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Biochemical and Biophysical Research Communications 350 (2006) 478–483
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Hepatocyte growth factor can substitute for M-CSF to support osteoclastogenesis

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Received 14 September 2006
Available online 25 September 2006

Abstract

Osteopetrotic mice lacking functional macrophage-colony stimulating factor (M-CSF) recover with ageing, suggesting that alternative osteoclastogenesis pathways exist. Hepatocyte growth factor (HGF) and M-CSF signal through tyrosine kinase receptors and phosphorylate common transducers and effectors such as Src, Grb2, and PI3-Kinase. HGF is known to play a role in osteoclast formation, and in this study we have determined whether HGF could replace M-CSF to support human osteoclastogenesis. We found that the HGF receptor, c-Met, is expressed by the $CD14^+$ monocyte fraction of human peripheral blood mononuclear cells (PBMC). HGF was able to support monocyte-osteoclast differentiation in the presence of receptor activator for nuclear factor κB ligand as evidenced by the formation of numerous multinucleated tartrate-resistant acid phosphatase and vitronectin receptor positive cells which formed F-actin rings and were capable of lacunar resorption. The addition of a neutralising antibody to M-CSF did not inhibit osteoclast differentiation. HGF is a well-established survival factor and viability assays and live/dead staining showed that it promoted the survival and proliferation of monocytes and osteoclasts in a manner similar to M-CSF. Our findings indicate that HGF can substitute for M-CSF to support human osteoclast formation.
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Keywords: Hepatocyte growth factor; Osteoclasts; Rheumatoid arthritis; Bone resorption

Mice homozygous for the recessive mutation osteopetrosis (op/op) do not produce functionally active M-CSF; these mice are severely deficient in mature macrophages and osteoclasts and exhibit impaired bone remodelling [1]. Although the administration of recombinant human M-CSF corrects this defect, op/op mice recover with age, suggesting that alternative osteoclastogenesis pathways exist [2–4].

M-CSF is known to stimulate RANK expression in early osteoclast precursors [5,6] and to act as a survival factor for osteoclast precursors and mature osteoclasts [7,8]. A number of growth factors, such as hepatocyte growth factor (HGF), Flt3 ligand, and vascular endothelial growth factor (VEGF), are known to influence osteoclast formation and function [9–11]. Flt3 ligand and VEGF have also been shown to support osteoclastogenesis in the absence of functional M-CSF [10,11]. HGF is a heparin binding glycoprotein, that initiates intracellular signalling by binding to its receptor, which is encoded by the c-Met proto-oncogene [12]. HGF regulates the growth and differentiation of many tissues and is synthesized and secreted by a number of cell types, including osteoblasts [9]. HGF secretion by osteoblasts has been shown to cooperatively stimulate the survival of haematopoietic progenitors [13]. HGF is thought to act as a coupling factor between osteoblasts and osteoclasts [9]. Osteoclast formation is stimulated by HGF in the presence of osteoblastic cells but the mechanism whereby this occurs is not known.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.076

Both HGF and M-CSF are known to signal through tyrosine kinase receptors, resulting in the phosphorylation of common transducers and effectors, such as Src, Grb2, and PI3-Kinase [14]. As both HGF and M-CSF act as survival factors and share common signalling pathways that have an osteoclastogenic function, we sought in this study to determine whether HGF can act as a substitute for M-CSF in inducing RANKL-dependent osteoclastogenesis.

Materials and methods

Reagents. All cell incubations were performed in alpha minimal essential medium (αMEM) (Invitrogen, UK), and glutamine (2 mM), benzyl penicillin (100 IU/ml) streptomycin (10 μg/ml), and 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere with 5% CO_2 at 37°C. Human soluble RANKL was kindly provided by Amgen Inc (Thousand Oaks, CA, USA). Human HGF, M-CSF, and α-M-CSF were purchased from R&D Systems (Abington, UK).

Isolation of human peripheral blood mononuclear cells (PBMCs) and magnetic cell sorting of CD14⁺ cells. The peripheral blood of four healthy male donors (age 25–35) was collected into tubes containing EDTA, diluted 1:1 in αMEM, layered over Ficoll–Hypaque (Pharmacia, UK), and centrifuged at 1800 rpm for 18 min at 4 °C. The peripheral blood mononuclear cell (PBMC) layer was removed. Cell sorting was performed using the MidiMACS separation kit (Miltenyi Biotec Ltd., UK). PBMCs were incubated with MACS CD14 MicroBeads (Miltenyi Biotec) for 20 min at 4 °C. The cells were washed in MACS running buffer (Miltenyi Biotec Ltd., UK) and passed through a MACS magnetic cell separator. The $CD14^+$ cell fraction was washed in αMEM and the cell pellet then resuspended in αMEM/FBS.

The number of PBMCs in the cell suspension was counted in a haemocytometer after lysis of red cells with a 5% v/v acetic acid solution. 5×10^5 PBMCs were added to culture wells containing 4 mm diameter dentine slices and 6 mm diameter glass coverslips. After 2 h incubation at 37 °C in 5% CO_2, non-adherent cells were removed by vigorous washing. Dentine slices and glass coverslips were then transferred to 16 mm diameter wells of a 24-well-tissue culture plate and incubated for up to 21 days in αMEM supplemented with the following factors: (1) HGF (25 ng/ml), (2) RANKL (10 ng/ml), (3) M-CSF (25 ng/ml), (4) HGF (10 ng/ml) + RANKL (30 ng/ml), (5) HGF (25 ng/ml) + RANKL (30 ng/ml), (6) HGF (60 ng/ml) + RANKL (30 ng/ml), and (7) HGF (25 ng/ml) + RANKL (30 ng/ml) + α-M-CSF (100 ng/ml). Negative controls consisted of PBMCs cultured alone with no added factors and positive controls consisted of PBMCs cultured in the presence of M-CSF (25 ng/ml) and RANKL (30 ng/ml). The culture medium and respective supplements were entirely replaced every 3 days.

Analysis of receptor expression by flow cytometry. $CD14^+$ cells were washed once in 1 ml wash buffer (PBS containing 0.5% BSA), resuspended in 100 μl wash buffer, and labelled with the c-Met antibody and its isotype control for 30 min. After washing, $CD14^+$ cells were labelled with a rabbit anti-mouse FITC-conjugated secondary antibody (Dako) for 30 min, washed twice in PBS, and resuspended in 300 μl PBS for flow cytometric analysis. The level of FITC fluorescence was measured using a FACS Calibur flow cytometer (Becton–Dickinson).

Cytochemical and functional assessment of osteoclast formation. Following incubation for 24 h and 14 days, $CD14^+$ cell cultures on glass coverslips were fixed and stained cytochemically for the osteoclast-associated enzyme TRAP, [15] and, using an indirect immunoperoxidase technique with the monoclonal antibody 23C6 (Serotec, UK), for the presence of VNR, an osteoclast-associated antigen [16]. In addition these cultures were immunohistochemically stained with the monoclonal antibody GRS1 for the presence of the macrophage-associated CD14 which is known not to be expressed by osteoclasts [17].

F-actin ring formation by osteoclasts formed on glass coverslips and dentine slices was also identified. To detect F-actin ring structure [18], cells were fixed with 4% formaldehyde for 5 min, permeabilised for 6 min in 0.5% Triton X-100 (in PBS), rinsed with PBS, incubated with TRITC-conjugated phalloidin (Sigma–Aldrich, UK) for 30 min, and observed using a fluorescence microscope (Nikon).

Functional evidence of osteoclast formation was determined by a resorption assay system using cell cultures on dentine slices. After 21 days the dentine slices were removed from the wells, rinsed in PBS, and placed in 0.25 M ammonium hydroxide and sonicated for 1 min. The slices were then washed in distilled water, stained with 0.5% (w/v) aqueous toluidine blue, and examined by light microscopy. Presence of lacunar resorption on dentine slices was also confirmed using scanning electron microscopy (SEM). Cells on dentine were fixed in 4% glutaraldehyde, dehydrated by passing through graded alcohols and then through graded (50–100%) hexamethyl-disilazane solution (Sigma–Aldrich, UK) before being air-dried. Dentine slices were then mounted onto aluminium stubs (Agar Scientific Ltd., UK), sputtered with gold, and examined using a Philips SEM 505 scanning electron microscope.

Live/dead cell viability assay. Following incubation for 3, 7, and 14 days, $CD14^+$ cell preparations on glass coverslips were stained using a LIVE/DEAD stain kit (Molecular Probes, Leiden, Netherland). The kit contained two fluorescent dyes: calcein to stain living cells green and ethidium homodimer-1 (Ethd-1) to stain damaged or dead cells red. Cells were rinsed twice using PBS and fixed in 4% formaldehyde (Sigma–Aldrich, Dorset, UK), stained using 4 μM calcein and 2 μM Ethd-1 (final concentration) in PBS for 30 min at 37 ± 1°C, and images were captured using a colour video camera (JVC 3-CCD, KY-F55B, Yokohama, Japan) at 100× magnification using the Optimas 5.1 software (Optimas Corp., Seattle, USA). For each sample, three images were captured. Images were opened in "OsteoPro" software imaging system.

AlamarBlue™. The ability of HGF to support the proliferation of $CD14^+$ cells was evaluated by the AlamarBlue™ proliferation assay at days 3, 7, and 14. After each time point, cells were cultured in 5% AlamarBlue™ in αMEM (Biosource, California, USA) for 2 h. The relative fluorescent units of cell degraded AlamarBlue™ was measured on SPECTRAmax GEMINI microplate spectrofluorimeter (Molecular devices, Berks, UK) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, with a cut-off at 570 nm.

Statistical analysis. Each experiment was carried out in triplicate. Statistical significance was determined using Student's t test and p values less than 0.05 were considered significant.

Results

Flow cytometry analysis (FACS)

Flow cytometry analysis detected the presence of c-Met in the $CD14^+$ fraction of PBMCs at levels of 6–10% (data not shown). These cells expressed the receptor strongly, suggesting that at least 6–10% of $CD14^+$ cells were able to respond to HGF signalling.

HGF supports human monocyte survival and proliferation

HGF in combination with RANKL was able to stimulate the survival and proliferation in $CD14^+$ cells as assessed by live/dead staining and proliferation assays after 3, 7, and 14 days of incubation. $CD14^+$ cells incubated with HGF and RANKL were alive after 14 day cultures on glass coverslips (Fig. 1a). A proportion of these cells had already differentiated to large multinucleated cells which stained positive for calcein indicating that these were live cells; only

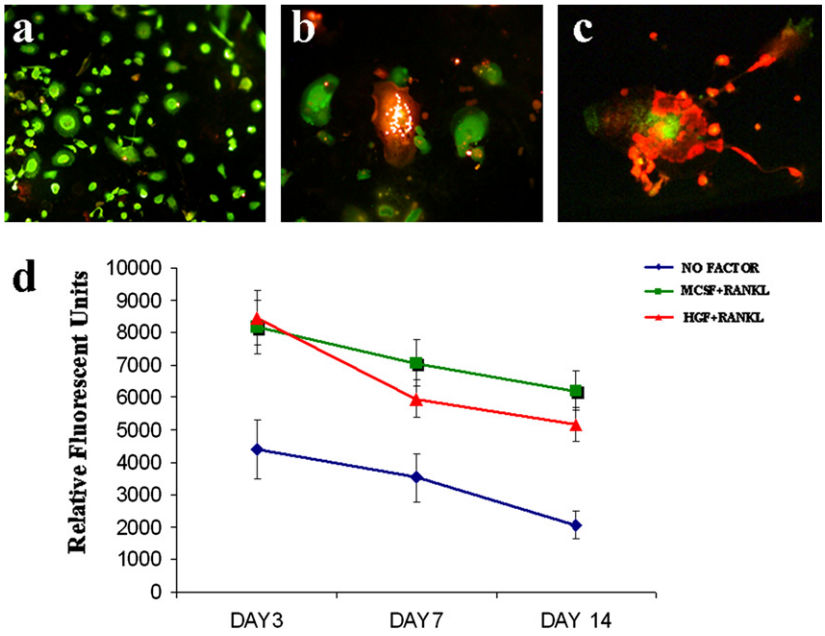

Fig. 1. HGF supports the survival and proliferation of hPBMCs. $CD14^+$ cells were cultured for 14 days with either M-CSF or HGF and RANKL and compared to control cultures where no factor had been added. Live/dead staining showing HGF and RANKL treated cells after 14 days in culture (a) and formation of live/dead multinucleated cells (b,c). Alamar blue proliferation assay (d) confirmed that HGF supports the survival and proliferation of hPBMCs similarly to M-CSF.

a small proportion of the cells were ethidium homodimer positive, indicating that they were dead or damaged cells (Fig. 1b and c). Similarly, cells treated with HGF and RANKL were able to proliferate to similar levels to those cells treated with M-CSF and RANKL after 3, 7, and 14 days in culture as shown by the results of the AlamarBlue assay (Fig. 1d). Addition of an M-CSF neutralising antibody did not inhibit proliferation.

Induction of human osteoclast formation in HGF/RANKL-treated cultures

In 14 day cultures of $CD14^+$ cells on glass coverslips, $TRAP^+$ and VNR^+ multinucleated cells were formed in the presence of HGF and RANKL (Fig. 2a and b). These multinucleated cells formed F-actin rings when cultured on dentine slices and glass coverslips for 21 days (Fig. 2c). Multinucleated cells were mainly large (50–100 μm) and contained up to 20 nuclei. The size of the cells formed in culture was confirmed by scanning electron microscopy. The cells were mainly round or ovoid, and spread over the dentine surface to which they were attached by fine microvilli (Fig. 3a). Some cells had cytoplasmic processes that extended for up to 10 μm over the dentine surface. It was also noted that small multinucleated or binucleated cells (less than 50 μm in diameter) were formed in these cultures. These cells also expressed TRAP and VNR, and were capable of F actin ring formation. The addition of an anti-M-CSF neutralising antibody to $CD14^+$ cell cultures did not inhibit the formation of $TRAP^+/VNR^+$ multinucleated cells.

Lacunar resorption in HGF/RANKL-treated cultures

Twenty-one day cultures of $CD14^+$ cells treated with HGF and RANKL resulted in the formation of numerous lacunar resorption pits on dentine slices. Both large compound areas of lacunar excavation and single resorption

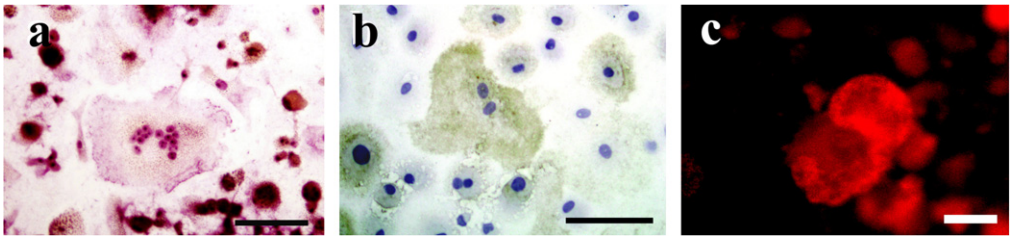

Fig. 2. HGF supports the formation of $TRAP^+/VNR^+$/F-actin ring forming cells. Fourteen day culture of human PBMCs treated with HGF, RANKL, and α-M-CSF on coverslips resulted in the formation of multinucleated (a) $TRAP^+$, (b) VNR^+ cells, which were capable of (c) F-actin ring formation. Bars represent (a,b) 100 μm, (c) 50 μm.

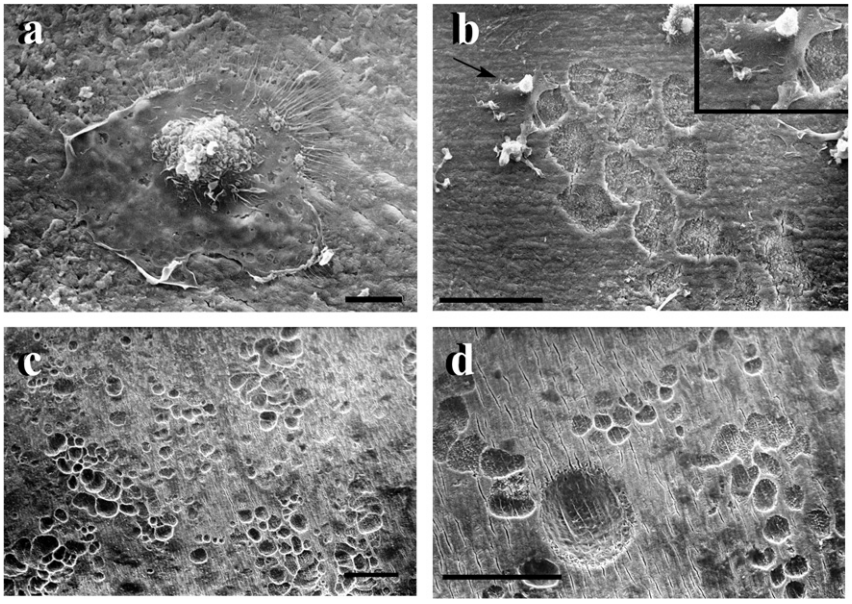

Fig. 3. HGF supports the formation of bone resorbing cells. Scanning electron micrographs of 21 day culture of human PBMCs treated with HGF, RANKL, and α-M-CSF on dentine slices, showing osteoclast-like cells on the dentine surface and associated lacunar resorption. Bars represent (a) 10 μm, (b) 50 μm, and (c,d) 100 μm.

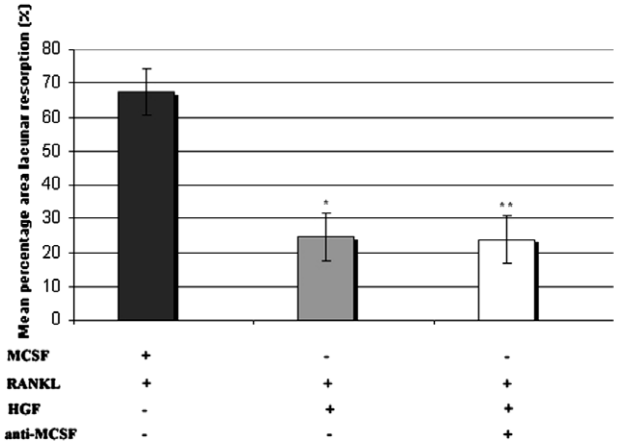

Fig. 4. HGF supports the formation of bone resorbing cells. Mean percentage area lacunar resorption of $CD14^+$ cells treated with M-CSF and RANKL or HGF and RANKL or HGF and RANKL and anti-M-CSF. The statistical differences between treated groups are given in the figure ($*p < 0.001$ and $**p < 0.005$) relative to M-CSF and RANKL control cultures.

pits were formed in these cultures (Fig. 3b and c). Small multinucleated cells (less than 50 μm) were also capable of lacunar resorption (Fig. 3d). Lacunar resorption was extensive in HGF/RANKL-treated cultures, up to 30% of the dentine slice surface area being resorbed. Dose–response experiments (10–100 ng/ml HGF) showed that the mean percentage area lacunar resorption was 10% at 10 ng/ml and that it increased up to 30 ng/ml beyond which there was little additional resorption. The addition of an anti-M-CSF neutralising antibody did not inhibit lacunar resorption (Fig. 4).

Discussion

Osteoclasts are formed from bone marrow-derived mononuclear precursors which circulate in the monocyte ($CD14^+$) fraction [6,19,20]. In this study we have shown that HGF can support the survival and proliferation of $CD14^+$ osteoclast precursors and that it can substitute for M-CSF to support RANKL-induced osteoclast differentiation. In the presence of RANKL, $CD14^+$ cells differentiated into multinucleated cells which exhibited all the cytochemical and functional characteristics of mature osteoclasts, including the expression of TRAP and VNR; these cells also formed F-actin rings and were capable of extensive lacunar resorption. This activity was not inhibited by an antibody to M-CSF, indicating that HGF acts independently of M-CSF to support osteoclast formation.

HGF is known to influence osteoclast formation and resorption and has been proposed as a coupling factor between osteoblasts and osteoclasts [9]. Both osteoblasts and osteoclasts are known to secrete HGF and to express its receptor c-Met [9,13]. HGF has been shown to promote human osteoblast differentiation from bone marrow stromal cells and to enhance the proliferation and survival of haematopoietic progenitor cells [13,20,21]. HGF also stimulates DNA synthesis in osteoblasts and enhances osteoclast motility and metabolic activity [9,22]. It has been shown that HGF stimulates the proliferation and differentiation of osteoclast precursors and that the addition of a neutralising antibody to HGF inhibits the formation of osteoclastic cells in co-cultures of mouse marrow cells and bone stromal cells [23]. Our results are in keeping with the findings of these studies and indicate that HGF stimulation of osteoclastogenesis may at least in part be due to

the fact that HGF can act as a substitute for M-CSF, a co-factor required for osteoclast formation. We found that HGF, like M-CSF, promotes the proliferation and survival of $CD14^+$ monocytes, the mononuclear phagocyte population from which osteoclast precursors are derived and that mature osteoclasts formed in monocyte cultures incubated with HGF and RANKL. RANKL was required for HGF-stimulated osteoclast formation, an observation that is in keeping with those of previous studies which found that HGF alone cannot induce osteoclast formation and that the presence of RANKL-expressing bone stromal cells is required for this to occur [23].

This is the first study to identify a growth factor which supports human osteoclastogenesis in the absence of M-CSF. VEGF and Flt3 ligand have been reported to support mouse osteoclast formation in marrow cultures in the absence of M-CSF [10,11]; the effect of these growth factors on human osteoclastogenesis is unknown. Niida et al. showed that in op/op mice, which lack M-CSF, osteoclast formation occurred when haematopoietic marrow cells were incubated in the presence of VEGF and RANKL [10]. Lean et al., however, found that VEGF could not act as an M-CSF substitute to induce osteoclast differentiation in normal or op/op mice and that VEGF did not stimulate RANK m-RNA expression in mouse marrow precursors [11]. They showed that adding Flt3 ligand to cultures of normal or op/op mouse haematopoietic marrow precursors (in the presence of RANKL) induced the formation of a small number of $TRAP^+$ multinucleated cells that were capable of a limited amount of lacunar resorption. It is of interest that, in both the above reports of osteoclastogenesis in VEGF and Flt3 ligand-treated marrow cultures, the osteoclasts which formed were significantly smaller in size and produced relatively smaller lacunar resorption pits than osteoclasts which formed in M-CSF and RANKL-treated cultures. This parallels our finding in HGF and RANKL-treated monocyte cultures where we noted that some of the osteoclasts were small and had only two or three nuclei. The amount of lacunar resorption produced in our cultures, however, was considerably greater than that noted in either VEGF or Flt3 ligand-treated cultures.

The c-Met proto-oncogene product is a high-affinity receptor for HGF and is known to be expressed by mature osteoclasts [9]. In our culture system, osteoclasts were formed from $CD14^+$ monocytes and we noted that 10% of these cells expressed c-Met. Despite this, osteoclast formation and lacunar resorption in HGF-treated cultures was very extensive and comparable to that seen in M-CSF-treated cultures. In previous studies it has been noted that a minority of $CD14^+$ monocytes differentiate into osteoclasts, and it is possible that these c-Met-expressing $CD14^+$ cells represent a primed population of circulating osteoclast precursors; osteoclast markers are known to accumulate on cells developing from human peripheral blood mononuclear precursors [24], and differences in the expression of HGF-regulated genes have been noted in murine mononuclear phagocyte populations exhibiting differences in the degree of differentiation [25].

An alternative explanation for the relatively low number of c-Met-expressing osteoclast precursors noted in our study is that HGF may be stimulating osteoclastogenesis through activation of tyrosine kinase receptors other than c-Met, such as c-fms or RON. The c-fms receptor is expressed by macrophages and experiments with c-fms/c-Met chimeric receptor where the extracellular domain of c-fms has been fused to the transmembrane and cytoplasmic domain of the c-Met receptor have shown that HGF can bind to the extracellular domain of c-fms [26]. RON is the receptor of a hepatocyte growth factor-like protein termed macrophage stimulating protein (MSP) which shares 45% homology with HGF. It has been shown that RON is expressed in osteoclast-like cells which form in cultures of human bone marrow cells [27]. The expression of RON is also induced by 1,25-dihydroxyvitamin D_3, which is known to stimulate osteoclastogenesis [27].

An osteoclastogenic role for HGF is consistent with its known activities as a physiological regulator of monocyte/macrophage differentiation [28]. Bone marrow stromal cells are known to produce abundant growth factors, including M-CSF, VEGF, Flt3 ligand, and HGF; production of these growth factors is stimulated by cytokines. Our findings and those of previous studies suggest that paracrine stimulation by growth factors, such as HGF, may play a role in modulating osteoclast differentiation by substituting for M-CSF to support mononuclear phagocyte–osteoclast differentiation. This indicates that there exists a degree of redundancy in the growth factor signalling required for osteoclast formation from mononuclear phagocyte precursors and is in keeping with a role for HGF as a coupling factor between osteoblasts and osteoclasts.

Acknowledgments

The authors wish to thank Dr. David Lacey, Amgen Inc., for providing the soluble RANKL. This work was carried out as part of the European network of excellence EuroBoNeT (LSHC-CT-2006-018814). This research was also funded by Research into Ageing, and The Rosetrees Trust.

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