Macrophage Colony-stimulating Factor Stimulates Survival and Chemotactic Behavior in Isolated Osteoclasts

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Summary

Macrophage colony-stimulating factor (M-CSF) is known to play an important role in osteoclast formation. However, its actions on mature cells have not been fully characterized. We now report that M-CSF dramatically stimulates osteoclastic motility and spreading; osteoclasts responded to a gradient of M-CSF with orientation, and random cell polarization occurred after isotropic exposure. M-CSF also supported the survival of osteoclasts by preventing apoptosis. Paradoxically, M-CSF inhibits bone resorption by isolated osteoclasts. We found that this was effected predominantly by reduction in the number of excavations. Thus, M-CSF showed a propensity to suppress resorption through a reduction in the proportion of cells that were resorbing bone. Our data suggest that apart from the established role of M-CSF in the provision of precursors for osteoclastic induction, a major role for M-CSF in bone resorption is to enhance osteoclastic survival, migration, and chemotaxis. It seems appropriate that during these processes resorptive functions should be suppressed. We suggest that M-CSF continues to modulate osteoclastic activity once osteoclasts are on resorptive sites, through regulation of the balance between resorption and migration, such that not only the quantity, but the spatial pattern of resorption can be controlled by adjacent M-CSF-secreting cells of osteoblastic lineage.

Materials and Methods

Recombinant human M-CSF was kindly provided by Dr. S. C. Clark, (Genetics Institute, Cambridge, MA). Mitramycin, cyclohexamide, and bovine serum albumin (BSA; used at 1 mg/ml) were purchased from Sigma Chemical Co. (Poole, UK). Medium 199 (Imperial Laboratories, Andover, UK) was used for osteoclast isolation and sedimentation, and Eagle's minimum essential medium (MEM; Imperial Laboratories) was used for subsequent incubations unless stated otherwise. Media were supplemented with 100 IU/ml benzylpenicillin and 100 μg/ml streptomycin (both from Imperial

1 Abbreviations used in this paper: CTR, calcitonin receptor; M-CSF, macrophage colony-stimulating factor; TEM, transmission electron microscopy; TRAP, tartrate-resistant acid phosphatase.
Laboratories). All incubations were performed at 37°C in 5% CO₂ in humidified air.

Slices of devitalized bovine cortical bone, used as substrates for osteoclastic resorption, were prepared as previously described (22). Bone slices (4 × 3 × 0.1 mm) were cut with a low-speed saw (Isomet Corp., Springfield, VA), cleaned by ultrasonication in sterile water, washed, immersed in alcohol, and stored dry at room temperature.

Isolation and Culture of Osteoclasts. Osteoclasts were disaggregated from neonatal rat or mouse long bones as previously described (23). Wistar rats or MF-1 mice from the St. George’s Hospital Medical School colony were killed by cervical dislocation within 3 d of birth. The femora, tibiae, and humeri were removed, freed of adherent soft tissues, and cut across their epiphyses. The bones were cut with a scalpel blade into 2 ml 199 and the fragments were vigorously agitated using a plastic Pasteur pipette. Larger fragments were allowed to sediment for 30 s and the resulting suspensions were used in the experiments described in the next section. All experiments were designed in such a way that all variables received osteoclasts from the same sample. All experiments were performed using rat osteoclasts unless otherwise specified.

Survival Studies. Bone cell suspensions were added either to three wells of a 100 × 18 mm multiwell Petri dish (Sterilin, Teddington, UK) containing bone slices, or to wells of a 96-well plate (Sterilin) containing Thermanox (Gibco, Uxbridge, UK) coverslips and incubated for 15 min. Bone slices and coverslips were then removed, washed in PBS, and placed into individual wells of a 96-well plate. Cells were incubated for 2 or 24 h in a total vol of 200 μl MEM/BSA in the presence of M-CSF or vehicle. Calcinonin receptor (CTR)-positive cells were identified using iodinated salmon CT (24). For this, coverslips were washed in 199 and transferred to fresh wells containing 200 μl 199/BSA with labeled CT (0.2 nM). After 60 min incubation at room temperature, coverslips were fixed in formalin, washed extensively, air dried, and then coated with K5 nuclear emulsion (Ilford Inc., Basildon, UK). They were developed after 7 d at 4°C, and counterstained with Meyer’s hematoxyl. Osteoclasts on bone slices were fixed for 2 min in formalin and stained for tartrate-resistant acid phosphatase (TRAP) (25). The osteoclasts were then washed with PBS, and placed in the incubation chamber of an Olympus IMT-373 inverted microscope (Gallenkamp and Co., Ltd., London, UK). Their video images were then recorded, and the survival of the same osteoclasts was assessed 1–24 h after the addition of M-CSF (5 ng/ml) or vehicle to the cultures.

Morphologic changes associated with apoptosis were assessed by acridine orange staining (Sigma Chemical Co.) (5 μg/ml) of coverslips fixed after incubation, and by transmission electron microscopy. Transmission electron microscopy (TEM) was performed on cells incubated for 6 or 24 h in the presence or absence of M-CSF (5 ng/ml). Cells adherent to 100-mm diameter petri dishes after incubation were washed with PBS and then fixed with 2 ml 5% glutaraldehyde in 0.2 M cacodylate buffer for 30 min, dehydrated through graded alcohols, and critical-point dried from CO₂. Specimens were sputter-coated with gold and examined in a Zeiss DSM 940 scanning electron microscope (Zeiss, Oberkochen, Germany).

Assessment of Osteoclast Appearance in the Scanning Electron Microscope. Osteoclasts were sedimented onto bone slices as previously described and incubated for 30 min. They were then incubated in the presence of M-CSF (5 ng/ml) or vehicle for 5–60 min. Bone slices were fixed in 5% glutaraldehyde in 0.2 M cacodylate buffer for 30 min, dehydrated through graded alcohols, and critical-point dried from CO₂. Specimens were sputter-coated with gold and examined in a Zeiss DSM 940 scanning electron microscope (Zeiss, Oberkochen, Germany).

Assessment of Osteoclast Polarization. Orientation was evaluated in a Zigmond orientation chamber as described by Zigmond (28). Osteoclasts were sedimented onto the central area of a 22 × 40 mm glass coverslip for 15 min. The coverslip was then washed and
incubated in 199/BSA for 30 min to allow osteoclast spreading. The cell-free area of the coverslip was wiped and the coverslip was inverted over the bridge. The chamber was placed into a 37°C incubator, and 100 µl 199/BSA was added to one well and 100 µl 199/BSA containing 0.1–10 ng/ml M-CSF was added to the other. After incubation for 30 min the osteoclasts present over the bridge were assessed for orientation with an inverted microscope using a ×25 objective. Osteoclasts were adjudged to show orientation when pseudopodia were evident on one side of the cell and retraction filopodia and lobopodia were visible on the opposite side. When orientated, osteoclasts that showed pseudopodia in the 180° sector towards the test well were scored positive, whereas the pseudopodia that were in the 180° sector towards the control well were scored negative (28). The same criteria were used for assessment of polarization of osteoclasts after incubation on coverslips in a uniform solution of M-CSF (0.05–50 ng/ml) for 30 min.

Statistical Methods. Differences between groups were analyzed with the Student's t test.

Results

In the absence of exogenous growth factor, isolated osteoclasts showed morphological changes suggestive of apoptosis that were prevented by M-CSF (Figs. 1–3). Within 24 h, most osteoclasts showed an abrupt change of behavior, in

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Figure 2. Fluorescence photomicrographs of osteoclasts stained with acridine orange after incubation with M-CSF (5 ng/ml) (a and c) or vehicle (b and d) for 6 or 24 h. (a) 6 h: relatively diffuse nuclear staining, with visible nucleoli; (b) a cell incubated without M-CSF for 6 h shows irregular nuclear staining; (c) osteoclast incubated for 24 h in M-CSF shows intact nuclear structure; (d) control cell after a 24 h incubation shows nuclear fragmentation. Bar, 10 µm.

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which previously well-spread cells became rounded, phase-granular, and formed blebs (Fig. 1). Osteoclasts detached on gentle agitation of the culture vessel in this state, or if left undisturbed, showed nuclear and cytoplasmic disintegration after a period of hours. Cells stained with acridine orange after fixation showed that changes in nuclear texture, with chromatin clumping, preceded the terminal rounding and blebbing phase identified in phase contrast observations, with nuclear changes becoming visible within 2 h of isolation (Fig. 2). Electron microscopical observations also revealed nuclear changes characteristic of apoptosis (Fig. 3). Osteoclastic apoptosis was neither prevented nor induced by cycloheximide (1 \( \mu \text{M} \)) or mithramycin (100 nM), suggesting that in osteoclasts, activation of a latent enzyme (29) may be involved in the process (data not shown).

Osteoclastic survival was considerably enhanced by addition of M-CSF to cultures, whether survival was measured as TRAP- or CTR-positive multinuclear cells (Fig. 4). Enhanced survival was also documented by observation of particular osteoclasts by time-lapse video microscopy for 24 h. We found that 2 out of 20 control cells, and 13 out of 20 osteoclasts incubated with M-CSF (5 ng/ml) survived. Apoptosis in serum-free medium appeared to be initiated and become irreversible relatively soon after osteoclast isolation, since M-CSF addition 2 h after isolation did not significantly increase survival compared to M-CSF-free cultures (Fig. 4). This action of M-CSF to sustain osteoclastic survival was reflected in suppression of apoptosis-associated changes in nuclear morphology (Figs. 2 and 3).

Because we have previously found (21) that M-CSF inhibits bone resorption, the improved survival of osteoclasts in M-CSF was unexpected. We therefore tested, in view of the close lineage relationship between macrophages and osteoclasts, for evidence of a possible action for M-CSF in diverting osteoclasts

Figure 3. Transmission electron micrograph of osteoclast incubated with M-CSF (5 ng/ml) (a) or vehicle (b) for 24 h. (a) Dispersed chromatin in two morphologically normal nuclei in M-CSF-treated cell; (b) condensed chromatin in nuclei of apoptotic osteoclast incubated without M-CSF. Bar, 10 \( \mu \text{m} \).

Figure 4. Number of CTR-positive cells or TRAP-positive cells per \( \text{cm}^2 \) after incubation for 2 or 24 h in the presence of M-CSF or vehicle. M-CSF was added at the initiation of incubation except where shown (at 2 h) to be added 2 h after the start of incubation. *\( p <0.05 \); **\( p <0.01 \) vs. 24-h control cultures. Each column is derived from five cultures.
towards an alternative, macrophagic role. We found, however, that rat osteoclasts incubated in M-CSF (5 ng/ml) for 24 h remained nonspecific esterase-negative, despite suppression of bone resorption by M-CSF during this time; and both rat and murine (which similarly were suppressed from apoptosis by M-CSF [data not shown]) osteoclasts remained negative for all the antigenic markers used, which are present on macrophages but not detected on osteoclasts (7, 26, 27). Moreover, osteoclasts incubated for 24 h with M-CSF (Fig. 4) remained strongly CTR-positive, not subjectively different in grain density from osteoclasts incubated for 2 h.

Since macrophages are unable to excavate bone (30), a further clue to a possible action of M-CSF to induce osteoclasts to undertake macrophagic differentiation might be reflected in a progressive decline in the ability of the cells to resorb bone. Thus, the ability of M-CSF to inhibit resorption would become greater with increasing incubation time. We found that the reverse was the case. Resorption-suppression was two orders of magnitude greater for resorption measured after 6 h of incubation (Fig. 5), than after 18 h (21), and resorption-suppression at 6 h showed a very similar sensitivity to M-CSF to the dose-responsiveness of osteoclasts for increased survival by M-CSF (Fig. 4). Moreover, if resorption was compared after 6 and 24 h in the same experiment (Table 1), it appears that while the resorption rate falls in control cultures, perhaps attributable to the decrease in osteoclast number during culture, the resorption rate was sustained in M-CSF-incubated cultures at a relatively steady rate through the incubation period. Thus, we found no evidence of a progressive increase with time in resorption-suppression by M-CSF, which might have reflected differentiation of osteoclasts towards a nonresorptive cell type.

Similar to our results on plastic, osteoclast survival on bone was enhanced by the presence of M-CSF (Fig. 4 and Table 1). This is unlike our experience in similar experiments which were performed in the presence of serum (21), which improves the survival of osteoclasts in vitro (data not shown). The lack of significant inhibition of bone resorption measured after 24 h in the present experiment is attributable to the impaired survival of osteoclasts in the absence of M-CSF and serum (Table 1).

**Table 1. Bone Resorption by Osteoclasts Incubated for 6 or 24 h in M-CSF or Vehicle**

| No. of excavations per bone slice | Plan area of excavations | Excavation depth | Plan area resorbed per bone slice | Multinucleate cells per bone slice | Rate of resorption per bone slice for periods 0–6 and 6–24 h |
|----------------------------------|-------------------------|-----------------|----------------------------------|-----------------------------------|----------------------------------------------------------|
|                                  | mean ± SEM              | μm² ± SEM       | μm ± SEM                         | μm² ± SEM                         | mean ± SEM                                              |
| Control 6 h                      | 5 ± 0.6                 | 813 ± 41        | 4.4 ± 0.2                        | 4084 ± 536                        | 6.3 ± 0.7                                                |
|                                  | (42)                    | (211)           | (63)                             |                                   |                                                         |
| 24 h                             | 5.9 ± 0.7               | 1300 ± 74⁰      | 4.5 ± 0.3                        | 7703 ± 1116⁰                      | 5.7 ± 1.0                                                |
|                                  | (42)                    | (248)           | (41)                             |                                   |                                                         |
| M-CSF 6 h                        | 2.2 ± 0.3⁴              | 749 ± 61        | 3.6 ± 0.2⁴                       | 1675 ± 275¹                       | 9.9 ± 1.1⁴                                               |
|                                  | (41)                    | (92)            | (43)                             |                                   |                                                         |
| 24 h                             | 5.5 ± 0.9⁰              | 1172 ± 64⁰      | 3.9 ± 0.2                        | 6062 ± 1004⁰                      | 10.2 ± 1.5⁵                                              |
|                                  | (42)                    | (229)           | (27)                             |                                   |                                                         |

Osteoclasts were incubated on bone slices for 6 or 24 h in M-CSF (50 ng/ml) or vehicle, before quantification of cell number and bone resorption. Numbers in parentheses represent the number of observations. Each group is derived from seven experiments, with six bone slices per variable per experiment. A random sample of excavations was assessed for excavation depth.

<sup>5</sup> p <0.05 vs. control; <sup>0</sup> p <0.01 vs. 6 h; <sup>↑</sup> p <0.01 vs. control.

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![Figure 5](image-url)  
**Figure 5.** Plan area resorbed per bone slice by osteoclasts incubated with M-CSF or vehicle for 6 h. *p <0.05 vs. control.
The reduction in excavation depth by M-CSF (Table 1) excludes the possibility (which would resolve the discordant actions of M-CSF to stimulate survival and suppress resorption), that the previously identified decrease in plan area of resorption (21) might have been associated with an increase in resorption depth.

In control cultures most osteoclasts produced an excavation (see Table 1). Since the number of excavations did not increase between 6 and 24 h, there appeared to be little initiation of fresh excavations after 6 h of incubation in control cultures (Table 1), consistent with the poor viability of these cells. However, osteoclasts incubated with M-CSF seemed to continue to initiate resorption after 6 h since the number of excavations was more than doubled at 24 h compared to 6 h. Excavation size (plan area and depth of each excavation) was only slightly reduced by M-CSF, while the proportion of osteoclasts resorbing bone appeared to have been reduced at 6 h from ~80% in control cultures to ~20% in M-CSF. Thus, the predominant explanation for the suppression of bone resorption by M-CSF appeared to be through a reduction in the proportion of osteoclasts that were resorbing bone, rather than through an equal suppression of the activity of each osteoclast.

The clear suppression of bone resorption by M-CSF seemed perverse for a cytokine that supported osteoclastic survival, and raised the possibility that M-CSF might stimulate some function in osteoclasts other than bone resorption. We found that addition of M-CSF to osteoclasts in serum-free medium was consistently followed within 30 s by a dramatic change in behavior (Fig. 6), consisting of a rapid increase in pseudopodial motility, with the formation of a peripheral phase-dense band that soon encircled the cell, associated with waves of pseudopodial activity starting at the cell periphery and passing towards the centre of the cell. This was accompanied by cell spreading. Motility then returned towards control levels, and cytoplasmic spreading was maintained, for at least the

Figure 6. Morphological changes induced in an osteoclast by exposure to M-CSF. Phase-contrast micrographs show appearance of osteoclast immediately before M-CSF (5 ng/ml) addition (a), and 1 (b), 2 (c), 5 (d), 10 (e), and 30 (f) min afterwards. A peripheral phase-dark margin develops rapidly, associated with intense local cytoplasmic motility and followed by cell spreading. Pseudopodial motility then abates but the cell maintains a relatively well-spread state (f). Bar, 10 μm.
duration of the experiments (24 h), at a higher level. The cell spreading phenomenon showed a dose-response relationship, and occurred on both plastic and bone substrates (Figs. 7 and 8). After incubation in M-CSF, osteoclasts showed an appearance similar to that seen in serum, and the morphological change induced by M-CSF was not consistent in the presence of serum (data not shown). No change in behavior was observed after addition of vehicle. The rapid, dramatic, transient change in behavior resembled a response to chemotactic agents (31). Such agents also characteristically induce orientation in gradients, and polarization in isotropic concentrations of the same agents (32, 33). We found that osteoclasts oriented towards an M-CSF source (Figs. 9 and 10), and an increased proportion of osteoclasts became polarized after 30 min of incubation in 0.05 and 0.5 ng/ml of M-CSF (percent ± SEM: vehicle 28 ± 18; M-CSF 0.05 ng/ml: 66 ± 17 [p < 0.01 vs. control]; 0.5 ng/ml: 46 ± 11 [p < 0.05] [six cultures per variable]). At concentrations of 5 ng/ml and above osteoclasts regularly showed active pseudopods over at least most, and generally the whole of their circumference, a phenomenon also observed after incubation of other target cells in high uniform concentrations of chemotactic agents (32, 33).

Discussion

The actions of M-CSF on cells of the mononuclear phagocyte system have been well characterized, and include promotion of survival, proliferation, differentiation, and several aspects of mature cell function (14–18). Although it has recently been shown that M-CSF is also important in osteoclast formation (8, 9), much less is known about the mechanisms by which M-CSF acts in osteoclast differentiation, or about its role, if any, in the regulation of the function of mature cells.

We now report that M-CSF promotes the survival of mature osteoclasts through suppression of apoptosis. Survival was enhanced at very low concentrations of M-CSF, suggesting that the phenomenon might have physiological significance. However, although this is the first identification of an osteoclastic survival factor, others may exist. Kodama et al. (34) have shown prolonged survival of osteoclasts in op/op mice, which are deficient in M-CSF, after a single injection of M-CSF.

The ability of M-CSF to support osteoclast survival makes it even more surprising that it inhibits bone resorption in vitro. Nevertheless, we found that the responsiveness of os-
teoclasts to resorption-inhibition was very similar to their sensitivity to M-CSF as a survival factor. It seems perverse that increased survival be associated with a reduction in the primary function of a cell. One possibility, in view of the shared origin of osteoclasts and macrophages, is that M-CSF might enhance macrophagic differentiation in osteoclasts.

Thus, it is known that in the presence of bone marrow stromal cells, hemopoietic precursors differentiate into osteoclasts, but in the presence of M-CSF alone, the same precursors differentiate only into macrophages (1, 3). Just as mononuclear phagocytes may dedifferentiate on removal of M-CSF (35), and have been found by some (36) (but not all [27]) workers to be capable of differentiation into osteoclasts in the presence of stromal osteoclast-forming activity (SOFA), it may be that, upon removal from SOFA, osteoclasts become capable of macrophagic differentiation in the presence of M-CSF.

Our results do not support this possibility. We found no evidence for macrophagic differentiation of osteoclasts in the presence of M-CSF. Moreover, if resorption inhibition by M-CSF were explicable as differentiation towards macrophages, which cannot excavate bone (30), we would anticipate a progressive decline in the rate of resorption by osteoclasts incubated in M-CSF. We found that bone resorption was inhibited to a similar degree early and late in the incubation period.

A second possible explanation for the inverse actions of M-CSF on osteoclastic survival and resorptive function might be that the stimulation of osteoclasts, implied by its potent action on survival, may be of some aspect of osteoclastic function that is not directly involved in the process of bone resorption itself.

Our results suggest that M-CSF plays a role in osteoclastic

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**Figure 9.** Number of osteoclasts oriented towards (open histograms) or away from (hatched histograms) source of M-CSF after incubation for 30 min in a Zigmond chamber. *p < 0.05; **p < 0.01 vs. oriented away. Each result is derived from at least 60 osteoclasts in seven separate experiments.

**Figure 10.** Phase contrast photomicrograph of osteoclast viewed on central bridge of a perspex Zigmond chamber after incubation with M-CSF (1 ng/ml) in the test chamber (on right of photomicrograph) that shows orientation towards source of gradient. Bar, 25 μm.
cytes: recently, osteoclasts have been shown to possess receptors for a chemotactic agent (31–33, 37). Also typical of a chemotactic response, osteoclasts showed positive orientation to M-CSF gradients, and an increase in the proportion of cells demonstrating random polarization in isotropic concentrations of M-CSF. Polarization of pseudopodial activity implies migration in the direction of the expanded, leading pseudopods in many cell types, including osteoclasts (38, 39; see Fig. 1, a–c).

Bone resorption occurs throughout development and adult life in intricate and dynamic patterns. There is much evidence that this spatial organization of osteoclastic activity is under the control of cells of the osteoblastic lineage (4, 40, 41), which have been shown to produce M-CSF (42). Our data suggest that the function of this M-CSF production may be to attract osteoclasts to resorptive sites, and to maintain their survival during migration.

A role for M-CSF to induce osteoclastic migration through tissues may be analogous to its role in the M-CSF–dependent migration through tissues of trophoblastic cells (43), monocytes (44, 45), and some c-fms–expressing breast cancer cells (46). There is evidence that suggests that osteoclasts might share other migratory mechanisms with mononuclear phagocytes: recently, osteoclasts have been shown to possess receptors for urokinase-type plasminogen activator (47), and to produce messenger RNA for collagenase (48). These neutral-pH active proteinases appear not to play a direct role in the excavation of bone (49), but they are required for bone resorption in intact bone tissue (50), perhaps to enable osteoclastic migration to resorptive sites.

Although M-CSF can stimulate some functions in macrophages as in osteoclasts, it is interesting that M-CSF suppresses activities that are directly related to the ultimate function of both cell types; bone resorption in osteoclasts, and superoxide generation in macrophages (51). Both activities may be inappropriate during migration, and this suggests that a primary role of M-CSF might be to induce migration of target cells through tissues.

It is intriguing to note that the dominant action of M-CSF on bone resorption by osteoclasts was not to suppress the rate of resorption by all osteoclasts equally, but to reduce the proportion of osteoclasts that were resorbing bone. This suggests that bone resorption and migration might represent alternative states of osteoclastic activity, that are to some extent mutually exclusive. Consistent with this, resorptive surfaces in vivo show a scalloped appearance, suggestive of episodic resorption and migration (52–54). We have previously found that contact with bone mineral induces resorptive behavior in osteoclasts; and because the mineral phase of bone is resorbed more rapidly than the organic phase, resorption itself progressively reduces mineral contact (55). Thus, on bone surfaces in vivo, as on our bone slices in vitro, mineral contact and M-CSF exposure might represent opposing stimuli that determine the behavioral state of osteoclasts, such that as resorption proceeds, migration becomes increasingly likely. Consistent with this thesis is the relative shallowness of excavations in normal animals in vivo, compared with the more irregular resorptive surface in SEM photographs of bone surfaces from op/op animals lacking M-CSF (56).

Our data thus suggest that the role of M-CSF in bone resorption is to enhance osteoclastic survival, migration, and chemotaxis. It seems appropriate that during these processes resorptive functions should be suppressed. We suggest that M-CSF continues to modulate osteoclastic activity once osteoclasts are on resorptive sites through regulation of the balance between resorption and migration, such that not only the quantity but the spatial pattern of resorption can be controlled by adjacent M-CSF-secreting cells of osteoblastic lineage.

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