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Multiple Roles of M-CSF in Human Osteoclastogenesis

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Abstract Although the critical role of M-CSF in osteoclastogenesis is well documented, there has been no detailed analysis of how it regulates human osteoclast formation and function in vitro. We used a human osteoclastogenesis model employing CFU-GM osteoclast precursors cultured for 14 days on dentine with RANKL, with varying exposure to exogenous human M-CSF. Short-term treatment of precursors with M-CSF (10–100 ng/mL) resulted in increased proliferation with or without RANKL. Treatment with M-CSF (1–100 ng/mL) for 14 days caused a biphasic concentration-dependent stimulation of formation, fusion, and resorption peaking at 10–50 ng/mL and almost complete abolition of resorption at 100 ng/mL. Time-course studies using M-CSF (25 ng/mL) showed that osteoclast size, nuclei/cell, and resorption increased with longer duration of M-CSF treatment. When treatment was restricted to the first 4 days, M-CSF (25–100 ng/mL) stimulated formation of normal numbers of osteoclasts that resorbed less. Blockade of endogenous M-CSF signaling with neutralizing M-CSF antibody during the first week of culture extensively inhibited osteoclastogenesis, whereas blockade during the second week produced only a small reduction in resorption. Treatment with M-CSF during the second week of culture caused a small increase in osteoclast number and a concentration-dependent increase in cytoplasmic spreading with inhibition of resorption. We have shown that M-CSF modulates multiple steps of human osteoclastogenesis, including proliferation, differentiation and fusion of precursors. In the later stages of osteoclastogenesis, M-CSF modulates osteoclast-resorbing activity, but is not required for survival. Modulation of M-CSF signaling is a potential therapeutic target for conditions associated with excess bone resorption. J. Cell. Biochem. 102: 759–768, 2007.

Key words: M-CSF; osteoclastogenesis; CFU-GM; proliferation; differentiation; cell fusion; bone resorption

Osteoclasts, the multinucleate cells responsible for bone resorption, are derived from myelomonocytic precursors [Ash et al., 1980; Scheven et al., 1986]. Evidence exists that osteoclast differentiation, survival, and activity are dependent on the presence of both M-CSF and RANKL [Fuller et al., 1993; Amano et al., 1998; Udagawa et al., 1999]. The critical role of M-CSF was demonstrated in osteopetrotic mice (*op*/*op*) that are M-CSF-deficient and lack functional osteoclasts [Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990], this defect being corrected by in vivo administration of M-CSF [Felix et al., 1990; Kodama et al., 1991]. Ample evidence exists that M-CSF is also required for osteoclast generation in vitro [Hattersley et al., 1991; Tanaka et al., 1993]. Nevertheless, the precise role remains unclear, with conflicting data reported in animal and human models.

In osteoclasts isolated from neonatal rat bone, M-CSF inhibits resorption [Hattersley et al., 1988] but promotes survival and chemotaxis [Fuller et al., 1993]. In the mouse, addition of exogenous M-CSF to bone marrow cells [Takahashi et al., 1991], co-cultures of spleen, and osteoblast cells [Perkins and Kling, 1995] or spleen cells treated with soluble RANKL [Yasuda et al., 1998] causes concentration-dependent inhibition of osteoclast formation. In contrast, studies using human monocytes [Matsuzaki et al., 1998; Fujikawa et al., 2001] found that M-CSF stimulated resorption in a concentration-dependent manner up to 100 ng/mL.

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In these cultures, osteoclast survival and resorption capacity was not affected by the removal of M-CSF (25 ng/mL) for the last week of the 3-week culture period. However, in cultures of osteoclasts isolated from human fetal bones, M-CSF increased survival in a biphasic manner between 25 and 100 ng/mL, with a peak effect at 50 ng/mL [Edwards et al., 1998].

We have established a human osteoclastogenesis model that uses CFU-GM derived from umbilical cord blood as precursors [Hodge et al., 2004a]. When treated with M-CSF and soluble RANKL, these precursors form osteoclasts at high efficiency, allowing the study of osteoclastogenesis in an environment free from the influence of stromal cells and lymphocytes. In view of the conflicting data concerning M-CSF in other models, we wished to characterize, in detail, its role in the human CFU-GM model. We now report that M-CSF has complex actions on human osteoclastogenesis that are dependent on both the timing of exposure and its concentration.

MATERIALS AND METHODS

Materials

Eagle's minimum essential media, penicillin/streptomycin and bromodeoxyuridine (BrdU) were purchased from Sigma (Castle Hill, Australia). Non-essential amino acids and fetal bovine serum were purchased from CSL Biosciences (Parkville, Australia). Recombinant human M-CSF was provided by Genetics Institute (Boston, MA). Soluble human RANKL coupled to GST fusion protein (sRANKL) was generously provided by Drs Matthew Gillespie and Julian Quinn, St Vincent's Institute of Medical Research, Melbourne, Australia. Polyclonal antibody to M-CSF was purchased from Chemicon (Boronia, Australia) and monoclonal antibody anti-BrdU-FITC was purchased from Becton Dickinson (North Ryde, Australia).

Osteoclast Precursors and Proliferation Assay

Human umbilical cord blood was obtained from healthy donors under a protocol approved by Barwon Health Research and Ethics Advisory Committee. The isolation of mononuclear cell (MoNCs) from cord blood and preparation of CFU-GM colonies has been previously described [Hodge et al., 2004a]. Freshly isolated CFU-GM osteoclast precursors were incubated for 24 h in media alone or with increasing concentrations of M-CSF (1–100 ng/mL) with or without RANKL (125 ng/mL, equivalent to ~50 ng/mL of soluble RANKL without GST tag). Cells were then pulse-labeled with BrdU as previously described [Gratzner, 1982]. The proportion of cells in S-phase was determined by FACS using a FACSCalibur (Becton Dickinson).

Osteoclastogenesis Cultures

The generation of osteoclasts from CFU-GM, and the quantitation of formation and resorption have been previously described [Hodge et al., 2004a]. The cultures were treated with sRANKL 125 ng/mL throughout, except where otherwise indicated, and dentine slices were included in all experiments. A polyclonal antibody to hM-CSF was added to some cultures at 1 μg/mL to neutralize endogenously produced M-CSF [Hodge et al., 2004b]. The number of nuclei per osteoclast was quantified in 50 randomly selected cells per treatment group using light microscopy. Changes in osteoclast plan area were discriminated from changes in cytoplasmic spreading by assessing nuclearity. Increases in plan area but not nuclei number was classified as cytoplasmic spreading, whereas increases in plan area and nuclei number were classified as increased size.

Statistics

Data are expressed as mean ± SEM. Differences between groups were determined using one-way analysis of variance (ANOVA) followed by Fisher's multiple comparison test, or by two-sample T-test. For all figures, groups with superscripts that do not include the same character are significantly different ($P < 0.05$), while groups with superscripts that do include the same character are not different: that is, "a" is significantly different to "b", "c", "b,c", etc., but not different to "a", "a,b", "a,c", etc.

RESULTS

Stimulation of Osteoclast Precursor Proliferation by M-CSF

When CFU-GM osteoclast precursors were exposed to increasing concentrations of M-CSF for 24 h, the percent of cells in S-Phase increased from 9.2 ± 0.45 in the control to 13.0 ± 0.79 at 10 ng/mL, 12.5 ± 0.49 at 25 ng/mL, 12.7 ± 0.60 at 50 ng/mL, and 14.7 ± 0.69 at 100 ng/mL (all $P = 0.000$ vs. control). The response was not

significantly different when the precursors were co-treated with RANKL 125 ng/mL.

Biphasic Concentration-Dependent Effect of M-CSF on Osteoclastogenesis

To determine the relationship between the concentration of M-CSF and osteoclastogenesis, cultures were treated for 14 days with sRANKL alone or with increasing concentrations of M-CSF (0.001–100 ng/mL) (Fig. 1). In the absence of exogenous M-CSF, small numbers of osteoclasts formed probably due to endogenous M-CSF production in these cultures [Hodge et al., 2004b]. However, compared to cultures treated with 25 ng/mL, the osteoclasts were only half the size and resorption was only ~10%. Treatment with exogenous M-CSF caused a concentration-dependant increase in osteoclast number, size, and resorption with a peak response at 10–25 ng/mL. The response was biphasic, particularly resorption, which was almost completely abolished at 100 ng/mL. The number of nuclei per osteoclast also showed a biphasic response to increasing concentrations of M-CSF. No significant relationship existed between nuclei per osteoclast and osteoclast area on regression analysis. However, the ratio plan area/nucleus was more than double in the presence of 100 ng/mL M-CSF (control 192 μm^2/nucleus, 100 ng/mL, 430 μm^2/nucleus).

In another experiment using this protocol, the osteoclast number, plan area and total resorption increased in a concentation-dependent manner with increasing concentrations of M-CSF from 0–25 ng/mL. In groups treated with M-CSF, 37.5 and 50 ng/mL these parameters where not statistically different to that seen with 25 ng/mL, but all where significantly reduced in the presence of 100 ng/mL.

These data suggest that when present for the entire culture period, high concentrations of M-CSF inhibit osteoclast generation and fusion, enhance cytoplasmic spreading and disproportionably inhibit resorption.

Time-Dependent Effect of M-CSF to Stimulate Osteoclast Generation, Fusion, and Resorption Capacity

To determine its role during the various phases of osteoclastogenesis, M-CSF (25 ng/mL) was added at the commencement of culture and then either removed at days 1, 2, 3, 4, 5, 7, 11, or maintained until day 14 (Fig. 2). Increasing duration of exposure to M-CSF caused a progressive increase in the number of osteoclasts, their size, and the number of nuclei per

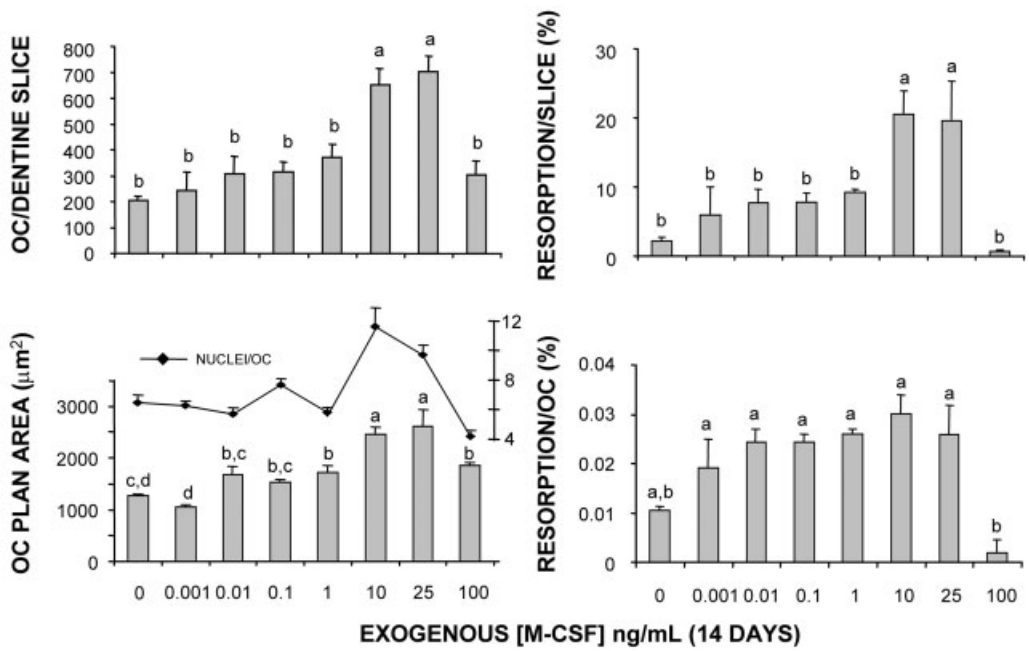

Fig. 1. Biphasic concentration-dependent effect of exogenous M-CSF on osteoclastogenesis. CFU-GM cultures were treated with sRANKL (125 ng/ml) and various concentrations of M-CSF for 14 days. n = 3 dentine slices/group; $P < 0.0001$.

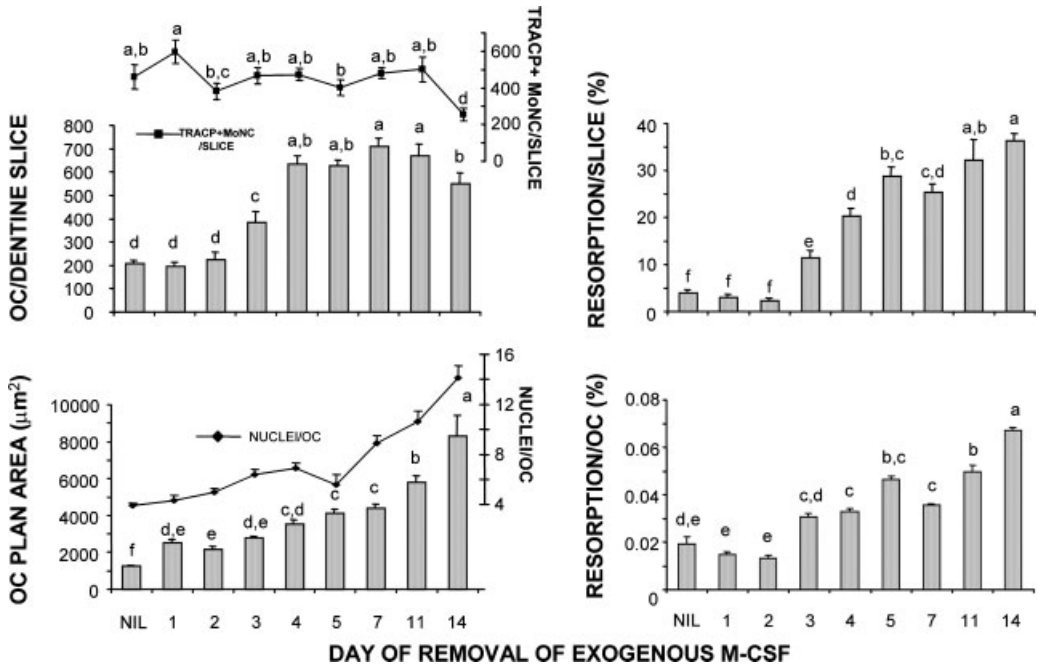

Fig. 2. Time-dependent effect of M-CSF to stimulate osteoclast generation, fusion, and resorption capacity. CFU-GM cultures were treated with sRANKL (125 ng/mL). M-CSF (25 ng/mL) was added from the commencement of culture and either removed on the days indicated, or continued until the cultures were terminated at day 14. n = 6 dentine slices/group; $P < 0.0001$.

osteoclast; the latter indicating progressive stimulation of cell fusion. Similarly, increasing duration of M-CSF exposure was associated with increasing total resorption and resorption per osteoclast suggesting a role for M-CSF in enhancing resorbing activity of osteoclasts. Interestingly, the maximal effect on osteoclast number was achieved by 4 days of treatment, after which a plateau was reached. Between 1 and 4 days of M-CSF treatment there was no significant increase in cell size, suggesting that a threshold of cell density exists after which enhanced cell fusion occurs. Regression analysis of data from this experiment revealed that the resorbing activity of osteoclasts is positively related to their log plan area (Resorption per osteoclast $= -0.26 + 0.8$ log osteoclast plan area; $r^2 = 70\%$, $P = 0.000$), which in turn is highly correlated to nuclei per osteoclast (log plan area $= 6.99 + 0.17$ nuclei per osteoclast, $r^2 = 76\%$, $P = 0.000$). In addition, resorption per osteoclast is highly correlated to nuclei per osteoclast (resorption per osteoclast $= 0.00212 + 0.00524$ nuclei per osteoclast, r^2 66%, $P = 0.00$). When the three variables were assessed by ANOVA, where resorption per osteoclast was the main outcome, a strong relationship exists ($P = 0.000$).

The TRACP + MoNC population varied little across the time course, except for a significant decrease at the last time-point when M-CSF treatment was extended from 11 to 14 days. This coincided with a rapid increase in osteoclast number and increase in nuclei per cell, suggesting that the decrease is due to fusion of TRACP + MoNC at a time when the generation of new TRACP + MoNC is waning.

Early, Short-Term Treatment with Higher Concentrations of M-CSF Promote Formation of Osteoclasts With Markedly Reduced Resorption Capacity

Previous time-course experiments with the cell-cycle inhibitor nocodazole established that proliferation is confined to the first 4 days of these cultures (results not shown). To investigate the effects of exogenous M-CSF during the proliferative versus later phases, osteoclast precursors were treated with sRANKL alone or together with increasing concentrations of M-CSF (0–100 ng/mL) from day 0 to 4 and with sRANKL only from day 5 to 14 (Fig. 3). This resulted in a concentration-dependent increase in the number of osteoclasts and their size. A maximal effect on cell number, not different to the control (i.e., M-CSF 25 ng/mL for 14 days),

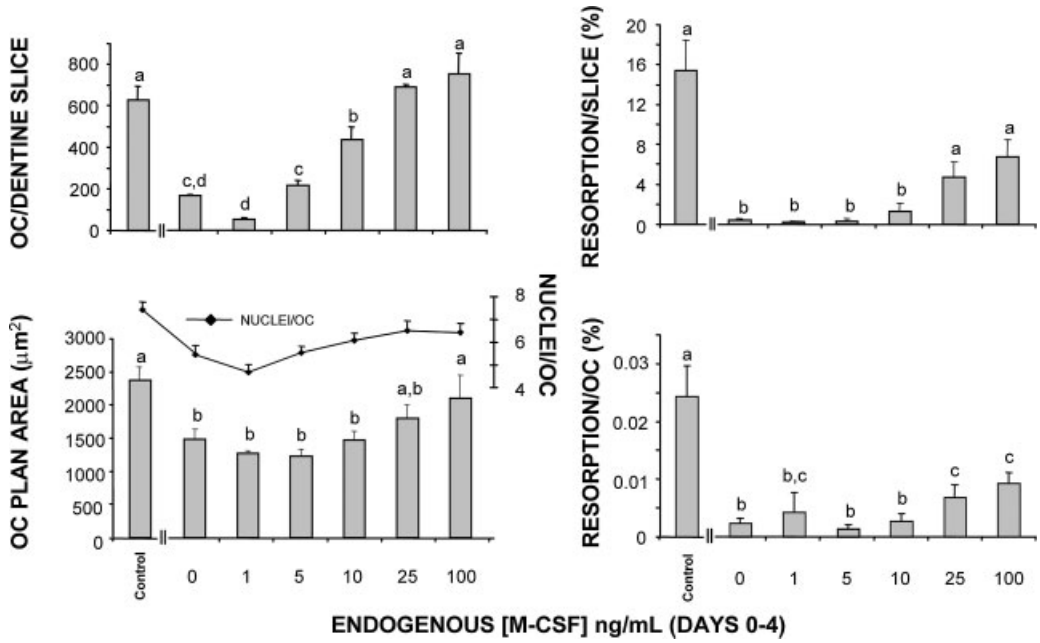

Fig. 3. Early, short-term treatment with higher concentrations of M-CSF promote formation of osteoclasts with markedly reduced resorption capacity. CFU-GM cultures were treated with sRANKL (125 ng/ml) and increasing concentrations of M-CSF for the first 4 days and then with sRANKL alone for a further 10 days. Control group was cultured with sRANKL and M-CSF (25 ng/mL) for 14 days. n = 4 dentine slices/group; $P < 0.0001$.

was seen with M-CSF 100 ng/mL for 4 days. A concentration-dependent effect on resorption was also seen although resorption was trivial with M-CSF 0–10 ng/mL and only about one third the control with 25 ng/mL. The "osteoclasts" formed under these conditions were the same size and had the same number of nuclei per cell as the controls. Thus, early, short-term M-CSF treatment at 25–100 ng/mL resulted in substantial, apparently "normal" osteoclastogenesis but these cells displayed reduced resorption capacity. This effect could be either due to dependence of efficient resorption on moderate concentrations of M-CSF, or due to incomplete differentiation of the cells, despite being multinuclear with high tartrate-resistant acid phosphatase activity.

Early Blockade of Endogenous M-CSF Signaling Markedly Inhibits Osteoclastogenesis but has Little Effect During the Second Week

We have previously shown [Hodge et al., 2004b] that CFU-GM cultures treated with sRANKL can generate small numbers of osteoclasts in the absence of added M-CSF, due to endogenous production of small amounts of M-CSF. This effect can be seen in the experiments depicted in Figures 1 and 2. Therefore, to determine the effect of absent M-CSF action during the different phases of osteoclastogenesis, endogenous M-CSF was neutralized with anti-human M-CSF antibody (1 μg/mL). Cultures of CFU-GM were treated with sRANKL and M-CSF antibody on days 0–4 or 0–7 and subsequently treated with sRANKL and M-CSF at 25 ng/mL until day 14 (Fig. 4A). When M-CSF antibody was present for the first 7 days, osteoclast formation was inhibited by 90%, the cells formed were 60% smaller, had fewer nuclei per cell (4.9 ± 0.3 vs. 7.4 ± 0.4; $P = 0.000$), and resorption was inhibited by 96%. Intermediate effects were seen when the antibody was present for the first 4 days only. These results indicate a strong dependence on M-CSF during the first week of in vitro osteoclastogenesis. However, when the M-CSF antibody was used during the second week of culture (Fig. 4B), there was no effect on osteoclast number, size or nuclei per cell. Resorption per slice was reduced significantly by 30–35% ($P = 0.031$) and resorption per osteoclast trended to be less ($P = 0.075$). Thus, osteoclasts formed in vitro are not dependent on M-CSF for survival, although the possibility of some residual

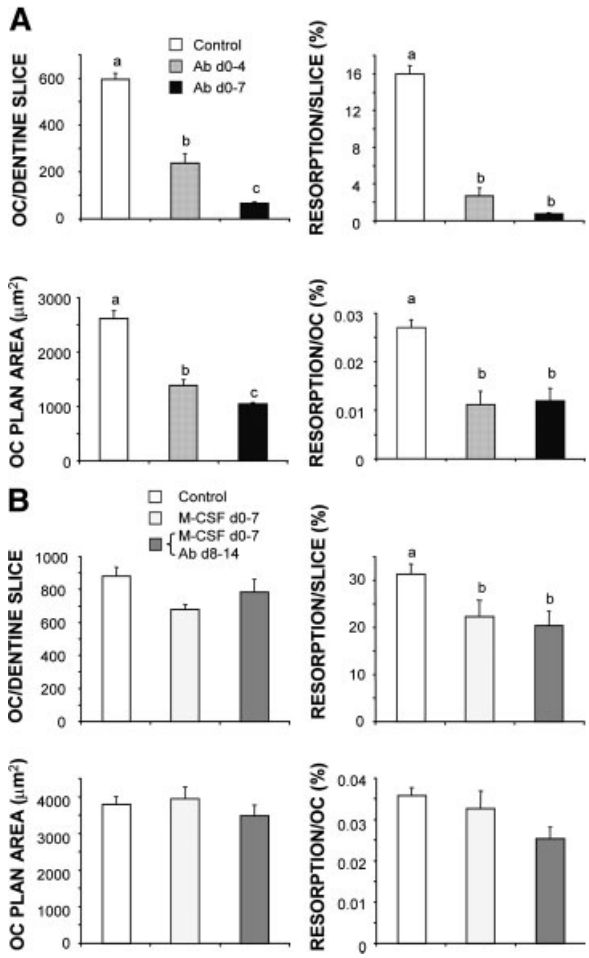

Fig. 4. Effects of blockade of M-CSF action on osteoclastogenesis. (A) CFU-GM cultures were treated with anti-M-CSF polyclonal antibody (1 μg/mL), for days 0–4 (Ab day 0–4) or days 0–7 (Ab day 0–7) and subsequently treated with exogenous M-CSF at 25 ng/mL until day 14; $P = 0.000$. (B) Cultures were incubated with M-CSF (100 ng/mL) for days 0–7 and subsequently incubated in the absence (M-CSF day 0–7) or presence of M-CSF antibody (M-CSF day 0–7 Ab d 8–14); $P = 0.031$. All groups in A and B were treated with sRANKL (125 ng/ml) and control cultures with M-CSF (25 ng/mL) for 14 days. n = 8 dentine slices/group; $P < 0.002$.

M-CSF signaling in this situation (perhaps related to membrane-bound M-CSF [Yao et al., 2003] cannot be excluded.

When Added During the Second Week of Culture, Exogenous M-CSF Inhibits Resorption, and Increases Osteoclast Cytoplasmic Spreading

Addition of M-CSF during the second week of culture had a borderline stimulatory effect on osteoclast number (+25%, $P = 0.05$) but doubled their size and reciprocally decreased resorption in a concentration-dependent manner, with approximately 33% inhibition of total resorption and 50% inhibition of resorption per osteoclast at 100 ng/mL (Fig. 5). The increase in osteoclast size was not associated with any change in the number of nuclei per osteoclast indicating that this effect was due to increased cytoplasmic spreading, rather than cell fusion.

DISCUSSION

Although both M-CSF and RANKL are required for osteoclast generation, recent literature including reviews of the topic [Chambers, 2000; Boyle et al., 2003] have focused on the regulatory role of RANKL (and related TNF-family members) with M-CSF being assigned a simple "permissive" role. However, the results presented here show that M-CSF has multiple regulatory functions in osteoclast generation and resorption that are dependent on timing of exposure and concentration. Continuous exposure to low to moderate concentrations of

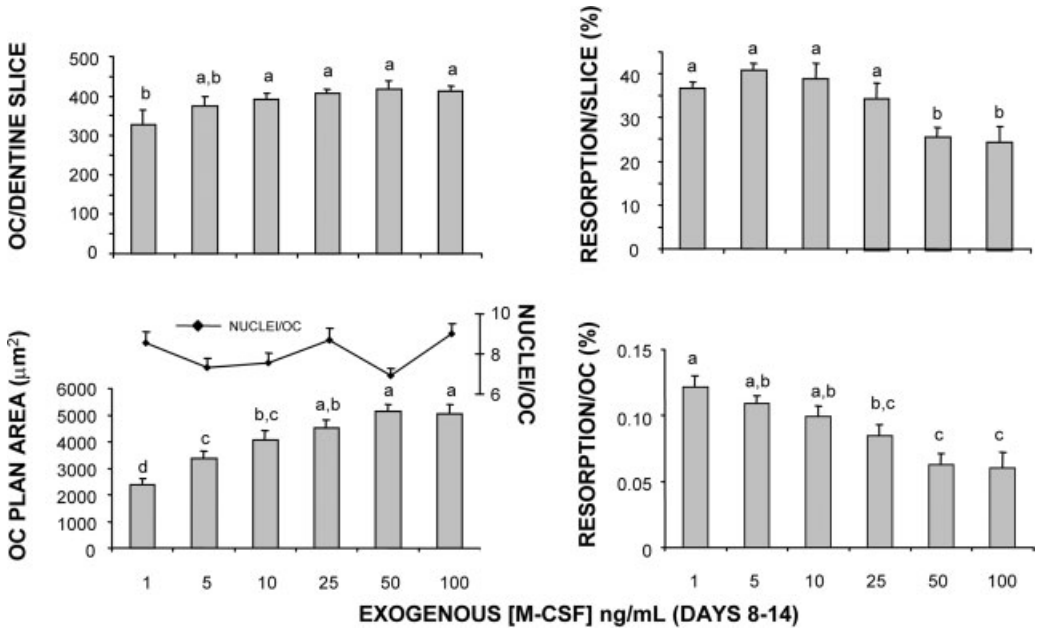

Fig. 5. Concentration-dependent inhibition of resorption and enhancement of spreading by M-CSF treatment during the second week. CFU-GM cultures were treated with sRANKL (125 ng/ml) and M-CSF (25 ng/mL) from day 0–7. The cultures were then treated from day 8–14 with various concentrations of M-CSF (1–100 ng/mL) in the presence of RANKL. n = 8 dentine slices/group; $P < 0.0001$.

M-CSF (≤25 ng/mL) promoted osteoclastogenesis. Short-term exposure to higher concentrations (25–100 ng/mL) during the proliferation phase (days 0–4) resulted in the generation of cells with reduced resorption capacity. Continuous exposure to concentrations >25 ng/mL inhibited osteoclastogenesis and high concentrations targeted to the resorptive phase of the assay (days 8–14) caused cytoplasmic spreading and reduced the resorbing activity of mature osteoclasts. This is consistent with an early report which showed that M-CSF stimulates the survival and chemotactic behavior of isolated rat osteoclasts but inhibits their capacity to resorb bone [Fuller et al., 1993]. These authors postulated that the major role for M-CSF, in addition to the provision of precursors, was to enhance osteoclast survival, migration, and chemotaxis and that it was appropriate that resorptive function should be suppressed under these circumstances. In contrast, our data suggest that in the human, M-CSF is not required for survival of osteoclasts. Udagawa et al. [1999] have reported that mature mouse osteoclasts did not survive 24–36 h culture in the absence of M-CSF or RANKL, and that their survival was markedly increased in the presence of either. However, only RANKL promoted resorption.

There have been a number of other reports of M-CSF-induced osteoclast spreading. In neonatal rat osteoclasts cultured on glass, Insogna et al. [1997] showed that 10 min treatment with M-CSF 2.5 nM (92 ng/mL) increased mean cell area by about 33% and caused redistribution of the actin cytoskeleton. These responses did not occur in osteoclasts derived from *c-src*-deficient rats, which do not have defined actin attachment rings. Teti et al. [1998] found that M-CSF 10 ng/mL increased the spreading of isolated mature rabbit osteoclasts cultured on glass by about 100% but had little effect when they were cultured on osteopontin-coated glass, in the presence of a vitronectin receptor antibody or on bone substrate, suggesting a role for the vitronectin receptor in the process. They also found that M-CSF inhibited bone-resorbing activity of rabbit osteoclasts by 70%. In our human model, this concentration of M-CSF increased spreading on dentine substrate by about 80% and had an insignificant effect on resorption.

Our experiments with neutralizing M-CSF antibody show that blockade of M-CSF action during the first half of the cultures essentially prevents the formation of bone-resorbing osteoclasts, which is consistent with the findings in M-CSF deficient *op*/*op* mice [Felix et al., 1990; Wiktor-Jedrzejczak et al., 1990; Yoshida et al.,

1990]. However, blockade of M-CSF during the second week had no effect on the number of osteoclasts formed, nor their size. Since substantial fusion occurs during the second week of these cultures (the mean plan area increases two to threefold) [Hodge et al., 2004a], these findings indicate that M-CSF is not involved in the fusion process, consistent with results obtained previously in a mouse bone marrow osteoclastogenesis model [Biskobing et al., 1995]. In contrast, in a mouse osteoblast and spleen cell co-culture model, treatment for the final 2 days of the 6-day culture period with either M-CSF antibody or *c-fms* antibody markedly inhibited the formation of osteoclast-like cells [Tanaka et al., 1993]. We found that blockade of M-CSF action with antibody during the second week, or treatment during this phase with exogenous M-CSF $\geq$25 ng/mL, reduced resorption by 30–35%. Thus, it seems that M-CSF has a biphasic effect on resorbing activity with low concentrations enhancing, but moderate concentrations causing inhibition. The mechanisms of regulation of osteoclastic resorbing activity by M-CSF are currently unknown, although we propose that these are most likely mediated by cross-talk modulation of signaling pathways downstream of RANK or by "inside-out" modulation of $\alpha_v\beta_3$ integrin function downstream of c-fms. Further investigation of this will require the use of a pure resorption assay employing short-term culture of mature osteoclasts, as previously described in a murine model [Fuller et al., 2006]. This model could also be used to investigate whether the cells generated with short-term (day 0–4) treatment with M-CSF that appear to be osteoclasts but resorb poorly have restored resorption capacity when M-CSF is re-introduced.

Circulating M-CSF levels in normal humans are approximately 2–9 ng/mL [Hanamura et al., 1988; Gilbert et al., 1989; Janowska-Wieczorek et al., 1991], which is the range where we observed a marked concentration-dependent stimulatory effect to increase osteoclast number, size, and resorption. The levels may be slightly higher in bone marrow serum [Denizot et al., 1996] and are significantly higher in a number of hematological [Hanamura et al., 1988; Gilbert et al., 1989; Janowska-Wieczorek et al., 1991; Fixe et al., 1995], colorectal [Mroczko et al., 2003], lung [Katsumata et al., 1996], breast [Scholl et al., 1996], and gynecological [Gadducci et al., 1998; Suzuki et al., 1998; van Haaften-Day et al., 2001] malignancies, as well as hemodialysis [Nitta et al., 2001], kidney allograft rejection [Le Meur et al., 2004], liver disease [Itoh et al., 1994], and ageing [Suehiro et al., 1999]. These conditions are generally associated with bone loss and it is possible that elevated M-CSF levels contribute to this. In rheumatoid arthritis, which is associated with increased local and systemic bone resorption, M-CSF is increased in synovial fluid [Smith et al., 1990], synovial fibroblasts [Seitz et al., 1994], and serum [Kawaji et al., 1995]. The levels of M-CSF are also increased in Still's disease [Matsui et al., 2003] and in loose hip prostheses [Takei et al., 2000], both of which are associated with bone loss. Our results show that although higher concentrations of M-CSF stimulate osteoclast formation, they also inhibit the resorbing activity of mature cells, which may be a counter-regulatory mechanism to limit bone resorption in pathological conditions associated with excess M-CSF production.

We have shown that M-CSF is able to regulate multiple steps of human in vitro osteoclastogenesis, including proliferation, differentiation, and fusion of precursors, and the resorbing activity and cytoplasmic spreading of mature osteoclasts. However, our results indicate that M-CSF is not required for the survival of osteoclasts. The potential exists to target M-CSF or its signaling pathways in the development of new anti-resorptive therapies.

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