Clin Oral Invest (2014) 18:1639–1646
DOI 10.1007/s00784-013-1129-6

ORIGINAL ARTICLE

TGF-βRI kinase activity mediates Emdogain-stimulated in vitro osteoclastogenesis

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Received: 17 July 2013 / Accepted: 17 October 2013 / Published online: 13 November 2013

Abstract
Objectives Emdogain, containing an extract of fetal porcine enamel matrix proteins, is a potent stimulator of in vitro osteoclastogenesis. The underlying molecular mechanisms are, however, unclear.
Material and methods Here, we have addressed the role of transforming growth factor-beta receptor type 1 (TGF-βRI) kinase activity on osteoclastogenesis in murine bone marrow cultures.
Results Inhibition of TGF-βRI kinase activity with SB431542 abolished the effect of Emdogain on osteoclastogenesis induced by receptor activator of nuclear factor kappa-B ligand or tumor necrosis factor-alpha. SB431542 also suppressed the Emdogain-mediated increase of OSCAR, a co-stimulatory protein, and dendritic cell-specific transmembrane protein and Atp6v0d2, the latter two being involved in cell fusion. Similar to transforming growth factor-beta1 (TGF-β), Emdogain could not compensate for the inhibition of IL-4 and IFNγ on osteoclast formation. When using the murine macrophage cell line RAW246.7, SB431542 and the smad-3 inhibitor SIS3 blocked Emdogain-stimulated expression of the transcription factor NFATc1.
Conclusions Taken together, the data suggest that TGF-βRI kinase activity is necessary to mediate in vitro effects of Emdogain on osteoclastogenesis.
Clinical relevance Based on these in vitro data, we can speculate that at least part of the clinical effects of Emdogain on osteoclastogenesis is mediated via TGF-β signaling.

Keywords Emdogain · Enamel matrix derivative · Osteoclast · TGF-β · Differentiation · SB431542 · SIS3 · Bone marrow

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Introduction

Osteoclasts, the exclusive bone resorbing cells, originate from hematopoietic progenitors [1, 2]. Under physiologic conditions, osteoclasts contribute to calcium-phosphate homeostasis and bone remodeling [1, 2]. Bone regeneration also involves osteoclastogenesis [3]. Under chronic inflammatory conditions, osteoclasts cause bone destruction, for example in periodontal disease, rheumatoid arthritis, and colitis [4]. It is thus of clinical relevance to understand the process of osteoclastogenesis and how it is modulated by local and systemic factors, including pharmacological therapies. In vitro models have traditionally provided insights into the process of osteoclastogenesis [5].

Osteoclastogenesis is controlled by the key factor, receptor activator of nuclear factor kappa-B ligand (RANKL), also known as tumor necrosis factor ligand superfamily member 11 [5]. Osteoclasts generated from bone marrow are characterized by histochemical staining of tartrate-resistant acid phosphatase (TRAP) and their multinucleated morphology [1, 2]. Moreover, these cells express other functional genes such as cathepsin K (CathK) and the calcitonin receptor (CTR). Osteoclasts express co-stimulatory molecules activating the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway

[6]. Osteoclast-associated receptor (OSCAR) and triggering receptor expressed in myeloid cells (TREM2) are receptors that are associated with the respective adaptor molecules Fc receptor common gamma chain (FcRγ) and DNAX-activating protein 12 kDa (DAP12). Downstream signaling pathways culminate in the expression and activation of the master regulator nuclear factor of activated T cells c1 (NFATc1), and genes regulating cell fusion such as dendrocyte expressed seven transmembrane protein (DC-STAMP) [7] and ATPase, H^+ transporting, lysosomal 38 kDa, V0 subunit d2 (Atp6v0d2) [8]. The expression levels of the respective genes, consequently, provide insights into osteoclastogenesis in vitro.

Emdogain is the trade name for the combination of enamel matrix derivative (EMD) isolated from the tooth germs of piglets and propylene glycol alginate (Institut Straumann, Basel, Switzerland, formerly Biora, Malmö, Sweden) [9, 10]. Emdogain can support periodontal tissue regeneration [11], however, also root resorption following surgical debridement was reported [12]. Emdogain can prevent root resorption after tooth replantation in rats [13, 14], with certain clinical translation [15–18]. In vitro, Emdogain clearly stimulates the differentiation of the mouse monocytic cell line RAW 264.7 and primary bone marrow cells into osteoclast-like cells [19, 20]. Chromatography further revealed fractions of enamel matrix derivatives responsible for the pro-osteoclastogenic activity of Emdogain, however, the molecular details have not been discovered so far [20]. Therefore, it is relevant to better understand the details on how Emdogain supports osteoclastogenesis in vitro.

Transforming growth factor-beta1 (TGF-β) signaling is among the main mechanisms that mediate at least part of the cellular response to EMD and Emdogain [21–26]. In vitro, TGF-β can increase osteoclastogenesis in the presence of RANKL or tumor necrosis factor-alpha (TNFα) [27]. TGF-β binds to the type II receptor, which in turn activates the type I receptor (TGF-βRI). TGF-β signaling supports osteoclastogenesis for example, by increasing the master regulator NFATc1 [28]. However, Emdogain contains not only TGF-β and TGF-β-like substances [10]. It is therefore not clear if TGF-β signaling mediates the effect of Emdogain on osteoclastogenesis [19, 20]. The mechanism through which Emdogain acts to stimulate osteoclastogenesis remains to be determined.

We therefore tested the hypothesis that osteoclastogenesis in the presence of Emdogain involves TGF-β signaling. To support this assumption, we blocked the TGF-βRI kinase with the pharmacologic compound SB431542 and studied osteoclastogenesis in murine bone marrow cultures. RAW 264.7 murine monocytic cells served as a model to study impact of SB431542 and SIS3, the latter being a smad-3 signaling inhibitor, on the regulation of NFATc1. Based on this in vitro setting, we report that TGF-βRI kinase signaling mediates the pro-osteoclastogenic effects of Emdogain at the level of cell morphology, expression of differentiation and fusion markers, and the master regulator NFATc1.

Material and methods

In vitro osteoclastogenesis in bone marrow cultures

Bone marrow cells were prepared by flushing the femur and tibiae of 4- to 6-week-old female mice (strain Balb/c,) and seeded at one million bone marrow cells per square centimeter in Eagle's Minimum Essential Medium—Alpha Modification (aMEM) supplemented with 10 % fetal calf serum (FCS), antibiotics. For osteoclastogenesis, medium was supplemented with macrophage colony-stimulating factor (M-CSF) at 30 ng/ml and soluble RANKL at 30 ng/ml. Cells were additionally exposed to Emdogain (Institut Straumann AG, Basel, Switzerland; 100 μg EMD/ml), human transforming growth factor-beta1 (TGF-β1) or human TNFα, both at 5 ng/ml. For indicated experiments, Emdogain from four different batches and reconstituted (0.1 % acetic acid) lyophilized EMD was used. In addition, Emdogain (10 mg/ml) was heat treated at 96 °C for 3 min as TGF-β resists high temperatures [29]. Also experiments with murine IL-4 and murine IFNγ were performed. Recombinant proteins were purchased from Prospec (Ness-Ziona, Israel). SB431542 was used at 10 μM (Santa Cruz Biotechnology, Santa Cruz, CA). After 5 days, histochemical staining for TRAP (Sigma Aldrich, St. Louis, MO) was performed.

Expression of marker genes in bone marrow cultures

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Rotkreuz, Switzerland). Reverse transcription (RT) was performed with Transcriptor Universal cDNA Master and PCR was done with TaqMan universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) or the FastStart Universal Probe Master Rox on a 7500 Real-Time PCR System (Roche). Probes for CTR, TRAP, CathK, OSCAR, TREM2, FcRγ, DAP12, and beta actin were obtained from the TaqMan Gene Expression Assays service (Applied Biosystems). The FastStart Universal SYBR Green Master Rox (Roche) was used for DC-STAMP (forward: aagctccttgagaaacgatca; reverse: cag gac tgg aaa cca gaa atg) and Atp6v0d2 (forward: aag cct ttg ttt gac gct gt; reverse: gcc agc aca ttc atc tgt acc). Primers were designed with the online Universal ProbeLibrary System. The mRNA levels were calculated by normalizing to the housekeeping gene beta actin using the ΔCt method.

Expression of NFATc1 in RAW 264.7

RAW 264.7, macrophage-like cells, were kindly provided by Jürg Gertsch (Institute of Biochemistry and Molecular Medicine, University of Bern). Cells were exposed to growth medium containing RANKL at 10 ng/ml with various combinations of Emdogain, TGF-β and SB431542 for 24 h. One experiment was performed with a TGF-β pan specific polyclonal Ab (AB-100-NA; R&D Systems, McKinley Place NE, MN) as reported recently [26]. Also the Smad3 inhibitor SIS3 at 10 μM (Calbiochem) was used in this setting. Total RNA was isolated and RT-PCR was performed for NFATc1 (forward: tccaaagtcattttcgtgga; reverese: ctttgcttccatctcccaga) according to the SYBR Green protocol.

Statistical analysis

Experiments were repeated in triplicates at least twice. Data are reported as mean and standard deviation of all data points. Statistical analysis was performed with ANOVA and post-hoc testing. p values less than 5 % were considered significant.

Results

Emdogain stimulates RANKL-induced osteoclastogenesis

To investigate the impact of Emdogain on osteoclastogenesis, we determined the formation of multinucleated cells staining positive for TRAP. RANKL and M-CSF induced the formation of osteoclasts. As expected [19, 20], Emdogain and TGF-β increased the number and size of osteoclast-like cells in vitro (Fig. 1a). Similar to recombinant TGF-β [29], heat-treatment of Emdogain [30] maintained its activity on osteoclastogenesis (data not shown). Emdogain considerably (greater than twofold) increased the mRNA level of TRAP, CathK and CTR, being in line with the morphological changes (Fig. 1b). Emdogain also increased OSCAR, while the other co-stimulatory molecules TREM2, FcRγ, and DAP12, remained unchanged (Fig. 1c). Together, the findings show that similar to TGF-β, Emdogain is a potent enhancer of RANKL-induced osteoclastogenesis.

Emdogain stimulates TNF-induced osteoclastogenesis

Besides RANKL, TNFα can also induce osteoclastogenesis in the presence of TGF-β [31]. Thus, we determined if Emdogain serves as cofactors for TNFα. Multinucleated cells staining positive for TRAP were found in cultures containing TNFα and TGF-β (Fig. 2a). When TGF-β was replaced by Emdogain, osteoclasts developed even though they were less in number and had fewer nuclei. These findings demonstrate that Emdogain can serve as a cofactor for TNFα-induced osteoclastogenesis, again, analogous to TGF-β.

Emdogain cannot overcome the inhibition of IL-4 and IFNγ on osteoclastogenesis

To further learn how Emdogain exerts its effect on osteoclastogenesis, we performed the bone marrow cultures in the presence of the potent inhibitors IL-4 and IFNγ (Fig. 3). As expected, IL-4 and IFNγ substantially diminished the formation of osteoclasts in vitro. Neither TGF-β nor Emdogain could compensate for the suppression of osteoclastogenesis, further suggesting a functional similarity of the two pro-osteoclastogenic factors.

SB431542 abolished osteoclastogenesis in the presence of Emdogain

Having shown that the effects of TGF-β and Emdogain on osteoclastogenesis are comparable, we went on to investigate if the cellular response to Emdogain involves TGF-β signaling. To do this, we performed the experiments in the presence of SB431542, an inhibitor of TGF-βRI kinase activity. Osteoclastogenesis was markedly decreased in the presence of SB431542 (Fig. 4a). These morphologic changes were accompanied by a reduction in the expression of the osteoclastogenic marker genes TRAP, CathK, and CTR (Fig. 4b). SB431542 also blocked the effects of TGF-β and Emdogain on the expression of DC-STAMP and Atp6v0d2 (Fig. 4c). However, SB431542 also blocks osteoclastogenesis in basic cultures containing RANKL and M-CSF, supporting the role of endogenous TGF-β in osteoclastogenesis (data not shown). Together, the data suggest Emdogain cannot overcome the essential role of the TGF-βRI kinase in osteoclastogenesis.

SB431542 and SIS3 suppressed the effects of Emdogain on NFATc1 expression

In the bone marrow culture, SIS3 also blocked osteoclastogenesis in the presence of TGF-β and Emdogain (Fig. 5a). We next took advantage of a murine macrophage cell line RAW246.7 and NFATc1, the latter being the master regulator of osteoclastogenesis, which is strongly increased by TGF-β [28]. RAW246.7 cells responded with increased mRNA levels of NFATc1 when activated with Emdogain (Fig. 5b). Importantly, SB431542 and SIS3 both blocked the effects of Emdogain on the expression of NFATc1. Together, these data further support the assumption that Emdogain mediates its activity via TGF-βRI kinase and smad3 signaling, targeting the key transcription factor of osteoclastogenesis, NFATc1.

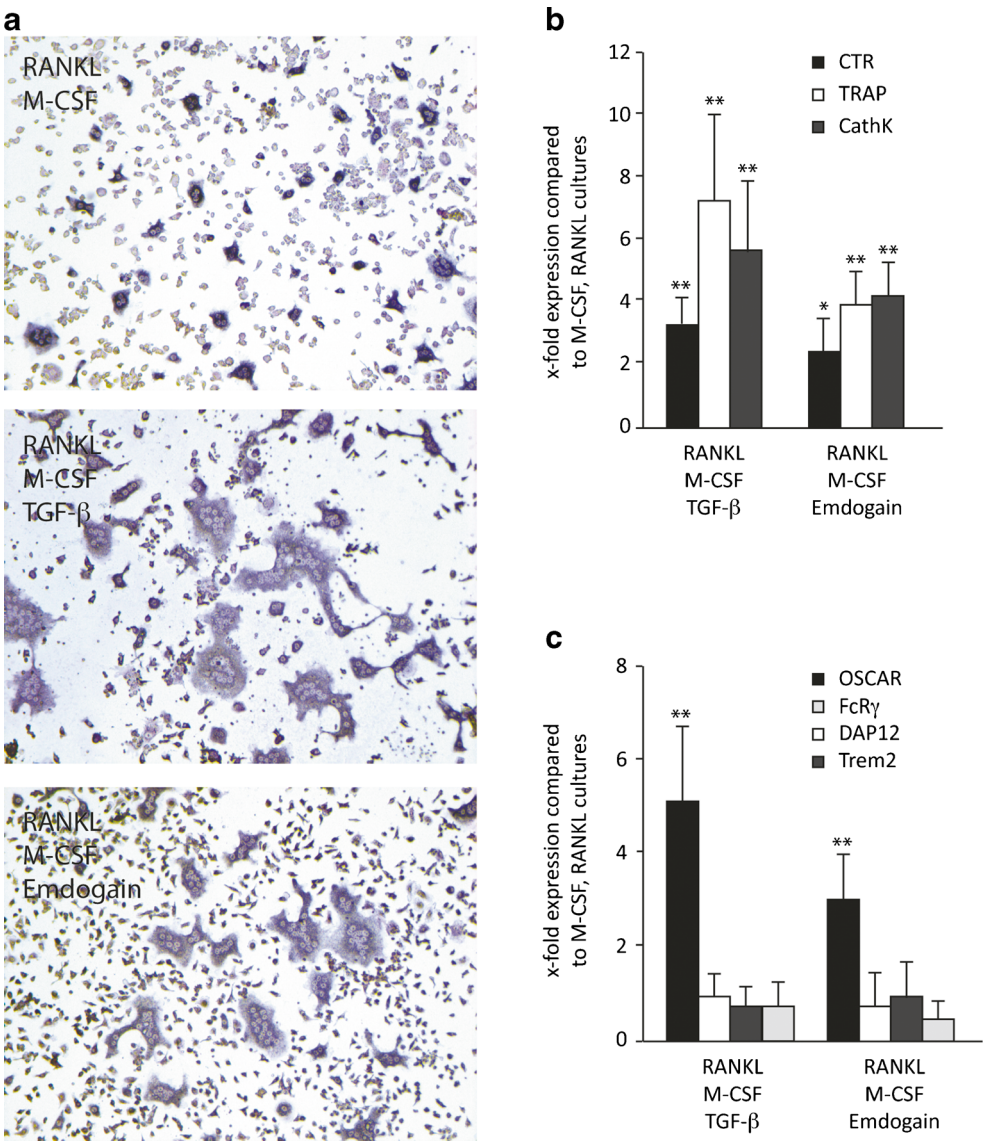

Fig. 1 Emdogain stimulates RANKL-induced osteoclastogenesis. Multinucleated cells staining positive for TRAP were considered osteoclast-like cells. **a** Emdogain and TGF-β substantially increased the number and size of osteoclasts. **b** Emdogain and TGF-β similarly increased the mRNA level of TRAP, CathK, and CTR. **c** Likewise, Emdogain and TGF-β increased OSCAR, while the other co-stimulatory molecules remained unchanged. Data represent the triplicate values of one out of two independent experiments. $^{**}p<0.01$ compared to cultures with RANKL and M-CSF

Discussion

The present study was based on two previous observations: First, similar to TGF-β [27], enamel matrix derivative can support in vitro osteoclastogenesis [19, 20]. Second, TGF-β can mediate at least part of the cellular responses to Emdogain in vitro [21–24]. Together, these data have raised the possibility that the stimulatory effects of Emdogain on osteoclastogenesis also involve TGF-β. The present in vitro study supports this hypothesis as blocking TGF-βRI kinase counteracts all supportive effects of Emdogain on in vitro osteoclastogenesis.

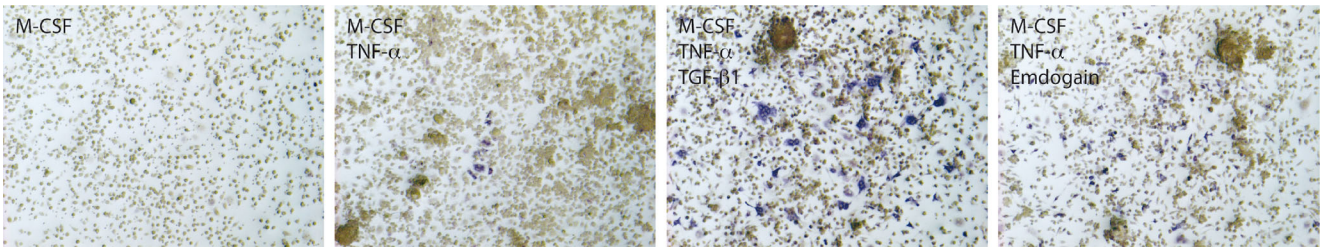

Fig. 2 Emdogain stimulates TNFα -induced osteoclastogenesis. Multinucleated cells which are TRAP positive (*violet*) were observed in bone marrow cultures containing TNFα and TGF-β. When TGF-β was replaced by Emdogain, osteoclasts developed, even though they were less in number and had fewer nuclei. Experiments were performed twice with similar results

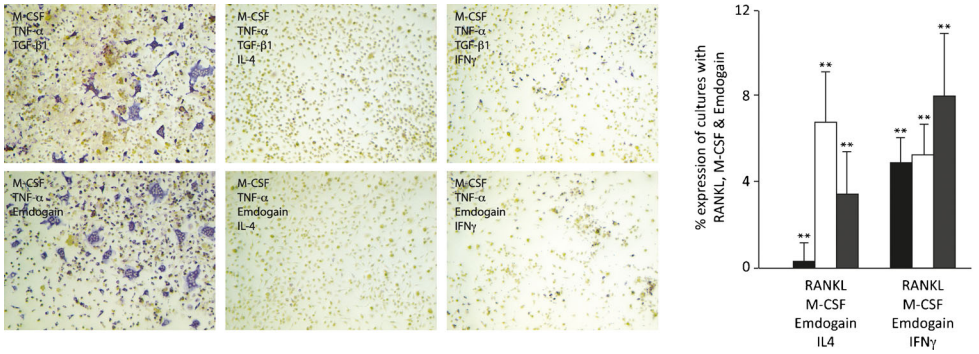

Fig. 3 Emdogain cannot overcome the inhibition of IL-4 and IFNγ on osteoclastogenesis. Osteoclastogenesis was performed in the presence of IL-4 and IFNγ. **a** Both factors diminished osteoclastogenesis in vitro. Emdogain and TGF-β could NOT compensate for the suppression of osteoclastogenesis. **b** Gene expression was reduced by IL-4 and IFNγ to less than 10 % of the respective controls. The data represent the triplicate data of one out of two experiments. $^{**}p < 0.01$ compared to cultures with RANKL, M-CSF, and Emdogain

Our findings extend previous observations that Emdogain has effects similar to TGF-β on osteoclastogenesis induced with RANKL and TNFα [27] and the regulation of NFATc1 [28]. Furthermore, the strong upregulation of genes involved in cell fusion, DC-STAMP and ADP6, is in line with the existing knowledge on TGF-β [32]. Together, our study adds to the current understanding that Emdogain causes a cellular response, similar to TGF-β1.

The question arises, if Emdogain mediates its activity on osteoclastogenesis exclusively via TGF-β signaling? This

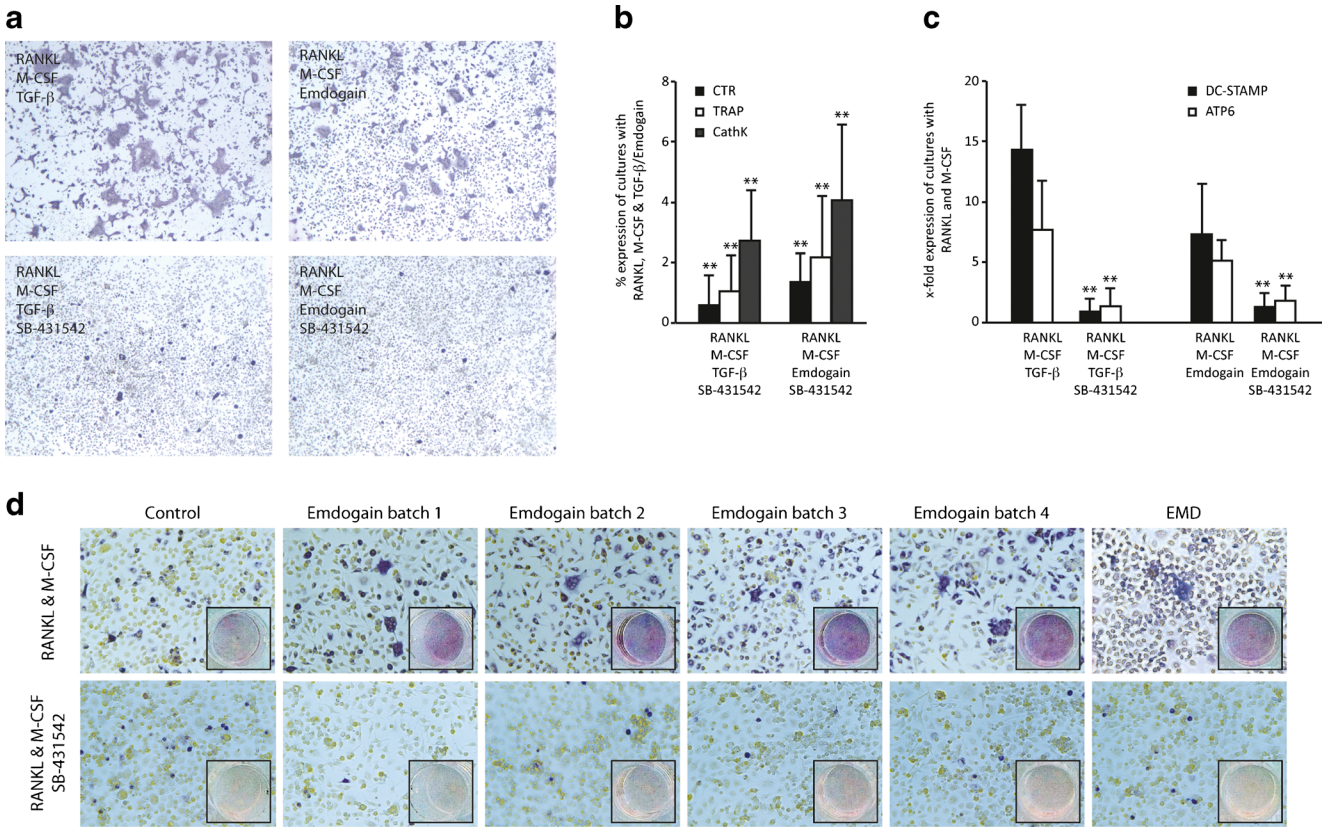

Fig. 4 SB431542 abolished osteoclastogenesis in the presence of Emdogain. Osteoclastogenesis was suppressed in the presence of SB-431542, an inhibitor of TGF-βRI kinase activity (**a**). These microscopic changes were accompanied by a reduction in the expression of the osteoclastogenic marker genes TRAP, CathK, and CTR (**b**). Moreover, also the genes that regulate cell fusion dendrocyte expressed seven transmembrane protein (*DC-STAMP*) and ATPase, H^+ transporting, lysosomal 38 kDa, V0 subunit d2 (*Atp6*) included in the analysis (**c**). SB431542 also suppresses osteoclastogenesis in the presence of various batches of Emdogain and EMD (**d**). The findings shown were conformed by another independent experiment. Expression data represent the mean of triplicate values. $^{**}p < 0.01$ compared to cultures with RANKL, M-CSF, and TGF-β1/Emdogain, respectively

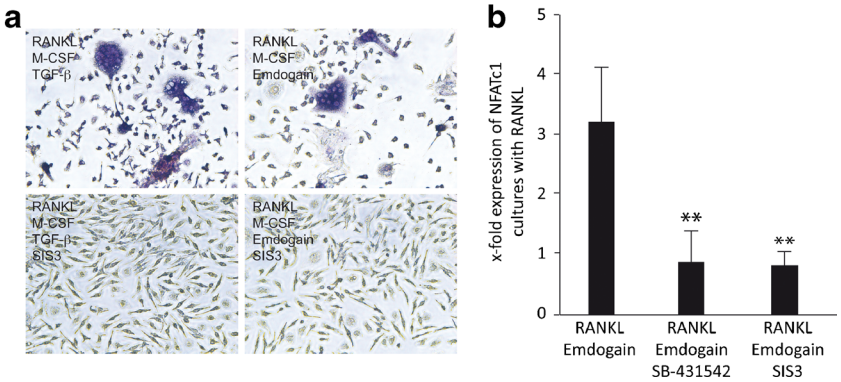

Fig. 5 SB431542 and SIS3 suppressed the effects of Emdogain on NFATc1 expression. SIS3, the inhibitor of smad3 signaling, abolished osteoclastogenesis in the presence of TGF-β1 and Emdogain in the bone marrow culture (**a**). NFATc1 is increasingly expressed when the murine macrophage cell line RAW246.7 is exposed to Emdogain. SB431542 and SIS3 both blocked the effects of Emdogain on the expression of NFATc1 (**b**). This experiment was performed two times with similar results. $^{**}p < 0.01$ compared to cells with RANKL and Emdogain

question is hard to answer because TGF-βRI kinase is obligatory for osteoclastogenesis, also when no extra TGF-β1 is added to the in vitro system. For example, in the presence of SB431542, RANKL-induced osteoclastogenesis is almost completely suppressed [33]. Nevertheless, we provide evidence that Emdogain mediates its activity via TGF-βRI kinase, e.g., SB431542 abolished the stimulatory effect of Emdogain on DC-STAMP, ADP6, and NFATc1 expression. We also show that blocking smad-3 signaling with SIS3 blunted NFATc1 expression and osteoclastogenesis. In support of these findings, smad3 is crucial for TGF-β1-induced osteoclast differentiation in giant cell tumor of bone [34]. Moreover, smad3 overexpression can reverse the inhibitory effect of SB431542 on in vitro osteoclastogenesis [33]. Emdogain also caused smad-3 phosphorylation in epithelial cell and mesenchymal cells, respectively [25, 35]. Together, these data support a direct involvement of TGF-βRI kinase signaling in the Emdogain-mediated cellular actions presented here.

Further support for the hypothesis comes from findings that Emdogain, similar to TGF-β, maintains its activity when heated to 96 °C [29, 30]. It remains however open if TGF-β or other factors that require the TGF-βRI kinase cause the effects of Emdogain on osteoclastogenesis. Emdogain presumably contains TGF-β1 or analogous molecules as suggested by studies with neutralizing antibodies raised against TGF-β1 [21–24] and the respective immunoassays [25, 26]. We also have data that a TGF-β1 neutralizing antibody reduced the potential of Emdogain to enhance NFATc1 expression in RAW246.7 cells (data not shown). Yet, others failed to show positive binding of a TGF-β1 antibody to Emdogain [36]. It thus remains a controversial subject if Emdogain contains TGF-β1. Also, other explanations for an involvement of TGF-β1 are possible. Emdogain can increase the expression of TGF-β1 in various cell types, pointing towards an autocrine mechanism [9, 10]. Overall, our data together with those of others support the assumption that Emdogain contains TGF-β1 and/or analogous molecules that requires the TGF-βRI kinase to support osteoclastogenesis in the murine bone marrow culture.

There remains the discrepancy with the in vivo data showing that Emdogain can prevent root resorption after tooth replantation [13, 14]. However, also in vitro, TGF-β1 inhibits osteoclastogenesis in the presence of stromal cells, which are forced to produce the key inhibitor of osteoclastogenesis, osteoprotegerin [37, 38]. On the other hand, in vivo inhibition of TGF-β1 by neutralizing antibody [39] and TGF-βRI kinase inhibitors [40] can reduce osteoclast differentiation. In vitro, Emdogain and TGF-β1 can also indirectly modulate osteoclastogenesis by stimulating cells to produce osteolytic factors such as IL-11 [41, 42]. Our ongoing studies indicate that Emdogain-induced IL-11 expression in oral fibroblasts also requires TGF-β signaling (Stähli et al.; manuscript in preparation). Thus, the in vitro data cannot be easily translated into the clinical scenario. Future in vitro studies should consider the possibility that Emdogain can decrease osteoclastogenesis in a co-culture model of hematopoietic progenitors and mesenchymal cells. The hypothesis is supported by data showing that Emdogain decreases the RANKL/OPG ratio in mesenchymal cells [43]. It will thus be worth investigating if the changes in the RANKL/OPG ratio caused by Emdogain also involve the TGF-βRI and the downstream smad-3 kinase.

Emdogain is a mixture of proteins with different peptides being responsible for its different biologic properties. The main component amelogenin has a role in osteoclastogenesis. Recombinant amelogenin inhibits in vitro osteoclastogenesis and root resorption [13], and in line with this finding, amelogenin-null mice experience elevated osteoclastogenesis [44]. Nevertheless, Emdogain supports in vitro osteoclastogenesis as indicated by the present study and recent observations [19, 20]. The data thus suggest that, at least in vitro, amelogenin cannot overcome the pro-osteoclastogenic activity of

Emdogain. The next steps would be to further characterize the possible pro-osteoclastogenic molecules previously basically purified by chromatography [19, 20]. Once the pro-osteoclastogenic activity is identified, Emdogain can be selectively modulated to control the respective in vitro properties. However, it should not be overlooked that the early transient osteoclastogenesis is part of the physiologic regeneration sequence. For example, TGF-β can enhance the osteoinductive activity of BMP-2 in vivo [45] and fracture healing is associated with strong expression of pro-osteoclastogenic genes [3]. The present study puts another piece into the mosaic to better understand the cellular response to Emdogain.

Acknowledgments We thank Catherine Solioz for her skillful technique assistance. This work was supported in part by the Straumann Institute.

Conflict of interest The authors declare to have no conflict of interest related to this study.

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