Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 277–280

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

The metabolism of 25-(OH)vitamin D_3 by osteoclasts and their precursors regulates the differentiation of osteoclasts☆

M. Kogawa[a], P.H. Anderson[b], D.M. Findlay[a,b], H.A. Morris[b], G.J. Atkins[a,b,*]

[a] Bone Cell Biology Group, Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia 5000, Australia
[b] Hanson Institute, Adelaide, South Australia 5000, Australia

ARTICLE INFO

Article history:
Received 19 October 2009
Accepted 13 March 2010

Keywords:
CYP27B1
25-Hydroxyvitamin D_3
Osteoclast
Metabolism
Autocrine
Paracrine

ABSTRACT

Current evidence suggests that levels of 25-(OH)vitamin D_3 (25D), rather than 1α,25-$(OH)_2$vitamin D_3 (1,25D), directly affect bone mineralization and that the skeleton is a site of extra-renal synthesis of 1,25D. Since cells of the monocyte lineage can also metabolise 25D, it is possible that osteoclasts participate in local production of, and the response to, 1,25D. In this study, we investigated the effects of vitamin D metabolism on osteoclastogenesis using both the murine RAW 264.7 cell line and the human peripheral blood mononuclear cell (PBMC) models. PBMC-derived osteoclasts expressed cytoplasmic cyp27b1 and nuclear vdr proteins. PBMC expressed CYP27B1 mRNA, levels of which increased during RANKL induced differentiation into osteoclasts in both cell types. While 1,25D elicited a robust CYP24 transcriptional response in PBMC, the response to 25D was approximately 100-fold less at the concentrations used. Using media devoid of pre-existing vitamin D metabolites, we found that 25D was metabolised by RAW 264.7 cells to 1,25D and resulted in significant elevation in the numbers of TRAP-positive, multinucleated osteoclasts when present in the cultures for the first 3–5 days. These results suggest that vitamin D metabolism by osteoclast lineage cells is an important regulator of osteoclast formation.

1. Introduction

Increasing evidence suggests that vitamin D plays a direct role in regulating the activity of bone cells, as recently reviewed [1]. A current view is that 1,25D maintains normocalcaemia by directly stimulating bone resorption and increasing the rate and extent of osteoblast-mediated osteoclastogenesis. However, while the precise mechanism of action is not clear, evidence suggests that 1,25D also has direct effects on osteoclast precursors [2–6]. We have recently demonstrated, using a defined serum-free culture system devoid of contaminating vitamin D metabolites, a direct effect of 1,25D on RANKL-induced osteoclast formation from the mouse osteoclast precursor cell line, RAW 264.7, where 1,25D in the co-presence of RANKL resulted in increased numbers of multinucleated TRAP-positive osteoclasts [7].

We [8,9] and others (reviewed in [1]) have demonstrated that osteoblasts are a source of extra-renal synthesis of vitamin D metabolism and convert 25D into functional 1,25D by virtue of their expression of CYP27B1. The intriguing possibility exists that osteoclasts also participate in local production of, as well as response to, 1,25D. Indeed, cells of the monocyte/macrophage lineage are known to express CYP27B1 and convert 25D into 1,25D [10,11]. The RAW 264.7 cell line has been shown to express CYP27B1 mRNA, levels of which increased during their differentiation into osteoclast-like cells [12].

In this study, we further examined the role of vitamin D metabolism during osteoclastogenesis. Our results indicate that the metabolism of 25D into 1,25D by RAW 264.7 osteoclast precursors, in a defined, otherwise vitamin D metabolite-free medium, results in enhanced formation of TRAP-positive, multinucleated osteoclasts. We conclude that the metabolism of vitamin D during osteoclastogenesis maybe an important intrinsic mechanism for controlling the osteoclast formation of these cells.

2. Materials and methods

2.1. Osteoclast formation

RAW 264.7 cells were cultured under osteoclast forming conditions essentially as described previously [7], with modifications. Briefly, RAW 264.7 cells were seeded into wells of a 96-well plate (1×10^4 cells/well) in serum-deprived medium (SDM) [7] and cultured overnight. Media were then replaced with fresh SDM

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author at: Discipline of Orthopaedics and Trauma, University of Adelaide, North Terrace, Adelaide, South Australia 5000, Australia.
Tel.: +61 8 8222 3054; fax: +61 8 8232 3065.
E-mail address: gerald.atkins@adelaide.edu.au (G.J. Atkins).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.03.048

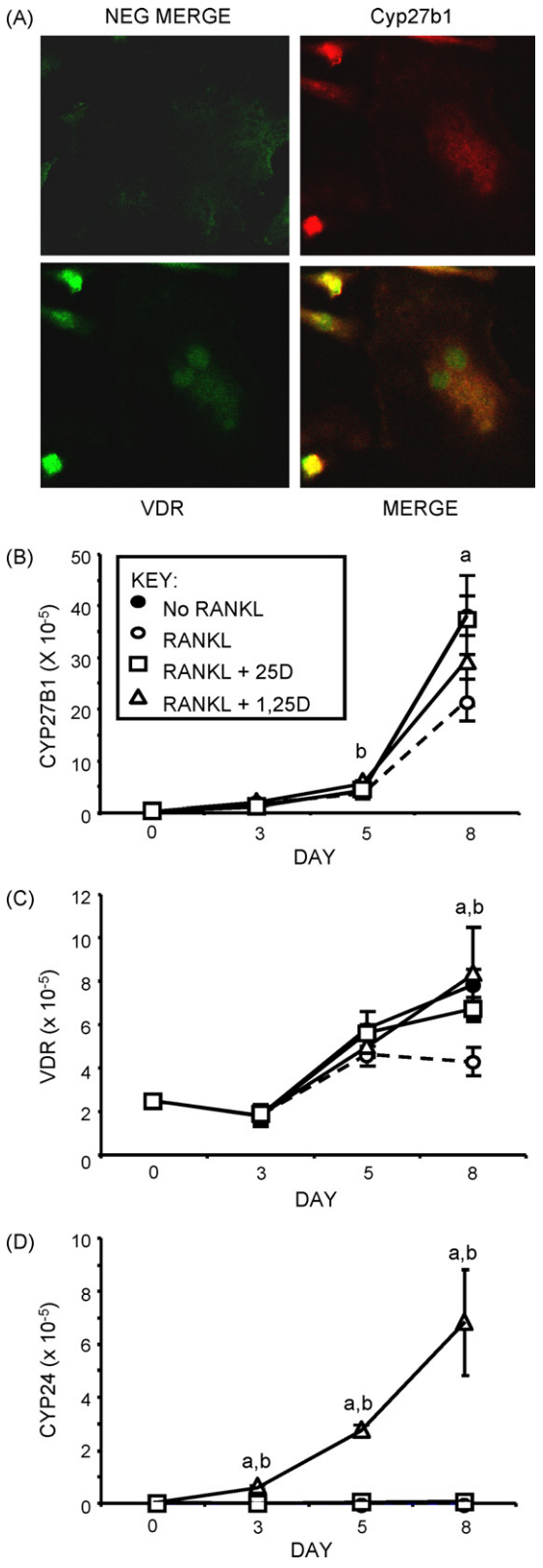

Fig. 1. Osteoclasts express the necessary components for 25D metabolism. (A) Human PBMC-derived osteoclasts were generated in response to culture with RANKL (50 ng/ml) and M-CSF (25 ng/ml) for 6 days. Cells were processed for immunofluorescence staining using anti-cyp27b1 antibody, anti-vdr antibody, or control IgGs, as described in Section 2. PBMC were cultured in α-MEM supplemented with 10% Charcoal/Dextran treated FCS containing RANKL (50 ng/ml) and M-CSF (25 ng/ml), in the absence or presence of 1,25D (1 nM) or 25D (50 nM). Total RNA was extracted at specified days and real-time RT-PCR was performed for the genes (B) CYP27B1, (C) VDR and (D) CYP24. Gene expression was normalised to that of 18S rRNA. Data shown are means ± SD of triplicate wells. Statistical difference in expression to control (RANKL) is indicated; a and b denote significant difference ($p < 0.05$) for 25D and 1,25D treatments, respectively.

with or without 25D or 1,25D (Wako, Osaka, Japan) and recombinant human RANKL (100 ng/ml, Chemicon, Temecula, CA), as indicated. Osteoclasts were derived from human PBMC essentially as described previously [13], with some modifications. PBMC were seeded into wells of a 96-well plate at 2.5×10^5 cells/well in α-MEM containing 10% charcoal stripped FCS (Hyclone Laboratories, South Logan, UT, USA), human recombinant M-CSF (25 ng/ml; Chemicon) and dexamethasone (10^{-8} M) and cultured overnight. After this time, media were replaced with either fresh medium, or with media containing combinations of 25D, 1,25D and human recombinant RANKL (50 ng/ml). Replicate cultures were stained for TRAP, as described previously [13]. Osteoclasts were defined as $TRAP^+$ cells containing 3 or more nuclei.

2.2. Preparation of RNA and real-time RT-PCR

Total RNA was prepared and gene expression analysed by real-time RT-PCR, as we have described previously [9]. Relative gene expression between samples was calculated using the comparative cycle threshold (C_T) method using 18S rRNA as a housekeeping gene. Oligonucleotide primers were designed in-house to flank intron-exon boundaries, and were purchased from Geneworks (Thebarton, SA, Australia). Sequences of oligonucleotide primers for the amplification of human CYP27B1, VDR and CYP24 are published elsewhere [9].

2.3. Immunofluorescence staining

To confirm that mature osteoclasts express VDR and CYP27B1, cell suspensions of human PBMC were seeded into wells of a chamber slide (Nalge Nunc, Naperville, IL, USA) and cultured for 6 days. Medium was aspirated and the cells were processed for immunostaining [14]. Primary antibodies to cyp27b1 (Binding Site, Birmingham, UK), VDR (Affinity Bioreagents, Rockford, IL, USA) or negative control IgGs were detected using Alexa Fluor 647 donkey anti-sheep and goat anti-rat IgG antibodies (Molecular Probes, Eugene, OR, USA), respectively. Slides were mounted in Prolong Gold-DAPI reagent (Molecular Probes), and examined by confocal microscopy (Radiance 2100; Bio-Rad, Hercules, CA, USA).

2.4. Statistical analysis

Statistical differences between parametric data sets were assessed using Student's *T*-test, or between multiple treatments by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A value for $p < 0.05$ was considered significant.

3. Results

3.1. CYP27B1 and VDR expression in osteoclastic cells

Cytoplasmic staining for CYP27B1 was observed in both mononuclear cells and multinucleated osteoclasts derived from PBMC and intranuclear staining was observed for VDR (Fig. 1A). These observations suggest that local production of 1,25D from 25D in osteoclasts has a potential effect on osteoclastogenesis in an autocrine or paracrine fashion.

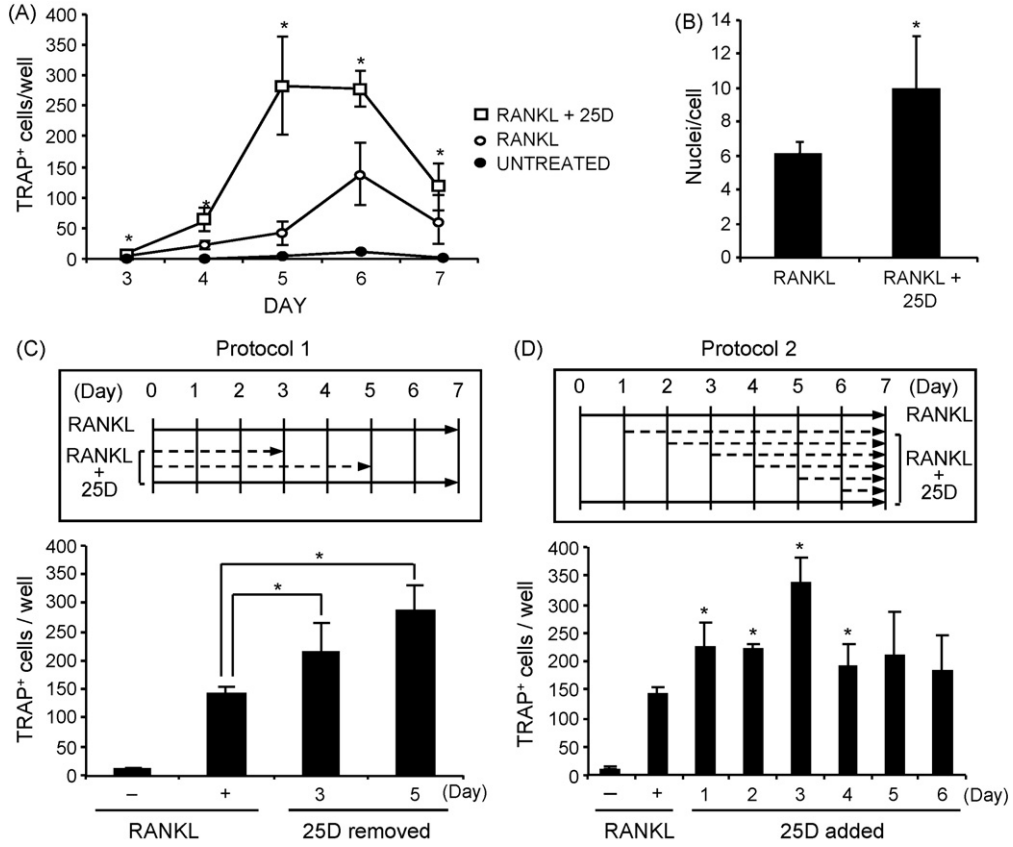

Fig. 2. Effect of 25D on RAW 264.7 osteoclast differentiation. (A) Cells were cultured in SDM containing RANKL (100 ng/ml) in the presence or absence of 25D (100 nM). Cells were stained for TRAP and the number of (A) TRAP-positive cells and (B) nuclei per multinucleated cell counted. The numbers of TRAP-positive cells were counted after 7 days of culture with RANKL (100 ng/ml) when 25D (100 nM) was either (C) added at day 0 and then removed at 3 or 5 days of culture (Protocol 1), or (D) added only after the times indicated (Protocol 2). Data shown are means ± SD of quadruplicate wells; significant difference to RANKL control indicated by * ($p < 0.05$).

3.2. Effect of 25D and 1,25D on expression of vitamin D metabolic pathway related genes

To further examine the relationship between 25D metabolism and osteoclastogenesis, we analysed CYP27B1 mRNA expression during human osteoclast formation. RANKL/M-CSF treatment of PBMC resulted in an increase in CYP27B1 mRNA expression, with a maximum on day 9 (Fig. 1B). In control PBMC (cultured in M-CSF only), CYP27B1 expression also increased, suggesting that CYP27B1 also plays a role during macrophage maturation. VDR mRNA expression also increased with time during the cultures, with only mild vitamin D-dependent effects (Fig. 1C). Whereas CYP24 mRNA expression was stimulated markedly by 1,25D (Fig. 1D), 25D elicited a much lesser CYP24 response (3.6-fold vs 358-fold at day 8, respectively), consistent with our findings in osteoblasts [9]. Similar results were obtained for RAW 264.7 cells (data not shown). Taken together, these results suggest that endogenous vitamin D metabolism has the potential to regulate osteoclast differentiation in a manner distinct from 1,25D.

3.3. Effect of 25D on osteoclastogenesis

We reported previously [7] that 1,25D has a positive effect on the formation of RANKL-induced osteoclast-like cell formation from RAW 264.7 cells. Treatment of cultures with 25D resulted in a striking increase in the number of TRAP-positive, multinucleated cells formed, to a greater extent than 1,25D (Fig. 2A) and also increased the number of nuclei per cell (Fig. 2B). To examine the stage, at which 25D metabolism influences osteoclastogenesis, we varied the exposure of cells to 25D. As shown in Fig. 2C, the numbers of TRAP-positive cells increased when 25D was present until day 5 of RANKL treatment. When 25D was added at specific times after the addition of RANKL, the number of TRAP-positive cells increased maximally when 25D was added at day 3 (Fig. 2D). Taken together, these results indicate that the presence of vitamin D between day 3 and day 5 is optimal for osteoclast differentiation from RAW 264.7 precursors, and is consistent with the notion that vitamin D in the form of 25D is important for 'optimising' osteoclastogenesis.

4. Discussion

In this study, we examined the effect of vitamin D metabolism on osteoclastogenesis, modeling the potential response of cells of the osteoclast lineage to the level of circulating 25D. We confirmed that human osteoclasts, derived from human PBMC, possess the molecular machinery to both metabolise and respond to 25D, since they express cytoplasmic CYP27B1 and nuclear VDR proteins. The latter observation is consistent with cells of the monocyte/macrophage lineage being a well-characterized site of extra-renal synthesis of 1,25D [10,11]. We also used the RAW 264.7 cell model to confirm that, in our hands, these cells could synthesise detectable 1,25D from exogenous 25D (data not shown), consistent with a previous report [12]. The finding that CYP27B1 mRNA expression increased in response to M-CSF/RANKL-induced differentiation of PBMC, suggested that 25D metabolism could play a role during osteoclast differentiation. To evaluate the effects of 25D metabolism, we used a defined, serum- and therefore vitamin D metabolite-free medium, SDM, for RAW 264.7 cell osteoclasts [7]. For human cells, we used charcoal-stripped serum to minimise the potential

metabolite content, since human osteoclast precursors do not differentiate in SDM in the absence of osteoblasts [15]. Extending our previous report that 1,25D stimulates RANKL-induced osteoclast formation from RAW 264.7 cells in SDM [7], physiological levels of 25D significantly increased TRAP-positive cell formation in response to RANKL. Furthermore, in RAW 264.7 cells, the number of nuclei and therefore size of the osteoclasts also increased in the presence of 25D. Of potential importance, the pharmacological doses of 1,25D used in this study strongly induced the expression of CYP24, the enzyme for inactivating 1,25D, whereas 25D, which was added at physiological concentrations, did so only after extended exposure and to a much lesser extent, consistent with slow intracellular accumulation of *de novo* 1,25D. Taken together, we suggest that the metabolism of 25D into 1,25D permits the cells to respond in a manner without down-regulation of intracellular levels of 1,25D by CYP24, resulting in enhanced differentiation into multinucleated osteoclasts. The biological significance of this gradual increase in the level of 1,25D in the cells remains to be addressed; however, it appears likely that low level intracellular exposure is an important regulatory pathway for osteoclast development.

We further investigated the effect, by which 25D modulates osteoclast differentiation in the RAW 264.7 model. Interestingly, the effect of physiologic 'replete' levels of 25D influenced most significantly the middle phase of RANKL-induced osteoclastogenesis, corresponding to the post-proliferation/fusion phase of osteoclast generation. This implies that serum 25D levels maybe important for optimizing osteoclast formation from a given precursor pool.

In summary, we have found evidence that 25D metabolism by osteoclasts maybe an autocrine mechanism for optimizing osteoclastogenesis, suggesting that the circulating level of 25D maybe a determinant of osteoclast differentiation. Together with our previous findings that osteoblasts also metabolise 25D [1], this implies that changes in 25D levels may have direct effects on bone cells and suggests the existence of paracrine networks of vitamin D metabolism in the bone microenvironment.

Acknowledgements

MK was supported by an Australian Endeavour (Post-Doctoral) Fellowship. GJA is a National Health and Medical Research Council of Australia (NHMRC) R. Douglas Wright Fellow. The authors thank Ms. S. Hay for her excellent technical help.

References

[1] P.H. Anderson, G.J. Atkins, The skeleton as an intracrine organ for vitamin D metabolism, Mol. Aspects Med. 29 (6) (2008) 397–406.
[2] G. Andersson, E.K. Johansson, Adhesion of human myelomonocytic (HL-60) cells induced by 1,25-dihydroxyvitamin D_3 and phorbol myristate acetate is dependent on osteopontin synthesis and the alpha v beta 3 integrin, Connect. Tissue Res. 35 (1–4) (1996) 163–171.
[3] H. Mimura, X. Cao, F.P. Ross, M. Chiba, S.L. Teitelbaum, 1,25-Dihydroxyvitamin D_3 transcriptionally activates the beta 3-integrin subunit gene in avian osteoclast precursors, Endocrinology 134 (3) (1994) 1061–1066.
[4] M.M. Medhora, S. Teitelbaum, J. Chappel, J. Alvarez, H. Mimura, F.P. Ross, K. Hruska, 1 Alpha, 25-dihydroxyvitamin D_3 up-regulates expression of the osteoclast integrin alpha v beta 3, J. Biol. Chem. 268 (2) (1993) 1456–1461.
[5] X. Cao, F.P. Ross, L. Zhang, P.N. MacDonald, J. Chappel, S.L. Teitelbaum, Cloning of the promoter for the avian integrin beta 3 subunit gene and its regulation by 1,25-dihydroxyvitamin D_3, J. Biol. Chem. 268 (36) (1993) 27371–27380.
[6] S. Kido, D. Inoue, K. Hiura, W. Javier, Y. Ito, T. Matsumoto, Expression of RANK is dependent upon differentiation into the macrophage/osteoclast lineage: induction by 1alpha,25-dihydroxyvitamin D_3 and TPA in a human myelomonocytic cell line, HL60, Bone 32 (6) (2003) 621–629.
[7] C. Vincent, M. Kogawa, D.M. Findlay, G.J. Atkins, The generation of osteoclasts from RAW 264.7 precursors in defined, serum-free conditions, J. Bone Miner. Metab. 27 (1) (2009) 114–119.
[8] P.H. Anderson, G.J. Atkins, D.M. Findlay, P.D. Oloughlin, K. Welldon, C. Vincent, H.A. Morris, RNAi-mediated silencing of CYP27B1 abolishes $1,25(OH)_2D_3$ synthesis and reduces osteocalcin and CYP24 mRNA expression in human osteosarcoma (HOS) cells, J. Steroid Biochem. Mol. Biol. 103 (3–5) (2007) 601–605.
[9] G.J. Atkins, P.H. Anderson, D.M. Findlay, K.J. Welldon, C. Vincent, A.C. Zannettino, D. O'Loughlin, P.H.A. Morris, Metabolism of vitamin D(3) in human osteoblasts: evidence for autocrine and paracrine activities of 1alpha, 25-dihydroxyvitamin D(3), Bone 40 (6) (2007) 1517–1528.
[10] J.S. Adams, T.G. Beeker, T. Hongo, T.L. Clemens, Constitutive expression of a vitamin D 1-hydroxylase in a myelomonocytic cell line: a model for studying 1,25-dihydroxyvitamin D production in vitro, J. Bone Miner. Res. 5 (12) (1990) 1265–1269.
[11] A.S. Dusso, J. Finch, A. Brown, C. Ritter, J. Delmez, G. Schreiner, E. Slatopolsky, Extrarenal production of calcitriol in normal and uremic humans, J. Clin. Endocrinol. Metab. 72 (1) (1991) 157–164.
[12] M. van Driel, M. Koedam, C.J. Buurman, M. Hewison, H. Chiba, A.G. Uitterlinden, H.A. Pols, J.P. van Leeuwen, Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells, FASEB J. 20 (13) (2006) 2417–2419.
[13] G.J. Atkins, P. Kostakis, C. Vincent, A.N. Farrugia, J.P. Houchins, D.M. Findlay, A. Evdokiou, A.C. Zannettino, RANK expression as a cell surface marker of human osteoclast precursors in peripheral blood, bone marrow, and giant cell tumors of bone, J. Bone Miner. Res. 21 (9) (2006) 1339–1349.
[14] C. Vincent, D.M. Findlay, K.J. Welldon, A.R. Wijenayaka, T.S. Zheng, D.R. Haynes, N.L. Fazzalari, A. Evdokiou, G.J. Atkins, Pro-inflammatory cytokines TNF-related weak inducer of apoptosis (TWEAK) and TNFalpha induce the mitogen-activated protein kinase (MAPK)-dependent expression of sclerostin in human osteoblasts, J. Bone Miner. Res. 24 (8) (2009) 1434–1449.
[15] G.J. Atkins, P. Kostakis, K.J. Welldon, C. Vincent, D.M. Findlay, A.C. Zannettino, Human trabecular bone-derived osteoblasts support human osteoclast formation in vitro in a defined, serum-free medium, J. Cell Physiol. 203 (3) (2005) 573–582.