Identification and Characterization of Osteoclast Progenitors by Clonal Analysis of Hematopoietic Cells

By Minako Y. Lee, Joan L. Lottsfeldt, and Karen L. Fevold

We have identified a distinct population of colony-forming cells that give rise to mononuclear cells expressing an enzyme marker and other features of the osteoclast in bone marrow cultures stimulated by conditioned medium of a murine tumor cell line. These colony-forming cells were defined as osteoclast colony-forming units (CFU-O). The tumor cell-derived activity was recently isolated and was named osteoclast colony-stimulating factor (O-CSF). To understand the development of osteoclast progenitors and to clarify the relationship of osteoclast progenitors to other hematopoietic progenitors, we examined CFU-O in hematopoietic tissues obtained from normal adult mice, mouse fetuses, and mice with 5-fluorouracil (5FU) treatment. CFU-O were present in the adult mouse bone marrow, adherent cell-depleted marrow, in the spleen, and in the day 14 fetal liver, with an incidence similar to other hematopoietic progenitors. The culture period required for the development of CFU-O-derived colonies in vitro and the manner in which CFU-O responded to 5FU suggested that CFU-O belonged to a relatively primitive progenitor population; they are clearly more immature than macrophage progenitors that respond to macrophage-CSF, but more mature than multilineage progenitors that respond to stem cell factor. Our studies have defined and characterized an osteoclast progenitor and distinguished it from other hematopoietic progenitors for the first time.

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MATERIALS AND METHODS

Mice. (Balb/c × CE)F1 mice were bred in the vivarium at the University of Washington. Parental male CE and female Balb/c mice, and C57Black6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Tumor cell cultures. A cloned cell line of a hypercalcemia- and granulocytosis-inducing murine mammary adenocarcinoma, designated CESJ13,14 was used. Each CESJ clone has been shown to produce bone-modulating activity in addition to producing granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF).11,12 Two other murine tumors were used as controls: a mammary carcinoma clone (Bo66) that does not induce neutrophilia nor hypercalcemia in mice,13 and produces M-CSF, but not G-CSF12; and a murine fibrosarcoma clone (NFSa-c9) that causes neutrophilia,14 but not hypercalcemia (M.Y. Lee, unpublished observations) and is a known source of murine G-CSF and M-CSF.12 All tumor cells were cultured and maintained in serum-free, HL-1 medium (Ventrex Division, Hycor Biomedical, Portland, ME), supplemented with 2 mmol/L L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.125 µg/mL amphotericin B (GIBCO, Grand Island, NY).

Tumor cell CM. The supernatant of culture medium in which tumor cells had been cultured for 7 to 8 days was concentrated approximately 500-fold by ultrafiltration using Minatan apparatus with 10,000 molecular weight cut off membranes (Millipore Corp, Bedford, MA), or Amicon apparatus with YM10 membranes (Amicon Corp, Danvers, MA), filtered (Millex GV, 0.22 µm; Millipore), and stored at −70°C in 2-mL aliquots. The CM of CESJ3 cells was designated as CESJ medium. For some experiments, O-CSF was semipurified by Sephacryl HR S-200 gel

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filtration column chromatography and reverse-phase high performance liquid chromatography (HPLC). Colonies were analyzed by colony formation in semisolid culture medium. Briefly, BM cells were obtained from the femurs of normal young adult mice by grinding the whole femur, as described. In some experiments, nonadherent BM cells were selected by passing the marrow cell suspension through Sephadex G10 columns. Marrow cells in single cell suspension were cultured in 35 × 10 mm petri dishes (Falcon; Becton Dickinson and Co, Oxnard, CA) or in 15 × 10 mm Linbro wells (Flow Laboratory, McLean, VA) at 10^5 cells/mL, in supplemented medium 199 containing 20% fetal calf serum (Sterile Systems, Logan, UT), 0.3% Bacto agar (Difco Laboratories, Detroit, MI), and various amounts of tumor cell CM or other test samples. Spleen cells were obtained by teasing the organ and were cultured using 5 × 10^5 cells/mL. Liver cells obtained from day 14 mouse fetuses were passed through Sephadex G-10 columns and were similarly cultured at various cell concentrations. Agar cultures were incubated at 37°C in a humidified atmosphere with 5% CO2 for 7 to 21 days.

**Analysis of colonies by cytochemical staining.** Colonies derived from putative osteoclast progenitors were identified by staining for TRAPase activity. The entire agar gel was transferred from the culture dish onto a glass slide, fixed by 10% (vol/vol) neutral buffered formalin, and dried. After slides were prehydrated for 1 hour at 37°C in a 0.2 mol/L Tris buffer solution (pH 9.0), TRAPase activity was demonstrated using Naphthol AS-TR phosphate (Sigma, St Louis, MO) as the substrate, hexazoniated pararosanilin as a coupling dye, and 10 mmol/L L(+)-tartaric acid (Sigma) as inhibitor. Slides were counter stained with toluidine blue. Mononuclear cells containing TRAPase activity stained an easily identifiable red color. Colonies were examined under an inverted microscope and three categories of TRAPase colonies were defined such that positive, mixed, and negative referred to colonies greater than 90%, 90% to 10%, and less than 10% of red staining cells in a colony, respectively. Colonies were defined as groups of 50 or more cells. All colonies appearing in an agar plate were scored and the result was expressed as colony numbers per unit of cell numbers.

**5FU treatment.** BM cells were obtained from groups of 12-week-old male (Balb/c × C57)F1 mice, and 4 days after a single intravenous injection of 5FU (Sokopak Laboratory, Franklin Park, IL) at 150 mg/kg body weight. BM cells of strain, age- and sex-matched, saline-injected mice were used as controls. Colony assays of BM cells were performed as described above.

**Demonstration of bone excavation.** Dentine slices (8 × 8 × 0.1 mm) were prepared from cow teeth using a low speed saw (Isomet saw; Buehler Ltd, Lake Bluff, IL), sterilized by 70% ethanol and UV irradiation, and placed in the bottom of Linbro wells. BM cells (5 × 10^4) were cultured over the dentine slices in 0.5 mL culture medium containing 20% fetal calf serum, the optimal concentrations of tumor CM and 0.3% agar. After 14 to 28 days of incubation at 37°C in 5% CO2 atmosphere, colonies in the agar portion as well as colonies developed over the dentine pieces were scored for TRAPase activity by cytochemical staining and the location of the colony in relation to the dentine slice was recorded. The dentine pieces were then immersed in sodium hypochlorite (50% vol/vol) for 30 minutes to remove cells, washed in distilled water and dehydrated in ethanol. The specimen was attached onto stubs, sputter-coated with gold/palladium, and viewed with a scanning electron microscope (Autoscan, ETEC Co).

**Growth factors.** Recombinant growth factors were from the following sources: murine G-CSF from Dr S. Nagata (Osaka Bioscience Institute, Osaka, Japan); murine granulocyte-macrophage-CSF (GM-CSF), murine interleukin-6 (IL-6) and murine IL-1 from Genzyme (Boston, MA); murine IL-3 from Biogen (Geneva, Switzerland). Purified murine M-CSF (Dr R. Shadduck, Pittsburgh, PA) or L-cell CM (LCM) was used as a source for murine M-CSF. Recombinant murine stem cell factor (SCF) was a kind gift from Dr S. Lyman (Immunex, Seattle, WA). Biologic activities of these CSFs were assessed by standard colony assays of BM cells.

**RESULTS**

Normal mouse BM cells cultured with concentrated CESJ medium formed distinct colonies predominantly composed of mononuclear cells that exhibit intense cytoplasmic red staining for TRAPase activity. As the concentration of CESJ medium in the culture increased, the number of TRAPase-positive colonies and the intensity of TRAPase reaction increased. At an optimal concentration of the CM in culture, more than 80% of colonies were in the TRAPase-positive or-mixed category. Most colonies in the TRAPase-positive category contained TRAPase-positive mononuclear cells mixed with TRAPase-negative macrophages or occasional granulocytes. At a higher concentration of the CM, colony numbers sharply decreased, while all colonies were strongly positive for the TRAPase-activity (Fig 1). In contrast, similarly prepared culture medium of control tumor cells, namely Bc66 (M-CSF-producing) and NFSA-c9 (G-CSF- and M-CSF-producing), stimulated many colonies, but virtually all of them were composed of macrophages that were negative for TRAPase (Fig 1).

As shown previously, coculture experiments of BM cells with dentin pieces in the presence of CESJ medium demonstrated rudimentary resorption sites corresponding to the locations where TRAPase-positive colonies or clusters were observed. These studies confirmed that some of the TRAPase-positive cells formed under our culture condition with CESJ medium have fundamental osteoclastic properties.

![Fig 1. Dose-related responses of TRAPase-positive colony-stimulating activity by CESJ medium. Concentrated, serum-free culture supernatant of CESJ, NFSA, or Bc66 cells was added at various concentrations to BM cultures, and day 14 colonies were stained for TRAPase activity. (I) TRAPase-positive colonies; (II) mixed colonies; (III) TRAPase-negative colonies. Vertical lines are the SD for each category of colonies determined from triplicate cultures of two experiments.](www.bloodjournal.org)
In time course studies, TRAPase-positive colonies were first observed on day 10 of culture, and the colony number reached the maximum level on day 14. Extension of the culture period to day 21 did not further enhance colony formation (Fig 2). Therefore, day 14 colonies were routinely evaluated. In contrast, colonies stimulated by NFSA-c9 or Bc66 tumor CM were TRAPase-negative on days 10, 14, and 21 (data not shown). Using the optimal concentration of CESJ medium, a cell dose relationship was examined using fresh whole BM cells or adherent cell-depleted BM cells. The number of TRAPase-positive colonies increased with increasing numbers of cells plated for both whole BM cells or adherent cell-depleted BM cells (Fig 3). Between 3 × 10^3 and 5 × 10^4 BM cells plated per 0.5 mL of culture, a linear relationship was observed with the regression line intercepting zero, for both whole and adherent cell-depleted BM cells, indicating each TRAPase-positive colony was clonally derived from a distinct progenitor. The incidence of progenitors that give rise to TRAPase-positive colonies was estimated to be about 1 in 10^5 normal BM cells. It is also likely that O-CSF acts directly on a target progenitor, and its action is stromal cell independent. There was no apparent strain specificity of O-CSF function because the time course experiments for TRAPase-positive colony formation suggested that osteoclast progenitors may belong to a relatively primitive progenitor population. In addition to the BM, osteoclast progenitors were also present in the adult mouse spleen with a ratio of about 3 in 10^6 spleen cells, and in the day 14 mouse fetal liver with a ratio of 2 in 10^5 fetal liver cells (Table 1). Thus, the incidence of osteoclast progenitors among hematopoietic cells is of a similar magnitude to that reported for various myeloid progenitors. The demonstration of osteoclast progenitors in these organs by stripped-bone coculture experiments.

Because the time course experiments for TRAPase-positive colony formation suggested that osteoclast progenitors may belong to a relatively primitive progenitor population, we examined the response of TRAPase-positive colony-forming cells to 5FU treatment to assess the immaturity of these cells in relation to other hematopoietic progenitors. BM cells obtained from mice 2 and 4 days after a single injection of 5FU were cultured in the presence of optimal concentrations of CESJ medium, LCM, or SCF. TRAPase-stained colonies were evaluated after 14 days of incubation for cultures stimulated by CESJ medium or SCF, and after 7 and 14 days of incubation for cultures stimulated by LCM. The results are summarized in Fig 4. In the BM of mice 2 days after 5FU injection, progenitors that give rise to TRAPase-positive colonies in response to CESJ medium were significantly (P < .005) reduced to approximately 17% of the saline controls, but were not completely abolished (Fig 4A). By day 4, a rapid recovery of these progenitors was observed. More than 90% of the colonies stimulated by the optimal concentration of CESJ medium were TRAPase-positive.

SCF stimulated a variety of multilineage colonies, most of which were TRAPase negative; however, a few contained TRAPase-positive cells. These multilineage progenitors were less sensitive to 5FU treatment than those responding to CESJ medium, and were reduced to about 30% of the saline controls (P < .005) on day 2 post-5FU treatment (Fig 4B). By day 4 post-5FU injection, progenitors that give rise to TRAPase-positive colonies in response to CESJ medium were significantly (P < .005) reduced to approximately 17% of the saline controls, but were not completely abolished (Fig 4A). By day 4, a rapid recovery of these progenitors was observed. More than 90% of the colonies stimulated by the optimal concentration of CESJ medium were TRAPase-positive.
Table 1. The Incidence of Progenitors That Form TRAPase-Positive Colonies in Mouse Hematopoietic Organs

<table>
<thead>
<tr>
<th>Organs</th>
<th>No. of Cells/Plate</th>
<th>TRAP-Positive</th>
<th>TRAP-Mix</th>
<th>TRAP-Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>10^5</td>
<td>67.3 ± 11.9</td>
<td>11.7 ± 4.2</td>
<td>6.3 ± 0.6</td>
<td>85.3 ± 12.9</td>
</tr>
<tr>
<td>Spleen</td>
<td>5 × 10^6</td>
<td>16 ± 5.7</td>
<td>3.5 ± 2.1</td>
<td>2.0 ± 2.4</td>
<td>21.5 ± 10.6</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>5 × 10^4</td>
<td>92.3 ± 5.0</td>
<td>19.7 ± 5.8</td>
<td>1.7 ± 0.6</td>
<td>117 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^4</td>
<td>59.3 ± 4.0</td>
<td>7.6 ± 3.5</td>
<td>0</td>
<td>66.3 ± 7.5</td>
</tr>
<tr>
<td></td>
<td>1.25 × 10^4</td>
<td>40.3 ± 12.7</td>
<td>3.7 ± 3.2</td>
<td>0</td>
<td>43.7 ± 9.6</td>
</tr>
</tbody>
</table>

Colonies were stimulated by CESJ medium, used at 10% (vol/vol) in cultures. Values are means ± SD obtained from four mice for BM and spleen, and four fetal livers.

missing in the BM, but day 14 macrophage colony-forming cells showed modest recovery, some of which showed TRAPase positivity. These studies suggest that progenitors that respond to O-CSF are distinctly different from those that respond to SCF or M-CSF in their maturational stages as well as in their ability to give rise to TRAPase-positive progenies. It is likely that osteoclast progenitors belong to a relatively primitive progenitor compartment in a hematopoietic stem cell hierarchy, clearly more immature than M-CSF responsive macrophage progenitors, but perhaps more mature than SCF-responsive primitive progenitors.

To further understand the relationship of osteoclast progenitors to other hematopoietic progenitors, we examined the effect of various recombinant murine hematopoietic growth factors on TRAPase-positive colony formation in vitro. Day 14 colonies formed by SCF, IL-3, IL-1, IL-6, GM-CSF, G-CSF, and M-CSF were stained for TRAPase expression and were compared with those stimulated by CESJ medium or purified O-CSF (Table 2). In support of our data obtained from control B66 and NFSA-c9 tumor CM, murine G-CSF, M-CSF, or a combination of both CSFs did not stimulate any TRAPase-positive colonies, suggesting a limited effect of these relatively lineage-specific CSFs. As expected, most of the TRAPase-negative colonies were granulocytes when cultures were stimulated by G-CSF, or by a combination of M-CSF and G-CSF. TRAPase-positive colonies were not stimulated by the doses of IL-6 (20 to 40 ng) or IL-1 (10^2 to 10^3 U) we tested. Optimal concentrations of GM-CSF or IL-3, and SCF at several concentrations, stimulated some TRAPase-positive and -mixed colonies. However, in contrast to discrete colonies stimulated by CESJ medium that consisted of single mononuclear cells exhibiting strong TRAPase positivity, TRAPase reaction of colonies stimulated by these growth factors was weak, mostly showing diffuse positivity in the center of a colony. SCF-induced TRAPase-mixed colonies contained TRAPase-positive cells among various TRAPase-negative cells, while the majority of colonies stimulated by SCF were TRAPase-negative showing multilineage morphology. Reverse-phase HPLC-purified O-CSF stimulated clusters of 8 to 50 cells, with the majority demonstrating TRAPase activity. This profile of responses indicates that osteoclast progenitors that respond to O-CSF may also be stimulated by some of the growth factors known to stimulate primitive hematopoietic cells.

Fig 4. Response of progenitors to 5FU treatment. BM cells were obtained on day 2 and day 4 post-5FU injections and cultured in the presence of CESJ medium (5% vol/vol) (A), SCF (100 ng/mL) (B), or LCM (1% vol/vol) (C and D). Day 14 colonies were evaluated for CESJ medium and SCF; day 7 (C) and day 14 (D) for LCM. Values are means ± SD of data obtained from four mice per experimental group. (■) TRAPase-positive colonies; (□) TRAPase-mixed colonies; (□) TRAPase-negative colonies.
Table 2. Comparative Responses of BM Cells to Growth Factors

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Doses*</th>
<th>TRAP-Positive</th>
<th>TRAP-Mix</th>
<th>TRAP-Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>10 ng</td>
<td>0</td>
<td>0</td>
<td>56 ± 8.7</td>
<td>56 ± 8.7</td>
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<tr>
<td>M-CSF (LCM) 10%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>80 ± 11.3</td>
<td>80 ± 11.3</td>
</tr>
<tr>
<td>G-CSF (5 ng) + LCM (10%)</td>
<td>0</td>
<td>0</td>
<td>239 ± 8.3</td>
<td>239 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>50 U</td>
<td>8 ± 2.8</td>
<td>13 ± 2.8</td>
<td>59 ± 3.5</td>
<td>80 ± 0.7</td>
</tr>
<tr>
<td>IL-3</td>
<td>12.5 U</td>
<td>3 ± 2.1</td>
<td>23 ± 2.1</td>
<td>29 ± 4.9</td>
<td>54 ± 9.2</td>
</tr>
<tr>
<td>SCF</td>
<td>50 ng</td>
<td>9 ± 4.2</td>
<td>36 ± 5.6</td>
<td>71 ± 12.4</td>
<td>116 ± 14.2</td>
</tr>
<tr>
<td>100 ng</td>
<td>17 ± 1.4</td>
<td>29 ± 7.0</td>
<td>69 ± 1.4</td>
<td>115 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>250 ng</td>
<td>29 ± 7.1</td>
<td>39 ± 4.2</td>
<td>73 ± 1.4</td>
<td>141 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>CESF medium</td>
<td>5.0%</td>
<td>116 ± 11.3</td>
<td>18 ± 11.3</td>
<td>19 ± 4.2</td>
<td>153 ± 4.2</td>
</tr>
<tr>
<td>O-CSF†</td>
<td>10%</td>
<td>153.2 ± 9.0</td>
<td>3.2 ± 3.9</td>
<td>45.2 ± 34.2</td>
<td>202 ± 36.0</td>
</tr>
</tbody>
</table>

Values are means ± SD of colony numbers grown in triplicate dishes.

*Doses shown are per milliliter of culture.
†The values given for purified O-CSF represent the number of clusters stimulated by samples from four separate reverse-phase HPLC preparations.

Clusters were defined as groups of cells greater than 8 but less than 50.

DISCUSSION

Osteoclasts play essential roles in bone resorption and in the development of medullary hematopoiesis. A number of experiments using BM transplantation and chimeric animals1-3 have clearly shown that osteoclasts are of hematopoietic origin. Like hematopoietic stem cells,24 embryonic osteoclast progenitors are distributed from the yolk sac to other tissues and hematopoietic organs through the vascular circulation.25 At the site of bone resorption, multinucleated giant osteoclasts are formed by the fusion of precursor mononuclear cells under the influence of osteotropic hormones such as vitamin D3 and parathyroid hormone (PTH).4 Studies of osteoclasts in early developmental stages have been hampered because these undifferentiated cells would not respond to osteotropic hormones or express osteoclast specific receptors. Nevertheless, accumulated evidence suggests the presence of progenitor cells in the BM that will develop into mature osteoclasts under appropriate conditions in vitro.4,5,7,25,26

Recently, we have isolated an O-CSF from proteins secreted by a hypercalcemia-inducing murine mammary tumor cell line.10 O-CSF stimulates murine BM cells to form colonies that stain positively for TRAPase in semisolid culture medium. O-CSF activity of the tumor cell medium was not attributable to known hematopoietic CSFs.10 We have shown that at least a few of these TRAPase-positive colony cells exhibit typical features of the osteoclast upon cocultivation with devitalized bone or dentine pieces, indicating that the factor present in the tumor CM or the purified protein indeed stimulates osteoclast progenitors. The target cell of O-CSF is thus defined as an osteoclast colony forming unit (CFU-O).

In this study, we have characterized the osteoclast progenitors using the serum-free culture supernatant of a cloned cell line of this tumor as a source of O-CSF. These progenitors were present in the adult mouse BM, spleen, and the day 14 mouse fetal liver, with an incidence similar to that found in other hematopoietic progenitors. Based on time course studies of TRAPase-positive colony development and the response of the progenitors to 5FU treatment, CFU-O seems to belong to a relatively primitive progenitor population, more mature than multilineage progenitors that respond to stem cell factor, but more immature than macrophage progenitors or mature macrophages. We propose a hypothetical position for CFU-O in the hematopoietic progenitor hierarchy as shown in Fig 5.

In our experimental system, we have classified colonies by cytochemical staining for TRAPase activity into three categories. Because resorption areas were observed only in locations where TRAPase-positive cells were located on the dentin surface, and not in locations with TRAPase-negative cells,10 we have categorically defined osteoclast progenitors by virtue of TRAPase-positive colony formation in this study. The colonies that were defined as TRAPase-mix contained both TRAPase-positive cells and -negative cells. These colonies may be formed from progenitors that have receptors for O-CSF as well as other CSFs, or may be due to a suboptimal concentration of O-CSF. Our studies were not designed to investigate differentiation of TRAPase-mixed colonies. Studies of O-CSF receptors will help to further understand the osteoclast lineage in the future.

As in the case for other hematopoietic cell lineages, we...
expect functional overlap and/or synergistic effects of O-CSF with other growth factors. Target cells of O-CSF may overlap with those of other growth factors. Formation of TRAPase-mix colonies and a few TRAPase-positive colonies in cultures stimulated by SCF, IL-3, or GM-CSF suggest that these CSFs, which are known to stimulate relatively primitive hematopoietic cells, can also stimulate osteoclast progenitors. In fact, several studies have shown generation of osteoclasts or osteoclast-like cells from the BM in the presence of IL-3 or GM-CSF and vitamin D3 in various culture systems. The production of vitamin D metabolites or PTH from the CE tumor has been ruled out; thus, O-CSF appears to stimulate osteoclast progenitors in the absence of added vitamin D3 in vitro. We have previously reported an increased number of osteoclasts in the bone of mice that were persistently stimulated by G-CSF, GM-CSF, or erythropoietin. Such in vivo stimulation of osteoclasts may be due to marrow perturbation that causes local production of O-CSF and heightened response to O-CSF and other factors by osteoclast progenitors. The appearance of some TRAPase-positive cells in the post-5FU BM stimulated by M-CSF in our study may be explained on the basis of this marrow perturbation.

Recently, Udagawa et al have shown the development of colonies containing TRAPase-positive cells from mouse BM, spleen, blood, and other tissues by culturing these cells on a marrow stromal cell monolayer in the presence of vitamin D3 and dexamethasone. They estimated a much higher incidence of osteoclast progenitors in the BM and in the spleen than that we observed in this study. It is conceivable that in their system, monocytes or macrophages that are more mature than our CFU-0 have been stimulated by stromal cells and/or osteotropic hormones. The incidence of CFU-O in our study seems comparable with that of other hematopoietic progenitors. The primitive nature of the osteoclast progenitors we observed in this study is corroborated by the study that showed the development of osteoclasts from blast cell colonies formed from the spleen of 5FU-treated mice, and the study that showed osteoclast generation from CD34+, GM-CSF-responsive progenitors in human marrow cultures. It should be noted that resorption sites created by our TRAPase-positive colony cells in our study are rudimentary compared with those created by fully mature, functional osteoclasts. This may be an indication of the primitive nature of the CFU-O. It is probable that these CFU-O-derived cells need additional factors to facilitate further differentiation and maturation. Such additional factors may well include osteotropic hormones, other growth factors, and/or stromal cell-derived factors.

A mutation in the coding region of the M-CSF gene has been shown to cause osteopetrosis in op/op in mice and important biologic roles of M-CSF have been postulated in osteoclast development and function. As shown in this study, M-CSF appears to be the factor that affects progenitors more mature than CFU-O; therefore, its role may be for further maturation of CFU-O. Because it is believed that osteoclast progenitors are present, but the lack of M-CSF in the marrow microenvironment leads to abnormal development of osteoclasts in op/op mice, it will be interesting to examine the CFU-O and their maturation in this animal model. Further molecular and functional studies of O-CSF will elucidate the relationship of O-CSF to SCF and M-CSF, and their target cells.

In conclusion, we have developed an in vitro technique useful for dissecting the mechanisms of osteoclast differentiation from hematopoietic stem cells. With this technique, we have characterized the osteoclast progenitor in murine hematopoietic tissues, and have defined its position in the hematopoietic stem cell hierarchy.

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