRAP-positive mononucleated pre-osteoclasts. However, no mature osteoclasts formed and no resorptions pits appeared unless RANKL was added to the culture media.

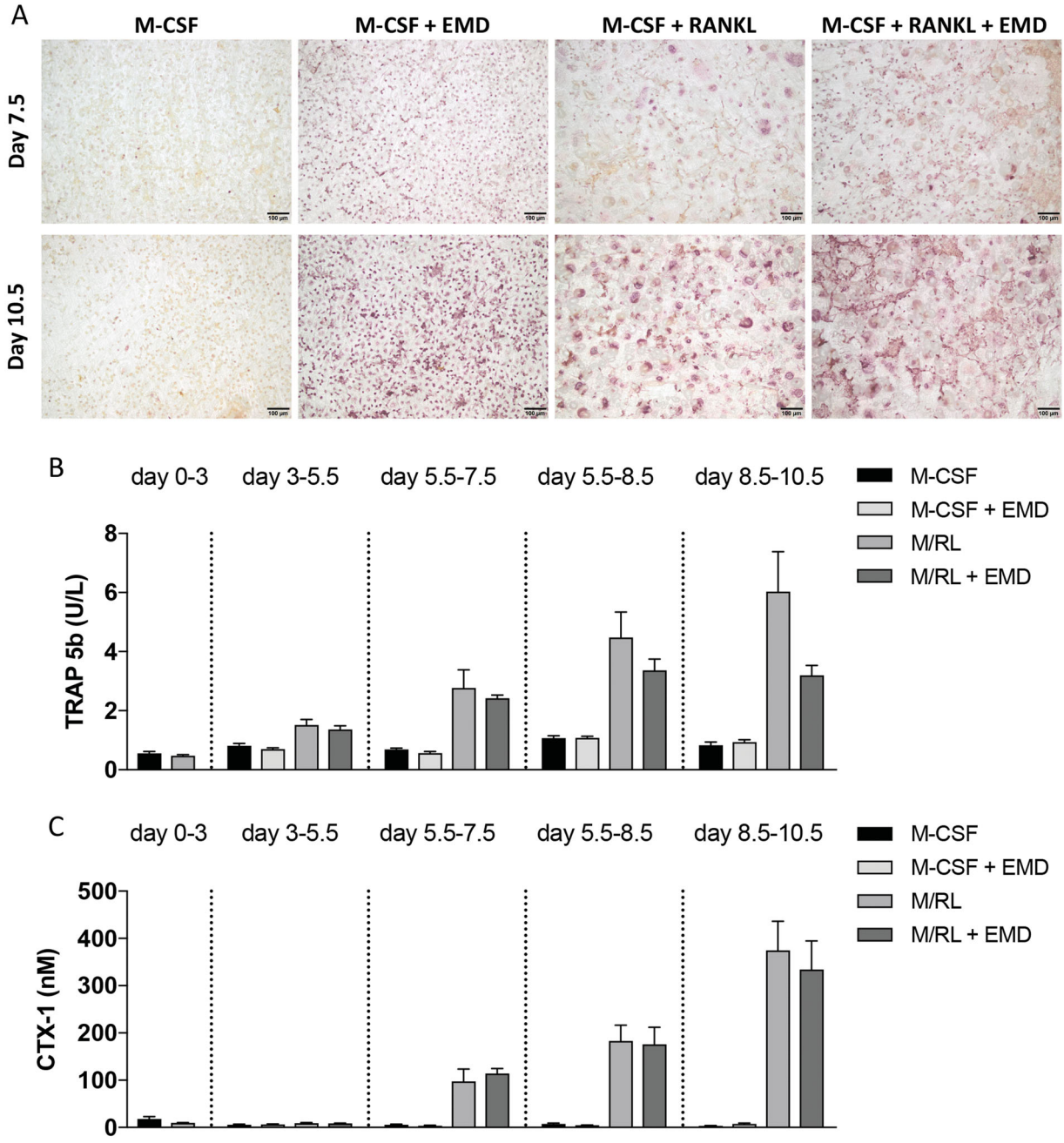

Figure 4. EMD does not affect RANKL-induced osteoclast formation in human monocyte cultures on bone slices. Human monocytes were cultured in the presence of M-CSF and RANKL, with or without EMD (100 μg/mL). The culture media were changed every third day, i.e. on days 3, 5.5, and 8.5. (A) After 7.5 and 10.5 d, adherent cells were stained for TRAP ($n = 4$ bone slices per treatment group and time-point). Representative images are shown. Original magnification ×100. Images show the total amounts of (B) TRAP 5b and (C) CTX-1 released in the culture media between days 0–3, 3–5.5, 5.5–7.5, 5.5–8.5 and 8.5–10.5. Data represent the mean ± SEM from four or eight observations.

During the experiment, culture media was changed every third day and the removed media was analysed for TRAP 5b and CTX-1 levels (Figure 4(B,C)). Both TRAP 5b and CTX-1 increased over time in cultures stimulated with M-CSF and RANKL. Cultures incubated with EMD showed slightly decreased TRAP 5b levels secreted between days 5.5–8.5 and 8.5–10.5, but these differences were not statistically significant. Addition of EMD to the culture media did not significantly affect CTX-1 release from bone slices. Overall, we found that EMD itself did not stimulate TRAP 5b secretion or CTX-1 release.

Discussion

In this study, we hypothesized that the positive effects of EMD on bone gain in surgical treatment of periodontitis [1,2] and peri-implantitis [16,17] could partly result from direct effects on osteoclast precursor cells, leading to decreased osteoclast formation or activity. If this is true, EMD could possibly be an important, but hitherto untested supplement for treatment of a variety of other clinical conditions that also include disturbed bone remodelling.

To test this hypothesis, we investigated whether EMD influenced osteoclast formation and bone resorption in pure primary mouse BMMs, and human peripheral monocyte cultures. Our results revealed that EMD itself did not affect osteoclast formation from mouse BMMs when cultured on a plastic surface or on bovine cortical bone slices. However, the addition of EMD (100 μg/mL) clearly suppressed osteoclastogenesis in cultures of mouse BMMs exposed to RANKL, based on decreases in both the size and number of osteoclasts formed on a plastic surface. To exclude that the EMD-mediated inhibitory effect was an artefact that occurred only on the plastic matrix, we also induced osteoclastogenesis by RANKL in mouse BMMs seeded on natural bone slices, which we and others [21] consider a more physiologically relevant system. After 6 d on bone slices, we did not detect any apparent effect of EMD on the size or number of osteoclasts formed. Moreover, EMD did not affect TRAP 5b levels in culture media, supporting that EMD did not affect the number of osteoclasts formed on bone slices. Moreover, EMD was not associated with any difference in CTX-1 levels released into culture media between days 3 and 6, clearly indicating that EMD also did not influence osteoclast resorbing activity.

Few studies have previously addressed the effects of EMD on osteoclastogenesis and bone resorption *in vitro*. In contrast to our present results, prior studies have all proposed that EMD can promote osteoclast formation [14,15,22]. However, the source of cells in this study is different from that in previous investigations. Herein, we used isolated mouse BMMs and human peripheral monocytes, while previous studies have used either crude mouse BMCs [14,15] or the mouse monocytic cell line RAW 264.7 [22].

The only report showing effects of EMD on osteoclastogenesis in pure osteoclast cultures was published by Itoh et al. The authors fractionated EMD using HPLC and demonstrated that one specific fraction increased RANK expression and enhanced RANKL-induced osteoclast formation in RAW 264.7 cells [22]. However, immortalized cells (such as RAW-cells) can behave differently from primary cells in culture and, thus, it is not surprising that the findings differ between this study and the study Itoh et al. performed in RAW 264.7 cells. Other notable differences between these studies are that in contrast to Itoh et al., we used the entire protein content of Emdogain®, and we cultured BMMs on natural bone slices, whereas the RAW 264.7 cells were cultured on a plastic surface or synthetic calcium phosphate thin films. Using the latter artificial substrate, Itoh et al. demonstrated increased calcium release in RAW cell cultures stimulated by RANKL and EMD, compared to in cultures stimulated only with RANKL. However, the cultures treated with RANKL and EMD also exhibited increased total numbers of osteoclasts, indicating that it was not necessarily increased osteoclast activity that was observed [22]. Our present results demonstrated that EMD had no effect on bone resorption when added to RANKL-stimulated BMMs or monocyte cultures on bone slices, providing evidence that EMD did not alter the resorption activity of primary osteoclasts.

BMC cultures contain a heterogeneous mixture of cells of haematopoietic and mesenchymal origin. Otsuka et al. [15] proposed that EMD induces osteoclast formation through RANKL expression on osteoblastic cells in BMC cultures [15]. In another study, Gruber et al. added Emdogain® to RANKL-stimulated BMCs and reported increased osteoclast formation that may have been mediated via transforming growth factor-beta receptor 1 type I (TGF-βR1) kinase signalling [14]. In surprising contrast, when we added Emdogain® (100 μg/mL) to BMM cultures, the pH dropped and the cells died (data not shown). Therefore, for our present study, we removed the acidic PGA carrier from Emdogain®, and then added the purified EMD fraction to cell cultures.

In this study, we compared the effects of EMD on mouse BMMs to the effects on isolated human peripheral blood monocytes. In cultures of human monocytes, RANKL-induced osteoclastogenesis was not affected by EMD. EMD exposure did not alter the apparent size of osteoclasts on a plastic surface, or the release of TRAP 5b or CTX-1 from cells on natural bone slices. When added to monocytes, EMD did stimulate differentiation of TRAP-positive mononucleated osteoclast progenitor cells, but the implication of this remains to be explored. Notably, no mature osteoclasts were formed unless exogenous RANKL was added.

A limitation of this study is that the results focus mainly on histological stainings and biomarkers in the culture medium supposed to reflect the number and bone-resorbing capacity of mature osteoclasts. In future studies, it would be interesting to also investigate the effect of EMD on mRNA expression of osteoclastic and osteoclastogenic genes. Moreover, further studies to evaluate the effect of EMD in osteoblast-osteoclast co-cultures are warranted.

In conclusion, our present results show that the addition of EMD, with or without RANKL, to osteoclast precursor cells cultured on bone substrate did not affect osteoclastogenesis or bone resorption. However, previous studies have shown that EMD increases the RANKL/OPG ratio, and enhances osteoclast formation in crude BMC cultures [14,15]. Overall, the previous *ex vivo* [14,15] and clinical studies [2,16,17] indicate that EMD likely plays a dual function as a supplement in the surgical treatment of periodontitis and peri-implantitis – partly stimulating bone formation, but also increasing osteoclastogenesis and bone resorption [23]. Our present results neither confirm nor contradict this possible dual function, but rather contribute to our present understanding of the mechanism by excluding a direct interaction between EMD and osteoclast precursor cells.

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Disclosure statement

The authors report there is no competing interest to declare.

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