0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 10
Printed in U.S.A.

A Combination of Osteoclast Differentiation Factor and Macrophage-Colony Stimulating Factor is Sufficient for both Human and Mouse Osteoclast Formation In Vitro*

Julian M.W. Quinn, Jan Elliott, Matthew T. Gillespie, T. John Martin

The University of Melbourne, Department of Medicine and St Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Australia.

ABSTRACT: Both human and murine osteoclasts can be derived in vitro from hematopoietic cells or monocytes that are co-cultured with osteoblasts or marrow-derived stromal cells. The osteoclastogenic stimulus provided by murine osteoblasts and marrow-derived stromal cells is now known to be mediated by osteoclast differentiation factor (ODF), a membrane-bound tumor necrosis factor-related ligand. This study demonstrates that mouse spleen cells and monocytes form osteoclasts when cultured in the presence of macrophage-colony stimulating factor (M-CSF) and a soluble form of murine ODF (sODF). Numerous multinucleated osteoclasts expressing tartrate resistant acid phosphatase (TRAP) and calcitonin receptor (CTR) formed within 7 days of culture and engaged in extensive lacunar bone resorption. Osteoclast number and bone resorption area was dependent on sODF concentration. Long-term cultured human monocytes also formed bone resorbing osteoclasts in response to co-stimulation by sODF and M-CSF, although this required more than 11 days in culture. This human osteoclast differentiation was strongly inhibited by granulocyte-macrophage colony stimulating factor. This study further characterises murine osteoclast differentiation caused by sODF and M-CSF co-stimulation in vitro, and shows that the same co-stimulation causes human osteoclast differentiation to occur. We propose that this methodology can be employed to investigate the direct effects of cytokines and other factors on human osteoclast differentiation.

Osteoclasts are multinucleated cells responsible for bone resorption. They form by fusion of precursors that are derived from the pluripotential hematopoietic stem cell [1] and circulate in the monocyte fraction [2,3]. Both human and mouse hematopoietic cells (or monocytes) form osteoclasts when co-cultured with osteoblastic or bone marrow derived stromal cells in the presence of an appropriate hormonal or cytokine stimulus. Macrophage colony stimulating factor (M-CSF) is essential for this process but not sufficient [3,4]. Recently, a second essential stimulatory factor provided by murine stromal cells has been identified and named ODF [5]. Here, we demonstrate that a combination of M-CSF and a soluble form of ODF stimulates human monocytes and mouse spleen cells to form osteoclasts in vitro, and show that these culture systems can be employed as a useful model for studying osteoclast differentiation.

Materials and Methods

Preparation and culture of mouse monocytes and spleen cells: Spleens from freshly killed 3 month old mice (strain c57BL/6J, Monash University, Clayton, Australia) were processed as previously described [6] to produce a suspension of spleen cells in alpha minimal essential medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum (CSL Biosciences, Parkville, Australia) (MEM/FCS). 10^6 spleen cells were added to 10mm diameter cell culture wells (Becton Dickinson, NJ). These wells contained either 6mm diameter glass coverslips (Lomb Scientific, Taren Point, Australia) or $10mm^2$ slices of bovine cortical bone [7]. To prepare monocyte cultures, peripheral blood mononuclear cells (PBMCs) were first prepared from diluted mouse blood (1:4 in Hanks Balanced Salt Solution (HBSS; Life Technologies, Grand Island, NY) which was layered over Ficoll-Paque® solution (Pharmacia Biotech, Uppsala, Sweden), centrifuged (693g), then washed and resuspended in MEM/FCS. 10^6 PBMCs were added to 6mm wells containing bone slices or coverslips and allowed to settle in MEM/FCS for 1 hour; coverslips and bone slices were then vigorously rinsed to remove non-adherent cells and placed in 10mm diameter culture wells. Spleen cells and monocytes were maintained (at 37°C, in a 5% CO_2 gassed incubator) in 10mm diameter culture wells in 0.4ml MEM/FCS for up to 11 days, both in the presence and absence of recombinant human M-CSF (25ng/ml) (a kind gift of Genetics Institute, Cambridge, MA, USA) and 0, 1, 3, 10 or 30 ng/ml recombinant murine sODF (a kind gift of Snow Brand Milk Products Co. Ltd, Tochigi, Japan) [5]. In all cultures, medium and added factors were entirely replaced every three days. After 0, 3, 7 and 11 days, bone slices and coverslips were removed for analysis as described below.

Preparation and culture of human monocytes: Monocytes were isolated from the peripheral blood of normal healthy subjects. Human PBMCs were prepared from diluted blood (1:1 in HBSS) by discontinuous gradient centrifugation as above, and then washed by centrifugation and resuspended in MEM/FCS. Monocyte cultures were prepared by adding 10^6 PBMCs to 6mm diameter culture wells containing bovine cortical bone slices or glass coverslips in MEM/FCS; after 1 hour, coverslips and bone slices were removed, vigorously rinsed to remove non-adherent cells, and placed in 10mm diameter culture wells. Monocyte cultures were maintained in these culture wells in 0.4ml MEM/FCS for up to 18 days, either in the presence or absence of recombinant human M-

*This work was supported by a program grant of the NHMRC of Australia and a project grant from Aza Research Pty Ltd, Australia. J. Quinn is a C.R. Roper Fellow.
Correspondence to: J. Quinn, The University of Melbourne, Dept. of Medicine, 41 Victoria Pde, Fitzroy, Australia 3065.
e-mail j.quinn@medicine. unimelb.edu.au

Received 05/01/98

CSF (25ng/ml) and 0, 1, 3, 10 or 30 ng/ml recombinant murine sODF. In some experiments, sODF was withheld for the first 7 days. Where indicated, 100ng/ml human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN) was also added. In all cultures, medium and added factors were entirely replaced every 3 days.

Determination of cell phenotypes: Following incubation for the appropriate period, cultures on coverslips were fixed and stained histochemically for the osteoclast-associated enzyme TRAP [6]. Using an indirect immunoperoxidase technique [8] human monocyte cultures were also immunostained for the osteoclast-associated vitronectin receptor (VNR), and the macrophage-associated antigens CD11b, CD14, and CD68 using monoclonal antibodies 23C6 (a kind gift of Dr M. Horton), ICRF44 (Serotec, Kidlington, UK), TUK4 and EBM11 (both Dako Pty Ltd, Botany, Australia), respectively [8]. Numbers of mononuclear and multinucleated cells (>2 nuclei) were determined. The presence of CTR, a specific marker for osteoclasts, was investigated by autoradiography using ^{125}I-labelled salmon calcitonin ligand binding [9]; in all experiments, the presence of 100ng/ml unlabelled calcitonin abolished all autoradiograph signal.

Analysis of bone resorption: Bone resorption on cortical bone slices was assessed by scanning electron microscopy (SEM) as previously described [7], on a Jeol JSM.5300. Data was obtained from five or more experiments, and is expressed as mean ± standard error.

Results

Mouse Monocyte and Spleen Cell Cultures: Spleen cells and monocytes were initially negative for TRAP. When spleen cells were cultured for up to 11 days either in the presence or absence of M-CSF (25ng/ml), cells expressing TRAP and/or CTR were not found and no bone resorption was evident. Spleen cells incubated for three days in the presence of 30ng/ml sODF and 25ng/ml M-CSF resulted in small numbers of CTR and TRAP positive mononuclear cells, but no bone resorption was found. After 7 and 11 days of incubation in the presence of sODF and M-CSF, many CTR/TRAP positive mono- and multinucleated cells were observed, as well as extensive bone resorption (Fig 1). The amount of resorption and number of CTR positive cells increased dose-dependently with sODF concentration (Fig 2). In cultures maintained for 7 and 11 days, numerous TRAP positive/CTR negative mononuclear cells were noted, but <1% of multinucleated cells were negative for CTR. Spleen cells stimulated by sODF alone formed a few CTR positive cells but did not resorb bone; however, the number of cells surviving to 11 days was often less than 10% of those in cultures in which M-CSF was present.

Mouse monocytes (approximately $3x10^4$ per coverslip after removal of non-adherent cells) treated for 11 days by M-CSF and sODF also formed CTR/TRAP positive multi-nucleated osteoclasts (25.3±8.0 per coverslip, 0.56% of cells present at 11 days), which resorbed up to 100% of the bone slice surface.

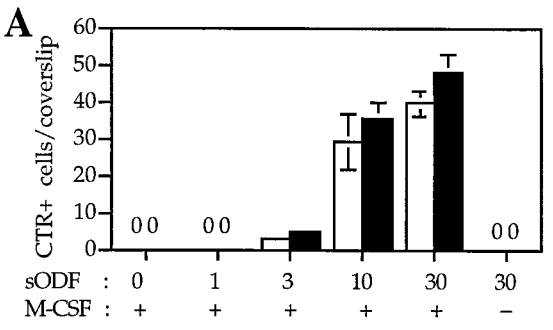
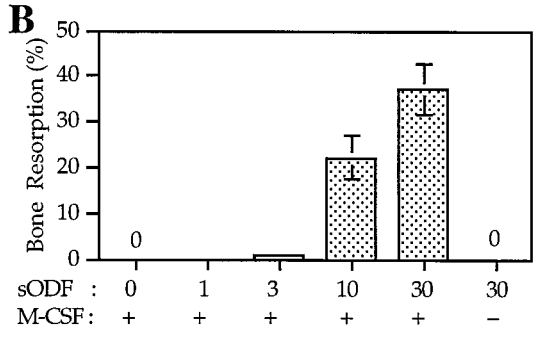


Figure 1. Osteoclastic differentiation of mouse spleen cells, after 11 days incubation, in response to increasing concentrations of sODF (shown in ng/ml), and the presence (+) or absence (–) of M-CSF (25ng/ml). **A**) CTR positive cell formation. Black columns indicates mononuclear cells, white columns indicate multinucleated cells. '0' indicates no CTR cells found (mean±standard error). **B**) proportion of bone slice surface area resorbed (mean±standard error). '0' indicates no resorption.



Human Monocyte Cultures: 6mm diameter coverslips initially contained approximately $5x10^4$ adherent human monocytes of which >95% expressed CD68, CD11b and CD14 macrophage-associated antigens. They were also entirely negative for TRAP, VNR and CTR. After human monocytes were cultured for 14 days in the presence of M-CSF alone, numerous mono- and multinucleated cells expressing TRAP were present on coverslips; indeed, frequently all cells were positive for TRAP in the presence of M-CSF alone. Numerous mono- and multinucleated cells expressing VNR and CD14 were found and all cells expressed CD11b (see Table 1) and CD68. However, no CTR positive cells or resorption pits were found after 14 days of culture. When 30ng/ml sODF was present in addition to M-CSF for 14 days, multinucleated cells positivefor CTR (6.22±0.76 per coverslip)(Fig. 3A), as well as VNR and CD14 were noted; many CD11b negative cells were also found (see Table 1). Extensive bone resorption was found after 18 days incubation with sODF and M-CSF (Fig. 3B). This was a dose-dependent response (Fig. 4). Much larger resorption pits were observed than in mouse spleen or monocyte cultures. Osteoclast differentiation from human monocytes occurred relatively late compared to mouse spleen cultures, with no CTR positive cells in cultures maintained for 11 days; one

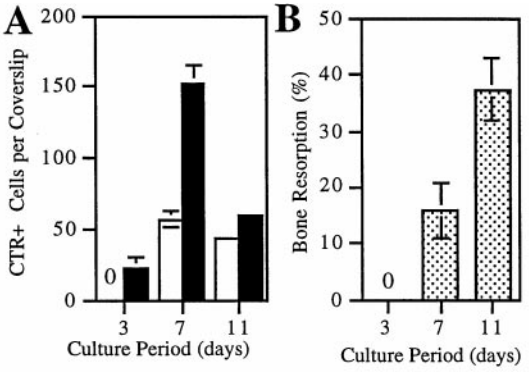


Figure 2. Osteoclastic differentiation of mouse spleen cells at 3, 7 and 10 days incubation in the presence of sODF (30 ng/ml) and M-CSF (25ng/ml). **A**) CTR positive cell formation (mean±standard error). Black columns indicates mononuclear cells, white columns indicate multinucleated cell numbers. '0' indicates no positive cells. **B**) proportion of bone slice surface area resorbed (mean±standard error). '0' indicates no resorption

CTR positive cells in cultures maintained for 11 days; one bone slice out of 15 showed evidence of resorption (11 pits,<0.1% of surface area) at 11 days, and none at 7 or 3 days. When sODF was omitted for the first 7 days, bone resorption at 18 days was variable and much reduced (3.7%±3.4 of bone surface area). No resorption or CTR positive cells were found in sODF-stimulated cultures in the absence of M-CSF.

Human monocytes treated with a combination of sODF (30ng/ml), M-CSF (25ng/ml) and GM-CSF (100ng/ml) formed no CTR positive cells (after 14 days) and formed very few resorption pits (<10 per bone slice) on 2 out of 15 bone slices (<0.1% of bone surface) after 18 days (Fig. 4).

Discussion

Mouse spleen cells and monocytes form osteoclasts when stimulated by long term co-culture with osteoblastic or pre-adipocytic cell lines in the presence of 1,25 dihydroxy vitamin D_3 [1,2]. It is now known that M-CSF and a stromal cell membrane-associated tumor necrosis factor-related molecule named ODF, TRANCE or RANKL [5,10,11] is required for osteoclast formation. A soluble form of ODF has been shown to promote osteoclast formation in spleen cells treated also with M-CSF [5]. This is confirmed in the present work, in which we also show that ODF treatment of mouse spleen cells (in the presence of M-CSF) results in a dose-dependent formation of TRAP and CTR positive cells and of bone resorption; the number of osteoclasts formed is similar to that formed in spleen cell/ osteoblast co-cultures [6]. We have also shown that osteoclasts form from mouse monocytes under these conditions.

Human monocytes also formed bone resorbing osteoclasts in long-term culture when stimulated with sODF and M-CSF. This is consistent with our previous findings that human

	M-CSF Alone		M-CSF + sODF	
	Mono-nuclear	MNCs	Mono-nuclear	MNCs
CD11b	100%	100%	79.1%±4.3	91.3%±1.0
CD14	28.2%±3.3	37.5%±7.8	33.2%±9.6	17.6%±7.1
VNR	26.7%±7.1	7.0%±1.1	59.9%±4.9	15.1%±3.4

Table 1. Immunohistochemical staining for CD11b, CD14 and VNR: proportion of mononuclear cells and multinucleated cells (MNCs) positive for these antigens at 14 days incubation. Coverslips incubated with M-CSF alone contained 279±63 MNCs and 15,560±1,494 mononucleated cells. Coverslips incubated with M-CSF and sODF contained 248±49 MNCs and 14,184±1,012 mononucleated cells. Results are from 4 experiments, 2 coverslips per experiment.

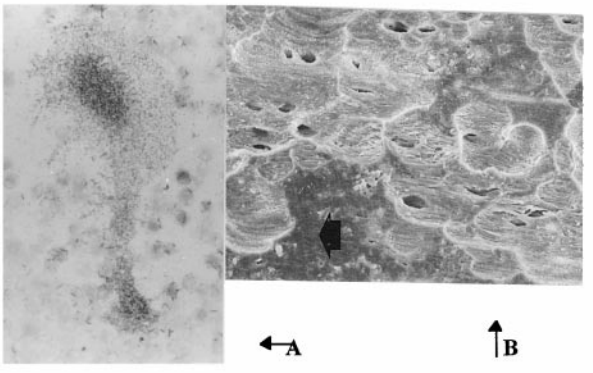


Figure 3. Cultured human monocytes stimulated by 30ng/ml sODF and 25ng/ml M-CSF. **A**) CTR positive multinucleated cell formed after 14 days incubation on a glass coverslip. Magnification: x213. **B**) Resorption pits on bovine cortical bone slice after 18 days. Arrow indicates unresorbed bone. Magnification: x200.

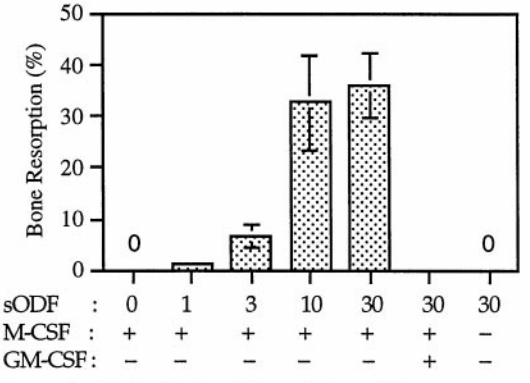


Figure 4. Osteoclastic differentiation of human monocytes: after 18 days incubation, in response to increasing concentrations of sODF (shown in ng/ml), the presence (+) or absence (–) of M-CSF (25ng/ml), and presence and absence of GM-CSF (100ng/ml): proportion of bone slice surface area resorbed (mean±standard error). '0' indicates no resorption.

monocytes form osteoclasts in long-term co-culture with 1,25 dihydroxyvitamin D_3-stimulated rodent osteoblastic cells when human M-CSF is added [3]. Relevant to this is the data on expression of CD11b, the alpha chain of the integrin $\alpha_m\beta_2$, which, like CD14, is expressed by human macrophages but not osteoclasts [8]. Monocytes treated with M-CSF alone resulted in only CD11b positive cells, but co-treatment with sODF resulted in numerous CD11b negative cells, consistent with the presence of osteoclasts. CD11b and CD14 immunostaining also indicates that substantial numbers of monocyte-derived macrophages persist in these cultures. When human monocytes were cultured for 14 days with M-CSF but without sODF, CTR expression and bone resorption were absent, but multinucleated cells expressing VNR and TRAP were found. Human monocytes respond to culture conditions by increased expression of TRAP; in addition, long term cultured monocytes have been shown to express VNR, which plays a role in the recognition and phagocytosis of apoptotic bodies [12,13]. It seems likely that many TRAP- and VNR- positive cells in these cultures are monocyte-derived macrophages, not osteoclasts. Therefore, in human monocyte cultured under these conditions, TRAP and VNR must be regarded as unreliable markers for mature or terminally differentiated osteoclasts.

It was notable that human in vitro osteoclastogenesis required longer stimulation by sODF than mouse osteoclastogenesis, and yielded fewer CTR positive cells. The reason for this difference is unclear; it may be due to the murine sODF employed, or perhaps to lower proliferation rates in cultured human osteoclast progenitors compared to cultured mouse progenitors. Also, fewer human osteoclasts were formed (6.22±0.76/coverslip) than in human monocyte/stromal cell co-cultures (39.3±4.6/coverslip, unpublished data; method of Fujikawa et al [3]). Further work is required to investigate differences in osteoclast yield in these culture systems.

We looked for a possible effect of GM-CSF on sODF/M-CSF induced human osteoclast formation because we have previously obtained evidence in studies of murine osteoclast formation that GM-CSF mediates the inhibitory effects of stromal-cell derived interleukin-18 on osteoclastogenesis [6]. GM-CSF appeared to do this through a direct action on the osteoclast progenitors, and the inhibitory effect of GM-CSF on human osteoclast formation seen in these experiments is consistent with such a direct action. We therefore suggest that the culture method described here, together with co-culture methodologies we have previously described [3] could be used to determine whether particular factors that affect bone resorption act directly on osteoclast progenitors or act through an effect on stromal cell elements. In this way, greater understanding could be obtained of the complex cellular and hormonal interactions that control osteoclast generation and bone resorption in health and disease.

Acknowledgements: The authors thank Wayne Holloway, Fiona Collier, Daphne Hards and Patricia Smith of the Department of Medicine, The University of Melbourne, for technical assistance, and Snow Brand Milk Co. Ltd for supply of recombinant sODF.

References

1. Suda T, Takahashi N, Martin TJ 1992 Modulation of osteoclast differentiation. Endocrine Rev **13**: 66-68
2. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Martin TJ, Suda T 1990 Origin of the osteoclast: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow derived cells. Proc Natl Acad Sci USA **87**: 7260-7264
3. Fujikawa Y, Quinn JMW, Sabokbar A, McGee JO'D, Athanasou NA 1996 The human osteoclast precursor circulates in the monocyte fraction. Endocrinology **137:** 4058-4060
4. Tanaka S, Takahashi N, Udagawa N, Tamura T, Akatsu T, Stanley ER, Kurokawa T, Suda T 1993 Macrophage colony stimulating factor is indispensible for both proliferation and differentiation of osteoclast progenitors. J Clin Invest **91**: 257-263
5. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/ osteoclast inhibitory factor and is identical to TRANCE/ RANKL. Proc Natl Acad Sci USA **95**: 3597-3602
6. Horwood N, Udagawa N, Elliott J, Grail D, Okamura H, Kurimoto M, Dunn AR, Martin TJ, Gillespie MT 1998 Interleukin 18 inhibits osteoclast formation via T-cell production of granulocyte-macrophage colony stimulating factor. J Clin Invest **101**: 595-603
7. Chambers TJ, Revell PA, Fuller K, Athanasou NA 1984 Resorption of bone by isolated rabbit osteoclasts. J Cell Sci **66**: 383-399
8. Athanasou NA, Quinn JM 1990 Immunophenotypic differences between osteoclasts and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. J Clin Pathol **43**: 997-1004
9. Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FAO, Martin TJ 1986 Abundant calcitonin receptors in isolated rat osteoclasts. J Clin Invest **78**: 355-365
10. Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Kalachnikov S, Bartlett F, Frankel W, Lee S, Choi Y 1997 Trance is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T-cells. J Biol Chem **272**: 25190-25194
11. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, Dubose RF, Cosman D, Galibert L 1997 A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic cell function. Nature **390**: 175-179
12. Bevilacqua MA, Lord DK, Cross NC, Whitaker KB, Moss DW, Cox TM 1991 Regulation and expression of type V (tartrate-resistant) acid phosphatase in human mononuclear phagocytes. Mol Biol Med **8**: 135-140
13. Stern M, Savill J, Haslett C 1996 Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by alpha v beta 3/CD36/thrombospondin recognition mechanism and lack of phlogistic response. Am J Pathol **149**: 911-921