Increasing membrane-bound MCSF does not enhance OPGL-driven osteoclastogenesis from marrow cells

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Fan, X., D. Fan, H. Gewant, C. L. Royce, M. S. Nanes, and J. Rubin. Increasing membrane-bound MCSF does not enhance OPGL-driven osteoclastogenesis from marrow cells. Am J Physiol Endocrinol Metab 280: E103–E111, 2001.—Macrophage colony-stimulating factor (MCSF) and osteoprotegerin ligand (OPGL), both produced by osteoblasts/stromal cells, are essential factors for osteoclastogenesis. Whether local MCSF levels regulate the amount of osteoclast formation is unclear. Two culture systems, ST-2 and Chinese hamster ovary-membrane-bound MCSF (CHO-mMCSF)-Tet-OFF cells, were used to study the role of mMCSF in osteoclast formation. Cells from bone marrow (BMM) or spleen were cultured with soluble OPGL on glutaraldehyde-fixed cell layers; osteoclasts formed after 7 days. Osteoclast number was proportional to the amount of soluble OPGL added. In contrast, varying mMCSF levels in the ST-2 or CHO-mMCSF-Tet-OFF cell layers, respectively by variable plating or by addition of doxycycline, did not affect BMM osteoclastogenesis: 20–450 U of mMCSF per well generated similar osteoclast numbers. In contrast, spleen cells were resistant to mMCSF: osteoclastogenesis required ≥250 U per well and further increased as mMCSF rose higher. Our results demonstrate that osteoclast formation in the local bone environment is dominated by OPGL. Increasing mMCSF above basal levels does not further enhance osteoclast formation from BMMs, indicating that mMCSF does not play a dominant regulatory role in the bone marrow.

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mMCSF 20-fold, i.e., to levels surpassing in vivo levels of expression, it does not increase osteoclast formation.

MATERIALS AND METHODS

Materials. 1,25-Hydroxyvitamin D₃, or 1,25(OH)₂D₃, was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). C57BL/6 male mice were purchased from the Frederick Cancer Center (Frederick, MD). Fetal bovine serum (FBS) was obtained from Atlanta Biological (Atlanta, GA). Mouse OPGL was obtained from Amgen (Thousand Oaks, CA). Other chemicals and media were obtained from Sigma (St. Louis, MO) except as noted.

Fixed ST-2 cell culture systems. ST-2 cells (Riken Cell Bank, Tsukuba Science City, Japan), original clones from murine bone marrow stromal cells, were plated in 12-well plates overnight and treated with 10 nM 1,25(OH)₂D₃ (1,25D) and 100 nM dexamethasone (Dex) for 2 days. ST-2 cell layers were fixed in 2.5% glutaraldehyde for 1 min, followed by the addition of 1.5% glycine. The cell layers were washed with PBS three times and covered with α-MEM containing 10% FBS (33). Nonadherent bone marrow cells (BMMs) were isolated from 3- to 4-wk-old C57BL/6 male mice. Briefly, BMMs were collected from mouse tibia and flushed bone marrow cavities. The cells were incubated in α-MEM containing 10% FBS at 37°C overnight. On the next day, nonadherent BMMs were collected and overlaid on prefixed ST-2 cell layers at a density of 5.5–6 million/well. After 7 days of culture, the cultures were fixed with ethanol-acetone and stained for tartrate-resistant acid phosphatase (TRAP; Sigma, St. Louis, MO). A TRAP-positive cell containing three or more nuclei was counted as a TRAP-positive multinuclear cell (TRAP⁺MNC).

mMCSF extraction. Live cell layers were washed with PBS and then treated for 10 min at 37°C with trypsin solution (1 mg/ml) to release mMCSF. Adding FBS for a final concentration of 15% stopped the reaction. The cell suspensions were centrifuged at 1,500 g for 5 min, and supernatants were stored at −30°C until bioassay (7, 24, 30).

MCSF bioassay. MCSF activity was determined by measuring the proliferation of MCSF-dependent M-NFS-60 cell line (American Type Tissue Collection, Rockville MD); 10,000 cells/well were cultured with test samples in a final volume of 100 μl as previously described (24). M-NFS-60 cell proliferation was assessed using MTT, a colorimetric assay to detect mitochondrial dehydrogenase levels. Recombinant human M-CSF (6.94 × 10⁵ CFU/mg; Cetus, Emeryville, CA) was used as the standard.

Blocking mMCSF. ST-2 cells were plated in 24-well plates at a density of 15,000/well and cultured with 10 nM 1,25D and 100 mM Dex for 2 days. ST-2 cell layers were treated at 50% confluence with 10 μg/ml of goat anti-mouse M-CSF neutralizing polyclonal antibody (pAb; R&D System, Minneapolis, MN) or 10 μg/ml goat IgG at 37°C for 2 h before glutaraldehyde fixation. To determine the dose of blocking pAb, 0.1, 1, 10, or 100 μg/ml of pAb or IgG were added to ST-2 cells before fixation. Spleen cells were plated over fixed layers, and [³[H]thymidine incorporation was determined. pAb at a dose of 1 μg/ml blocked one-half of the mMCSF-dependent proliferation, and complete blocking was seen by 100 μg/ml (no further decrease by 100 μg/ml).

Preparation of spleen cells. Spleen cells were isolated from 3- to 4-wk-old C57BL/6 male mice. Briefly, spleens were washed, minced, and suspended in PBS. Contaminating erythrocytes were eliminated from the cell pellet by adding 0.83% NH₄Cl in 10 mM Tris buffer (pH 7.4). The cells were washed three times with PBS, suspended in α-MEM containing 10% FBS, and cultured in 75-cm² flasks overnight. The nonadherent spleen cells were collected and overlaid on prefixed ST-2 or CHO-MCSF Tet-OFF cell layers.

TRAP assay. After culture for 7 days, the cell layers were washed twice with PBS and lysed with 0.5% Triton X-100. The cell lysates were centrifuged at 10,000 g for 15 min. The supernatants were stored at −30°C. For assay, 20 μl of cell lysate and 180 μl of substrate buffer [0.48 M acetate buffer pH 5, 2 mM methylumbelliferone phosphate (MUP), 83 mM tartaric acid] were added to each well in Microtiter 96-well white plates (Dynex Technologies, Chantilly, VA). After incubation at 37°C for 30 min in the dark, 100 μl of 0.5 M glycine solution containing 50 mM EDTA (pH 10.4) were added to stop the reaction. Fluorescence was measured on an LB 50 Plate Reader (Perkin Elmer, Buckinghamshire, England) at excitation wavelength 366 nm and emission wavelength 456 nm. A serial dilution of methyl-umbelliferone (0–800 μM) was used to generate a standard curve. The enzyme activity was represented as micromoles of MUP hydrolyzed per milligram of protein per min.

Cell proliferation. [³[H]thymidine incorporation per million cells) was added to cultures on the specific day. After 24 h, cells were lysed in 20 mM NaOH containing 1% SDS, transferred to scintillation vials, and counted by a PACKARD 2500 TR Liquid Scintillation Analyzer (Downers Grove, IL).

RT-PCR. Total RNA was prepared in TRIzol (Life Technologies, Gaithersburg, MD). For measurement of cathepsin K, 0.5 μg of total RNA was added to a RT reaction containing 1 mM dNTP, 0.5 μM forward primer, 100 U of MMLV reverse transcriptase, and 20 U of RNAsin. The RT reaction was incubated for 30 min at 37°C. For quantitation of PCR products, 1 μCi of [α-³²P]dCTP was used in each standard PCR reaction (cathepsin K PCR forward primer 5'-CCAGT-GAA-GAAGTTGTTCCG, reverse primer 5'-TATCTTCTTCTGAGAAGAGTTCG). PCR products were chromatographed and phosphorimages captured on a Molecular Dynamics instrument. The cathepsin K density was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) density obtained from the same sample.

Tet-OFF system. The mMCSF cDNA (1.6 kb) plasmid was the generous gift of Dr. Martine Roussel (St. Jude’s Children Research Hospital, Memphis, TN). The region coding for mMCSF (i.e., exon 6) was obtained by PCR with adapters added to mutate two base pairs on each PCR primer (forward primer 5’-CCCCG-GCG-GGC-GGC-GCC-GCT-GCT-GCG-ATG-GAGG-C-3’ and reverse primer 5’-TATCTTCTTCTGAGAAGAGTTCG). PCR products were chromatographed and phosphorimages captured on a Molecular Dynamics instrument. The cathepsin K density was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) density obtained from the same sample.

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10:1 ratio. Stable cell lines were selected under 400 μg/ml G418 and 200 μg/ml hygromycin (Sigma). Thirty-six clones were isolated and tested by MCSF bioassay in the presence and absence of doxycycline (Dox). Clone 4 produced the highest mMCSF expression in the absence of Dox vs. low expression with 1 μg/ml Dox. The doubly stable CHO-mMCSF cell line was maintained under 200 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml Dox constraints.

Statistical analysis. Data were analyzed by Tukey ANOVA by use of Prism software.

RESULTS

mMCSF stimulates bone marrow macrophage and spleen cell proliferation. ST-2 cells were plated at densities from 7,500 to 120,000 per well. After culture with 1,25D and Dex for 2 days, cell number and mMCSF were assayed. The ST-2 cell doubling time of 28 h was highly reproducible, as was the final mMCSF level achieved. Figure 1A shows that mMCSF levels as measured by bioassay before fixation increased from 3.5 to 138 U/well as final cell numbers increased from 56,000 to 266,000/well. Glutaraldehyde treatment at this time can thus ensure a controlled amount of mMCSF for study.

We next evaluated the ability of the glutaraldehyde-fixed mMCSF units in each well to support dose-dependent proliferation of MCSF-responsive BMMs. Nonadherent BMMs were cultured on fixed ST-2 cell layers containing different amounts of cells with variable units of mMCSF in proportion to the cell number. [3H]thymidine incorporation assay showed that cell proliferation increased threefold in those wells with the highest levels of mMCSF (93 ± 6 U/well) compared with wells containing one-third as many cells (36 ± 3 U/well) (Fig. 1B), demonstrating as well that functional mMCSF remained in ST-2 cell layers after glutaraldehyde fixation. Increasing ST-2 cell number was thus associated with proportional increases in both mMCSF expression and proliferation of bone marrow macrophages.

To verify that bone marrow cell proliferation on fixed ST-2 cells was dependent on mMCSF, mMCSF was blocked with goat anti-MCSF pAb before fixing ST-2 cells. As shown in Fig. 2, spleen cell proliferation decreased to 57.8 ± 7% (P < 0.05) after treatment with pAb compared with ST-2 cultures pretreated with a control IgG. pAb to murine MCSF added to the media with spleen cells did not further decrease proliferation in cultures pretreated with anti-MCSF pAb, but it did in the IgG-pretreated cultures. These data indicate that mMCSF on fixed ST-2 cell layers was fully blocked by the pAb before fixation and that MCSF was responsible for more than one-half of the ensuing proliferation. The mMCSF-independent proliferation indicated that BMMs were able to respond to factors other than mMCSF enmeshed in the glutaraldehyde-fixed cell layer or the extracellular matrix, e.g., vascular endothelium growth factor, granulocyte-macrophage colony-stimulating factor, or transforming growth factor-β (TGF-β) (19, 28).
CHO cell numbers were not affected by the Dox treatment (data not shown). The CHO-mMCSF cells were thus treated with Dox for 3 days before the glutaraldehyde fixation procedure. As shown in Fig. 3B, the proliferation of spleen cells plated over these fixed CHO-mMCSF cell layers decreased in a dose-dependent fashion as Dox shut off mMCSF expression.

**OPGL regulation of osteoclast formation is dose dependent.** ST-2 cells were treated for 2 days with 1,25D (10 nM) and Dex (100 nM), which induces maximal expression of both mMCSF (22) and OPGL (10, 34). At this time, cells were fixed with 2.5% glutaraldehyde. To test whether the fixed ST-2 cells were able to support osteoclastogenesis, nonadherent BMMs were plated over the fixed ST-2 layers in the presence or absence of murine sOPGL (0–10 ng/ml). After 7 days of culture, no TRAP$^+$ MNCs appeared when BMMs were cultured over ST-2 cells alone, but they were induced with the addition of exogenous OPGL. Because ST-2 cells are well known to display OPGL and support osteoclastogenesis under the culture conditions used here (34), the glutaraldehyde fixation procedure blocked or destroyed the OPGL molecule on the cell surface. In the presence of exogenous OPGL, however, glutaraldehyde-fixed layers were able to support osteoclast formation. This result suggested that the process of glutaraldehyde fixation removed the contribution of endogenous OPGL and allowed us to study the effects of mMCSF availability on osteoclast formation.

Osteoclast formation increased in an sOPGL dose-dependent fashion as seen both visually (Fig. 4A) and by counting TRAP$^+$ MNCs (Fig. 4B). As a corroborative measure of increase in the osteoclast phenotype, TRAP activity was measured using a sensitive methylumbiliforone assay. In Fig. 4C, TRAP activity dose-dependently increased in proportion to the added sOPGL. ST-2 cells express mMCSF in the absence of 1,25D or Dex, as we have previously shown (11). To assure that a lower level of mMCSF expression also supported osteoclastogenesis, sOPGL was added to cocultures of ST-2 cells and BMMs in the absence of 1,25D or Dex. sOPGL caused a dose-dependent increase in osteoclast formation.

![Graph of progenitor proliferation on fixed ST-2 layers](http://ajpendo.physiology.org/)

**Fig. 2.** Progenitor proliferation on fixed ST-2 layers is diminished by pretreatment of cell layers with anti-MCSF antibody. ST-2 cells were treated with goat anti-murine MCSF neutralized polyclonal antibody (pAb) or goat IgG before fixation. Spleen cells were cultured on the fixed ST-2 layers for 6 days, and [³H]thymidine (2 µCi/well) was added for the final 24 h of culture. Cells were harvested and [³H]thymidine uptake was counted. [³H]thymidine uptake by spleen cells decreased to 57% in the presence of blocking antibodies to mMCSF (n = 3, P < 0.05). Addition of pAb (10 µg/ml) to the culture medium decreased proliferation by 50% in the IgG-pretreated group but had no further effect in groups pretreated with pAb. Data are means ± SE from 3 separate experiments.

**Fig. 3.** mMCSF expression in Chinese hamster ovary (CHO)-mMCSF Tet-OFF cells. CHO cells were doubly transfected with pTRE-MCSF and tetracycline regulator plasmids. Stable cell lines were selected in the presence of G418 and hygromycin. CHO-mMCSF cells were treated with doxycycline (Dox: 0–2 µg/ml) for 3 days. A: mMCSF on cell surface was released by trypsin and measured by MCSF bioassay. mMCSF expression in the absence of Dox was >10-fold higher than that in the presence of maximal 2 µg/ml Dox treatment. B: spleen cells were grown over fixed CHO-mMCSF cell cultures for 5 days. [³H]thymidine was added to cultures during the last 24 h. mMCSF-dependent proliferation can be seen in the presence of decreasing Dox. Values are means ± SE (n = 3) and are representative of ≥3 experiments.
numbers as follows: sOPGL at 0 ng/ml = 6 ± 2 osteoclasts, at 2.5 ng/ml = 74 ± 7 osteoclasts, at 5 ng/ml = 353 ± 16 osteoclasts, and at 10 ng/ml = 783 ± 43 osteoclasts. Although these data cannot be compared directly with the results obtained when ST2 cells are incubated in the presence of 1,25D and Dex, they serve to show that basal levels of mMCSF are adequate to support osteoclastogenesis in the presence of sOPGL.

Cathepsin K has been previously used as a positive measure of osteoclast phenotype (1). Both BMMs and spleen cells express cathepsin K in the absence of OPGL in our culture system, decreasing the specificity for assaying osteoclast transformation of the semi-quantitative RT-PCR assay. Thus, although sOPGL dose dependently increased cathepsin K mRNA expression, the dynamic range was very poor (the ratio of cathepsin K to GAPDH was 0.054 ± 0.0001 in the presence of 8 ng/ml sOPGL compared with 0.031 ± 0.001 in the absence of sOPGL). These data showed that cathepsin K mRNA measurement was neither specific nor sensitive enough to evaluate osteoclast formation in this culture system.

Role of mMCSF in regulation of osteoclast formation from bone marrow progenitors. To evaluate whether mMCSF might play a dominant role in regulating osteoclast precursor differentiation, we varied concentrations of the presented growth factor before fixation. In the first system, the ST-2 cell-plating density regulated mMCSF availability. In the second, mMCSF expression was regulated in the tetracycline-sensitive CHO-mMCSF clone with Dox.

The range of mMCSF in fixed ST-2 cell layers was between 35 and 120 U/well. Soluble OPGL was added to stimulate osteoclastogenesis: 5 ng/ml sOPGL represented the ED50 for sOPGL-induced osteoclast formation in the fixed ST-2 culture system (see Fig. 4). After 7 days of culture with BMMs, cultures were fixed and stained for TRAP. As shown in Fig. 5A, TRAP+ MNCs did not increase when mMCSF availability rose from 34 to 120 U/well. The sensitive TRAP activity assay also revealed no changes as mMCSF units increased, as shown in Fig. 5A as TRAP activity. There was even a trend toward decreased osteoclast formation at the higher mMCSF availability. In addition, experiments
were performed with 2.5 ng/ml sOPGL, which confirmed that increasing mMCSF did not increase TRAP activity (data not shown).

To confirm the observations with ST-2 cell layers, BMMs were plated over tetracycline-sensitive CHO-mMCSF Tet-OFF cell layers. In these cultures, the addition of Dox from 0.01 to 2,000 ng/ml reduced mMCSF from nearly 500 to 25 U/well as measured by bioassay (see Figs. 3A and 5B). In the presence of sOPGL, TRAP$^+$MNC numbers were not significantly changed despite the >20-fold increase in mMCSF. Thus raising mMCSF had no apparent effect on the number of cells entering the osteoclast lineage in this unique culture system.

Requirements for MCSF and OPGL during spleen progenitor to osteoclast differentiation. Spleen cells can proliferate with added MCSF and differentiate into the osteoclast lineage when sOPGL is added to the culture medium in the absence of accessory stromal cells (34). We were not able to generate osteoclasts from spleen cells over fixed ST-2 cell layers; however, the CHO-mMCSF-Tet-OFF system, which displays higher amounts of mMCSF, was successful. TRAP$^+$MNCs were present at the highest doses of fixed mMCSF (632 and 320 U/well), disappearing when Dox decreased mMCSF expression below 100 U/well (Fig. 6). Figure 6, which compiles two separate experiments, did show a statistical trend toward an increase in osteoclast numbers when mMCSF rose above 300 U/well, suggesting that spleen cells respond differently than do BMMs. Although it is not possible to compare amounts of membrane-bound and soluble ligands directly, spleen cell resistance to MCSF during osteoclast differentiation can also be demonstrated using sMCSF. BMM and spleen cell cultures were set up in standard fashion to promote osteoclastogenesis, and soluble MCSF (sMCSF; 125–2,000 U/ml) and sOPGL were added. As shown in Fig. 7 in spleen cell cultures, addition of 250 U/ml of sMCSF was required before the appearance of TRAP$^+$MNCs. TRAP$^+$MNC numbers reached a plateau at 1,000 U/ml sMCSF. In comparison, BMMs cultured with sOPGL formed osteoclasts even in the absence of added sMCSF, reaching plateau immediately upon addition of 200–250 U/ml of sMCSF. Interestingly, sMCSF above 500 U/ml inhibited osteoclast formation, as we have previously reported (6). Although it is not possible to compare osteoclast numbers directly between the BMM and spleen cell cultures, Fig. 7 demonstrates that the MCSF requirement for osteoclastogenesis is greater in spleen cell cultures.
These results also indicate that the inability of fixed ST-2 cell layers to support spleen cell-to-osteoclast transformation is due to inadequacy of mMCSF.

**DISCUSSION**

In the work presented here, we wished to clarify whether mMCSF levels could regulate the level of osteoclast formation. During the past decade, MCSF elaborated by stromal cells has been recognized to be an essential factor for osteoclast precursor proliferation and differentiation and osteoclast survival (27). Stromal cells from op/op mice that are deficient in MCSF do not support osteoclastogenesis in the absence of exogenous MCSF (32). Antibodies that completely block the MCSF receptor inhibit the development of osteoclasts in vitro (9). Furthermore, even with the addition of substantial amounts of sOPGL, monocytic precursors do not appear to generate osteoclasts in the absence of MCSF (3, 15, 34).

Osteoclastogenesis is known to be modulated by systemic hormones and local stimulatory factors, such as 1,25D, parathyroid hormone (PTH), interleukins, TNF-α, and TGF-β, among others (8, 19, 25, 27). Many of these factors stimulate the expression of MCSF, leading to the hypothesis that the level of MCSF present within the local bone environment might dose dependently direct osteoclast formation. For instance, 1,25D, TNF-α, and PTH, all of which potently induce osteoclast formation, cause increased secretion of sMCSF (11, 24, 31). Additionally, several investigators have suggested that estrogen deficiency might promote osteoclastogenesis by increasing the production of sMCSF by bone stromal cells (12, 26). However, very high levels of added sMCSF inhibit recruitment of progenitors into the osteoclast lineage despite increased proliferation of the predominant clone (6, 20, 29, 34). Further confounding these issues is a lack of data demonstrating significant transcriptional control within the murine MCSF promoter by osteoclastogenic factors known to affect sMCSF expression (11, 23). We and others have suggested that sMCSF secretion might be controlled posttranscriptionally (5) or posttranslationally via the trafficking of the secreted protein within the stromal cell (22). In sum, the role of sMCSF in regulating osteoclastogenesis, directly or indirectly, has never been firmly established.

Alternatively, mMCSF, which is directed to the cell membrane after posttranscriptional excision of exon 6 (16), might represent a more finely tuned and significant regulatory molecule as expressed by cells within bone. Glucocorticoids, which promote bone resorption, increase the expression of mMCSF in bone stromal cells while decreasing secretion of the soluble form (22). As well, estrogen deficiency may specifically increase the expression of mMCSF, as opposed to sMCSF, within the bone marrow (17). Recent studies have shown that mMCSF is sufficient to support osteoclast formation in vitro (33), suggesting a role for this isoform in regulating osteoclast lineage selection.

To investigate effects of mMCSF on regulation of osteoclastogenesis, we used both fixed ST-2 cells and osteoclast cultures. BMMS (2 million/well) were plated in 24-well plates and treated with 2.5 ng/ml OPGL and recombinant human MCSF (rh-MCSF, range 0–2,000 U/ml) for 7 days. TRAP+ MNCs reached peak level in 250 U/ml rhMCSF and decreased significantly at addition of 2,000 U/ml rhMCSF. a = Mean value significantly different from other groups (n = 4, *P < 0.05). Data are representative of 2 independent experiments.

![Fig. 7. Addition of exogenous soluble MCSF in BMM and spleen cell cultures. BMMS (2 million/well) were plated in 24-well plates and treated with 2.5 ng/ml OPGL and recombinant human MCSF (rh-MCSF, range 0–2,000 U/ml) for 7 days. TRAP+ MNC numbers reached plateau in the presence of rhMCSF above 1,000 U/ml. a = Mean value significantly different from group without rhMCSF treatment (n = 4, P < 0.01). For spleen cell cultures, 1.5 million spleen cells were plated in 24-well plates, treated with 10 ng/ml sOPGL and rhMCSF (0–2,000 U/ml), and stained for TRAP after 7 days. TRAP+ MNC numbers reached plateau in the presence of rhMCSF above 1,000 U/ml. b = Mean value significantly different from that of cells without rhMCSF treatment (n = 4, P < 0.01).](http://ajpendo.physiology.org/DownloadedFrom/10.220.32.246/10.220.32.246)
CHO-McMCSF Tet-OFF cells overlaid with osteoclast progenitors. In both systems, proliferation of the osteoclast progenitors was proportional to McMCSF availability, as expected (6). Neither cell system, however, supported osteoclastogenesis in the absence of exogenous OPGL. This was unexpected in the case of the ST-2 cells, which express OPGL under the control of hormones (13), indicating that OPGL was destroyed by our fixation procedure or was fixed in an inactive conformation. In contrast, Kong et al. (14) reported that fixed activated T cells expressing OPGL could trigger osteoclastogenesis; this difference may be due to differences in fixation or in aspects of lymphocyte presentation of the molecule. Nonetheless, our system allowed investigation of the role of McMCSF in directing osteoclast formation.

Our experiments showed that BMMs cultured on fixed ST-2 cell layers displaying varying amounts of McMCSF (34–120 U) showed no increase in osteoclast formation as McMCSF availability increased. The concentration of sOPGL, on the other hand, was intrinsically related to the amount of osteoclastogenesis. In confirmation of this finding, experiments using fixed CHO-mMCSF cell layers showed that BMM osteoclastogenesis was regulatable by sOPGL and that osteoclast formation was not changed as McMCSF was increased 20-fold. These data indicate that increasing McMCSF expression stimulates precursor proliferation but does not enhance osteoclast formation. Thus, whereas McMCSF undoubtedly has an important role in the recruitment of monocytes and may have effects on both osteoclast activity and fusion (2, 18), its regulatory function during osteoclast recruitment from marrow cells appears to be minor. MCSF’s ability to enhance the expression of RANK, the receptor for OPGL (3), suggests a competence role for MCSF, rather than one that predicates final selection of the osteoclast phenotype.

Both marrow and spleen cells represent sources of osteoclast precursors. In our experiments, these two cell sources were not interchangeable. Whereas BMMs formed osteoclasts in the presence of minimal amounts of McMCSF (34 U/well) and sOPGL (2 ng/ml), spleen cells required nearly ten times as much McMCSF and 10 ng/ml sOPGL to induce osteoclast formation. This could be due to the differentiation state of the spleen cells, which may harbor fewer cells capable of proliferating and responding to sOPGL, perhaps suggesting that fewer spleen cells express either RANK or MCSF receptors. In addition, spleen cells may not provide other stimulatory factors that enhance osteoclast development; for instance, Sells Galvin et al. (25) reported that TGF-β had a direct stimulatory effect on osteoclastogenesis in hematopoietic cells treated with sOPGL/ODF and MCSF. Furthermore, our data did show that increasing McMCSF far above levels that would generally be expressed in bone would further enhance osteoclast formation from spleen progenitors. The source of osteoclast progenitors thus represents a significant variable in studies that evaluate the effects of stimulators and inhibitors of osteoclast development. Because marrow cells, and not spleen or peripheral blood cells, are precursors for bone osteoclasts in most organisms, lower levels of McMCSF are likely adequate to support osteoclastogenesis.

Thus the major limiting factor for osteoclast induction in marrow culture is OPGL. Previous results showing that very high levels of sMCSF were associated with decreased entry of proliferating cells into the osteoclast lineage can now be reinterpreted as being limited by the fixed OPGL potential presented by stromal cells (6, 20). Furthermore, it is unlikely that small changes in McMCSF induced by resorptive factors such as TNF-α (33) and 1,25D (24) or physical factors such as hydrostatic pressure (21) have significant effects on osteoclast recruitment. Alternatively, BMMs cultured with increasing amounts of sOPGL on fixed ST-2 cell layers providing equivalent McMCSF showed a clear sOPGL dose dependence in terms of osteoclast formation. In summary, our results suggest that the osteoclastogenic potential of the marrow environment is dominantly regulated by OPGL, whereas McMCSF serves as a competence factor. It is likely that agents regulating osteoclastogenesis do so directly or indirectly through OPGL.

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