Growth of osteoclast precursor-like cells from whole mouse bone marrow: inhibitory effect of CSF-1

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Summary

We studied the growth of mononuclear phagocytes (MPs) and mononuclear osteoclast precursor-resembling (OCP-like) cells from freshly isolated whole mouse bone marrow. Expression of tartrate-resistant acid phosphatase (TRAcP) served as a general marker for the identification of OCP-like cells. Bone resorbing capacity of the cultured cells was studied in a coculture assay with periost-free fetal bone rudiments. Freshly isolated mouse bone marrow contained approximately 30 OCP-like cells and 1100 MPs per 10\textsuperscript{6} nucleated bone marrow cells. OCP-like cell numbers did not increase in suspension cultures containing macrophage-colony stimulating factor (CSF-1), in contrast to the number of MPs which increased strongly. OCP-like cell numbers however did increase in monolayer cultures, which also allowed anchorage-dependent growth of bone marrow fibroblasts. Strongest increase of OCP-like cells occurred in monolayer cultures in the absence of CSF-1. Dermal fibroblasts of fetal mice did not enhance OCP-like cell growth. OCP-like cell density was strongly correlated with the number of osteoclast nuclei formed in cocultures with periost-free fetal bone rudiments. These data indicate that mononuclear cells, cytochemically and functionally resembling osteoclast precursors, may be grown from mouse bone marrow. CSF-1 inhibited growth of OCP-like cells, indicating that osteoclast precursors differ from mononuclear phagocytes in growth requirements.

Key words: Osteoclast precursor; Bone marrow culture

Introduction

During bone growth and remodeling, mineralized bone and cartilage matrix is re-
sorbed by osteoclasts, large multinucleated cells which form by fusion of mononuclear precursor cells. It is generally accepted that these precursor cells are of hematopoietic origin [1] and are related to the mononuclear phagocyte system [2–5]. The exact identity of the osteoclast precursor (OCP) is, however, still unknown. Even less understood are the conditions controlling OCP development. Major impediments to the study of osteoclast differentiation have been the lack of methods by which large numbers of osteoclast precursors can be isolated and the lack of identification markers, i.e., characterization assays, to determine the true osteoclastic nature of the isolated cells.

In earlier studies [2,6] we demonstrated the formation of multinucleated, mineral resorbing osteoclasts from bone marrow cells which had been precultured in the presence of CSF-1, and were subsequently cocultured with periost-free fetal bone rudiments. No data were presented on the identity of the osteoclast precursors, or the conditions controlling OCP cell growth, as OCPs could not be identified during preculture.

Since 1971, tartrate-resistant acid phosphatase (TRAcP) has been used as a marker to identify multinucleated osteoclasts [7–10]. Recently the enzyme was also demonstrated in mononuclear osteoclast precursors homing in the periosteum of fetal mouse bone rudiments, and in hitherto unidentified mononuclear cells of adult mouse bone marrow [11]. By contrast mouse mononuclear phagocytes showed only tartrate-sensitive acid phosphatase (TSAcP) activity.

In the present study, TRAcP-activity was used as a parameter to identify mononuclear osteoclast precursor-like cells cultured from freshly isolated mouse bone marrow. Bone resorbing capacity of OCP-like cells was tested in a bone resorption assay, using periost-free fetal bone rudiments [2]. Growth conditions of OCP-like cells were compared with those of mononuclear phagocytes which were also grown from freshly isolated mouse bone marrow.

Materials and Methods

Experimental animals
The study was performed with specific pathogen-free Swiss albino mice (Central Institute for the Breeding of Laboratory Animals, TNO Zeist, The Netherlands), i.e., 10–12 week old males weighing between 30 and 35 g, and fetuses from 17-day-old pregnant females.

Bone marrow cell cultures
Cell isolation Femora were isolated and removed intact from the hind limbs of adult males. After the bones were cleaned of adherent muscle both ends were severed in the region of the metaphysis. Bone marrow cell suspensions were prepared by flushing the shaft with 2 ml culture medium (see below) by means of a syringe fitted with a 0.4 gauge needle. Single cell suspensions were prepared by repeated gentle aspiration in a Pasteur pipette. Viability and cell numbers were determined in a Burker counting chamber by trypan blue (0.5%) exclusion.
Table 1
Inoculation scheme: number of nucleated bone marrow cells added per culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Teflon culture bag ('suspension')</th>
<th>15 mm well ('monolayer')</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/HS/EMFCM</td>
<td>$2 \times 10^7/10$ ml</td>
<td>$5 \times 10^5$/ml</td>
</tr>
<tr>
<td>aMEM/RS/EMFCM</td>
<td>n.d.</td>
<td>$2 \times 10^7/0.5$ ml</td>
</tr>
<tr>
<td>aMEM/RS</td>
<td>$2 \times 10^7/10$ ml</td>
<td>$5 \times 10^5/0.5$ ml</td>
</tr>
<tr>
<td>Fisher/HS</td>
<td>n.d.</td>
<td>$5 \times 10^7$/ml</td>
</tr>
</tbody>
</table>

n.d., not done.
In aMEM and in the absence of EMFCM cells were seeded at higher density than in DMEM/HS/EMFCM because cell proliferation was lower (see Table 2). In monolayer cultures the number of seeded cells was further reduced to allow growth of mononuclear phagocytes as separate colonies [14].

Culture media After appropriate dilution (see Table 1), cells were cultured in the following media.

(1) Dulbecco’s modification of Eagle’s Minimal Essential Medium (DMEM, Gibco, Grand Island, NY), containing 20% heat inactivated (HI) horse serum (HS, Flow Laboratories, Irvine, Scotland) and 30% embryonic mouse fibroblast conditioned medium (EMFCM, see below) (DMEM/HS/EMFCM) for 7 days at 37 °C in a humidified atmosphere of 5% CO₂ in air. The culture medium was not renewed during culture.

(2) Fisher’s medium (Gibco) containing 25% HI HS (Flow) (Fisher/HS), for 10 days at 37 °C in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂. Half of the culture medium was renewed after 7 days of culture. The gas phase was flushed every 3 days, according to Schooley et al. [12].

(3) Alpha’s modification of Eagle’s Minimal Essential Medium (αMEM, Gibco) containing 20% HI rat serum (RS, TNO Zeist, The Netherlands) (αMEM/RS) and either with or without 30% EMFCM for 7 days at 37 °C in a humidified atmosphere of 5% CO₂ in air. The culture medium was renewed after 4 days of culture.

Culture vessels were respectively: (1) Teflon culture bags (TCB, non-toxic hydrophobic Teflon fluorinated ethylene propylene resin, gauge 25 μm, supplied by Janssens M and L, St. Niklaas, Belgium) [13]; ‘suspension cultures’; (2) 24-well tissue culture plates (15 mm dia., Falcon plastics, Becton and Dickinson and Co., Oxnard, USA) provided with plastic coverslips (13 mm/dia., Thermanox, Miles Scientific, Naperville, IL); ‘monolayer cultures’.

Conditioned medium of embryonic mouse fibroblasts (EMFCM): source of colony stimulating factor (CSF-I)
EMFCM was prepared according to the method initially described by Goud [14]. For the preparation of EMFCM, 13 to 15-day-old mouse fetuses were incubated for 20 min at room temperature in a solution of 0.25% trypsin (Difco Laboratories, Detroit, MI) in phosphate-buffered saline (PBS), after the limbs, skull, liver and spleen had been removed. After centrifugation (125 × g) of the cell suspension the precipitate was suspended in Waymouth medium (Gibco) containing 5% HI new-
born calf serum (Gibco), 50 μg/ml streptomycin (Mycofarm, Delft, The Netherlands), and 2000 U/ml penicillin G (Mycofarm). A 10-ml aliquot of this suspension, containing about $3 \times 10^6$ embryonic cells, was incubated in a petridish (100 mm dia., Falcon) at 37 °C in a humidified atmosphere of 10% CO$_2$ in air. The conditioned medium was collected at regular intervals, usually on the 4th, 8th and 12th day of incubation, after which new culture medium was added to the dishes. After collection, the EMFCMs were filtered (pore size 0.45 μm: Millipore SA, Buc, France) and stored in 5-ml vials at −70 °C. The capacity of the EMFCMs to stimulate the growth of mononuclear phagocytes was determined by counting the number of colonies grown from mouse bone marrow cells in viscous methyl cellulose after 7 days of incubation.

**Fibroblast cell cultures**

To investigate the capacity of fetal dermal fibroblasts to stimulate the growth of mononuclear OCP-like cells, precultured bone marrow mononuclear phagocytes (BMMP's) were inoculated on confluent layers of dermal fibroblasts and cultured for 6 days (secondary cultures) at 37 °C in 5% CO$_2$ in air. Fibroblasts were obtained from the minced trunkal skin of 17-day-old mouse fetuses after incubation with collagenase (Worthington, Millipore Corp., USA, 300 U/ml) in aMEM for 30 min at 37 °C. After repeated washing in αMEM, cells were resuspended in Medium 199 (Gibco) containing 20% HI fetal calf serum (Gibco) and inoculated in (1) individual wells of 24-well tissue culture plates (Falcon) at $5 \times 10^5$ cells per ml culture medium or (2) 250 ml culture flasks at $2.5 \times 10^7$ cells per 50 ml culture medium and grown to confluency within 1 week. Fibroblast cultures were incubated at 37 °C in a humidified atmosphere of 5% CO$_2$ in air. Culture medium was renewed after 3 days of culture.

**Cell isolation for secondary cultures**

(1) *From 'suspension' cultures*: after gentle kneading of the teflon culture bag the culture medium was withdrawn by means of a 19 gauge needle attached to a syringe. Cell suspensions were dispersed by gentle aspiration, and subsequently washed twice in DMEM. (2) *From 'support' cultures*: adherent cells were flushed off their support after incubation for 2 min at 37 °C with trypsin solution (0.25%, Gibco), followed by repeated washing in culture medium. Viability and cell numbers of isolated cells were determined in a Burker counting chamber by trypan blue (0.5%) exclusion.

**Assessment of bone resorbing capacity of precultured bone marrow cells**

The capacity of precultured bone marrow cells to form multinucleated osteoclasts capable of mineral resorption was tested as described earlier [2,6]. Briefly, bone marrow cells were cocultured with non-invaded, periost-free long bone rudiments from 17-day-old fetal mice, and the number of osteoclasts present after 7 days coculture assessed in histological sections. Modifications of the original method were as follows: (1) The coagulum, containing $3 \times 10^5$ precultured bone marrow cells and one fetal bone rudiment, consisted of 5 μl chick embryo extract (EE) (Gibco Labo-
ratories, Chagrin Falls, USA) and 5 μl chicken plasma (ChP) (Difco Laboratories, Detroit, USA). (2) The semi-solid culture medium consisted of 30 μl RS, 30 μl EE, 60 μl ChP and 180 μl αMEM. The semi-solid culture medium was renewed every 2 or 3 days. (3) In control cultures, periost-free bone rudiments were cocultured with isolated dermal fibroblasts or cultured without added cells.

**Histology**

Cytocentrifuge preparations (prepared from suspension cultures) and monolayer cultures on coverslips were fixed for 15 min at 4 °C in a mixture of freshly prepared 36% formalin/1% CaCl₂/dextran 70 000 (Macrodex, dextran poviet 70; Organon Teknika B.V., Oss) (10/10/80 v/v). Cocultures of bone marrow cells and fetal bone rudiments were fixed for 2 h at 4 °C in 5% paraformaldehyde in 0.1 M phosphate buffer containing 2% sucrose, pH 7.4, and embedded in hydroxyglycol methacrylate [11]. Serial sections were cut at 3 μm.

**Enzyme histo(cyto)chemistry**

Acid phosphatase [15,16], tartrate-resistant acid phosphatase [11], non-specific esterase [17] and Sudan III staining [18] were all performed as originally described. For alkaline phosphatase staining, 15 mg naphtol AS-TR phosphate (Sigma) was dissolved in 0.25 ml N,N-dimethylformamide. After dissolving, 50 ml 0.1 M Tris-buffer (pH 8.9), 100 μl 10% MgSO₄ and 30 mg Fast Red Violet LB salt (Sigma) were added. The mixture was filtered before use. Coverslip cultures were incubated for 15 min and plastic sections for 2 h at room temperature.

**Results**

**Effects of culture conditions on the growth of OCP-like cells and mononuclear phagocytes from whole mouse bone marrow**

In all experiments, approximately 2 × 10⁷ nucleated bone marrow cells (viability >98%) were isolated per femur. The fresh isolate contained about 30 mononuclear TRAcP-positive OCP-like cells and 1100 mononuclear phagocytes (MP) per 10⁶ nucleated cells. MPs were identified by the presence of many cytoplasmic inclusions and the expression of strong tartrate-sensitive acid phosphatase (TSAcP) and non-specific esterase (nSE) activity. OCP-like cells were identified as cells staining dark red after TRAcP incubation. Low numbers of lightly staining, pink cells were usually present in the cultures, but were not considered because distinction from TRAcP-negative cells was difficult.

The effects of various culture conditions on the growth of mouse bone marrow cells are summarized in Table 2. After 7 days of culture, mononuclear phagocytes, granulocytes, and low numbers of OCP-like cells were always present. Fibroblast-like cells grew in monolayer, but not in suspension cultures, while adipocyte-like cells grew only under low pO₂ conditions, in agreement with the data of Schooley et al. [12]. Most erythroid and lymphoid cells died in the first 3–4 days of culture. Culture of bone marrow in DMEM/HS/EMFCM resulted in extensive prolifer-
ation of MPs (Table 2, Fig. 1a) in agreement with the data of Goud et al. [14] and Van der Meer et al. [13,19,20]. The rate of proliferation in teflon bags (suspension cultures) was similar to that of cultures on coverslips (monolayer cultures). On coverslips however, the MPs grew in typical colonies which allowed distinction between monoblasts, promonocytes, and macrophages, according to the criteria described by Goud et al. [14].

Growth of mononuclear TRAcP-positive OCP-like cells in DMEM/HS/EMFCM cultures was very limited (Table 2). Only in monolayer cultures we observed a five-fold increase in OCP-like cells. They grew adjacent to large fibroblast-like cells, characterized by a large ovoid nucleus with five to ten nucleoli, weak TSAcP activity and weak to strong alkaline phosphatase activity. In cytocentrifuge preparations of suspension cultures OCP-like cells were very rare (Fig. 1b).

Culture of bone marrow in Fisher/HS medium stimulated the growth of small fibroblast-like cells, and allowed low to moderate growth of MPs. OCP-like cell growth was slightly enhanced (Table 2). Fisher/HS cultures also contained many TSAcP- but not TRAcP-positive multinucleated cells and cells characterized by an accumulation of a lipid-like material which stained strongly with Sudan III and non-specific esterase. MPs could again be separated into monoblasts, promonocytes and macrophages.

Table 2
Effect of culture conditions on the number of osteoclast precursor-like cells (OCP-like, TRAcP*) and mononuclear phagocytes (MP, TRAcP*) in 7 day cultures of whole mouse bone marrow

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Total cell number ( \times 10^5 ) per 5 x ( \times 10^5 ) plated cells</th>
<th>% distribution of cells after culture</th>
<th>Fold increaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OCP-like</td>
<td>MP</td>
</tr>
<tr>
<td>DMEM/HS/EMFCM suspension</td>
<td>530.1 ± 23.7</td>
<td>0.003</td>
<td>94.0</td>
</tr>
<tr>
<td>DMEM/HS/EMFCM monolayer</td>
<td>512.5 ± 27.2</td>
<td>0.016</td>
<td>94.8</td>
</tr>
<tr>
<td>Fisher/HS monolayer</td>
<td>126.1 ± 4.8</td>
<td>0.090</td>
<td>85.0</td>
</tr>
<tr>
<td>aMEM/RS/EMFCM monolayer</td>
<td>84.5 ± 4.4</td>
<td>0.054</td>
<td>80.4</td>
</tr>
<tr>
<td>aMEM/RS suspension</td>
<td>15.2 ± 0.8</td>
<td>0.140</td>
<td>81.1</td>
</tr>
<tr>
<td>aMEM/RS monolayer</td>
<td>18.5 ± 0.9</td>
<td>2.300</td>
<td>76.7</td>
</tr>
</tbody>
</table>

a Suspension cultures were evaluated by counting all cells in cytocentrifuge preparations made after appropriate dilution. Monolayer cultures were evaluated by counting all cells within one quadrant of the round coverslip. To show the net effect of cell loss and gain during culture, total cell numbers are expressed per 5 x \( \times 10^5 \) nucleated plated cells. For actual cell numbers plated, see Table 1. Values are means ± S.E.M. of six cultures.

b Calculated by dividing the cell number found after culture by the number observed at inoculation.

Fresh bone marrow contained 15 OCP-like cells and 550 MP's per 5 x \( \times 10^5 \) nucleated cells, counted in cytocentrifuge preparations stained for TRAcP and non-specific esterase.

c Fisher/HS cultures were maintained for 10 days, according to Schooley et al. [12].

GR, granulocytes; Adip, adipocyte-like cells; Fibr, fibroblast-like cells.
Fig. 1. Cytocentrifuge preparations of cells from DMEM/HS/EMFCM suspension cultures, incubated for acid phosphatase in the absence (1a) or presence (1b) of tartrate. In the presence of tartrate, TSACP activity of the mononuclear phagocytes is completely inhibited, while a mononuclear OCP-like cell (arrow) remains positive (1b). Bar = 50 μm.

Fig. 2. αMEM/RS support culture, incubated for TRACP; 2a, no counterstain; 2b, lightly counterstained with haematoxylin to show fibroblast-like cell nuclei. OCP-like cells, positive for TRACP, grow in small colonies (2a, arrows). At higher magnification (2b), the TRACP positive cells are often found in close association with fibroblast-like cells (2b, arrowheads, OCP-like cells; asterisks, fibroblast-like cells).

2a, bar = 160 μm; 2b, bar = 100 μm.
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Fig. 3. Growth curve of OCP-like cells in αMEM/RS monolayer culture, either with (---) or without (-- ---) change of medium after 4 days of culture. $5 \times 10^5$ nucleated bone marrow cells were plated and cultured for 7 days. Values are means ± S.E.M. for five cultures.

Culture of bone marrow in αMEM/RS strongly reduced the growth of mononuclear phagocytes, even in the presence of EMFCM (Table 2). In the absence of EMFCM, we observed a considerable growth of OCP-like cells, but only in mono-

Table 3
Lack of effect of dermal fibroblasts on the formation of OCP-like cells in secondary bone marrow cultures.a

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Dermal fibroblasts presentb</th>
<th>Fold increase of OCP-like cellsc</th>
<th>p d</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/HS/EMFCM</td>
<td>no</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>DMEM/HS/EMFCM</td>
<td>yes</td>
<td>1.0</td>
<td>NS</td>
</tr>
<tr>
<td>αMEM/RS</td>
<td>no</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>αMEM/RS</td>
<td>yes</td>
<td>1.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Precultured for 7 days in Teflon culture bags in DMEM/HS/EMFCM; precultured cells were subsequently seeded in 24-well plates ($10^6$ cells per well) with or without an adherent layer of dermal fibroblasts, and cultured for an additional 6 days.
b The fibroblastic nature of the adherent cell layer was confirmed by the expression of alkaline phosphatase activity.
c Values are means of 12 cultures from three experiments.
d Student’s t-test; NS, not significant.
layer cultures, i.e., under conditions which allowed fibroblast-like cell growth (Table 2) (Fig. 2a,b). In these monolayer cultures OCP-like cells displayed a variety of sizes and morphologies (Fig. 2b). They included small rounded cells, 15–20 \( \mu \text{m} \) in diameter, similar to those seen in fresh bone marrow isolates, and larger cells, 20–50 \( \mu \text{m} \) in diameter, often with long cytoplasmic extensions (Fig. 2b). OCP-like cells were found as solitary cells or in small colonies (Fig. 2a), which grew in close association with fibroblast-like cells and also contained TSACP-positive macrophages and mature and immature granulocytes. Multinucleated cells, be it TRAcP- or TSACP-positive, were seldom observed in these 7-day cultures.

The growth curve of OCP-like cells is shown in Fig. 3. During the first 4 days of culture there is a rapid exponential growth of OCP-like cells. The population doubling time, determined from the slope of the growth curve, amounts to 30 h (mean of four cultures). During the last 3 culture days, growth of OCP-like cells levelled off, even in the presence of fresh culture medium. Renewal of the culture medium after 4 days of culture was necessary to prevent a strong loss of OCP-like cells during the last 3 culture days.

Effect of dermal fibroblasts on the formation of mononuclear OCP-like cells from precultured bone marrow

Secondary culture of DMEM/HS/EMFCM cultures on confluent layers of fetal dermal fibroblasts, with or without EMFCM, did not result in an increase of the number of OCP-like cells (Table 3). The confluent fibroblast cell layer itself did not express any TRAcP activity nor did it contain any TRAcP-positive OCP-like cells.

Assessment of bone resorbing capacity of precultured bone marrow cells: cocultivation with periost-free fetal bone rudiments

Culture of periost-free (stripped) bone rudiments for 7 days either with precultured bone marrow cells or with OCP-like cells grown in monolayer cultures of either DMEM/HS/EMFCM suspension cultures, Fisher/HS monolayer cultures or aMEM/RS monolayer cultures was performed as described above.

Table 4

<table>
<thead>
<tr>
<th>Cocultured cell population</th>
<th>No. of OC-nuclei per coculture(^b)</th>
<th>Cell growth during preculture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCP-like</td>
<td>MP</td>
</tr>
<tr>
<td>No cells added</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Dermal fibroblasts</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bone marrow cells of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM/HS/EMFCM suspension cultures</td>
<td>138 ± 33</td>
<td>×1</td>
</tr>
<tr>
<td>Fisher/HS monolayer cultures</td>
<td>562 ± 17(^c)</td>
<td>×7</td>
</tr>
<tr>
<td>aMEM/RS monolayer cultures</td>
<td>864 ± 62(^d)</td>
<td>×29</td>
</tr>
</tbody>
</table>

\(^a\) 3 \( \times 10^5 \) precultured bone marrow cells were added per rudiment.

\(^b\) Counts were made in 3 \( \mu \text{m} \) sections, collected at intervals of 15 \( \mu \text{m} \) and incubated for acid phosphatase in the presence of tartrate. Values are means ± S.E.M., \( n = 5 \) for two experiments.

\(^c\) Student’s \( t \)-test, \( P < 0.001 \), compared with DMEM/HS/EMFCM suspension cultures.

\(^d\) Student’s \( t \)-test, \( P < 0.001 \), compared with Fisher/HS monolayer cultures.
Fig. 4. Central part of stripped bone rudiments cocultured for 7 days with dermal fibroblasts (a), cells from DMEM/HS/EMFCM suspension cultures (b), or cells from αMEM/RS monolayer cultures (c). Incubated for acid phosphatase in the presence of tartrate. Hematoxylin counterstain. In the coculture with dermal fibroblasts no osteoclasts are present. Coculture with bone marrow cells from DMEM/HS/EMFCM (b) or αMEM/RS (c) cultures has resulted in the formation of few (b) or many (c) TRAP-positive osteoclasts (arrows). Part of the mineralized matrix has been resorbed. For all figures: ca, mineralized cartilage matrix; arrowheads, bone collar; asterisks, semi-solid medium with precultured cells. Bar = 40 μm.
dermal fibroblasts (Fig. 4a) or without added cells did not result in the formation of mono- or multinucleated TRAcP-positive cells (Table 4). Cocultures remained healthy during culture as mineralization proceeded and no pycnosis of nuclei was observed. Culture of stripped bone rudiments with precultured bone marrow cells resulted in the formation of multinucleated TRAcP-positive osteoclasts from all bone marrow cultures tested (Fig. 4b,c). Cell counts made in serial histological sections are summarized in Table 4. Development of osteoclasts was positively correlated with the growth of OCP-like cells during preculture, but negatively with the growth of mononuclear phagocytes. Most osteoclasts were formed after coculture of bones with cells from αMEM/RS monolayer cultures. They were multinuclear, expressed strong TRAcP activity and were formed along the bone shaft (Fig. 4c). Part of the bone shaft and the mineralized center of the rudiments had been resorbed. In the surrounding coagulum some granulocyte colonies and TSacP-positive macrophages were observed and, in cocultures with cells from Fisher/HS support cultures, many fibroblast-like and adipocyte-like cells. Lowest numbers of osteoclasts were formed after coculture of bone rudiments with DMEM/HS/EMFCM suspension cultures. Osteoclasts were small and were mainly observed in the partially eroded bone center (Fig. 4b).

Discussion

We investigated methods to culture mononuclear cells, cytochemically resembling osteoclast precursors, from freshly isolated mouse bone marrow. Expression of tartrate-resistant acid phosphatase (TRAcP) activity served as marker for OCP-like cell identification. In the mouse, TRAcP activity is specifically associated with osteoclasts and their mononuclear precursors, while macrophages show only tartrate-sensitive acid phosphatase (TSacP) activity [11]. The present study shows that macrophage progenitors of the mouse are also negative for TRAcP and express only TSacP activity.

Functional evidence for the osteoclastic nature of the OCP-like cells was provided by coculture with periost-free long bone rudiments. Formation of multinucleated osteoclasts from precultured bone marrow cell populations was positively correlated with the growth of OCP-like cells during preculture. Unlike others [21,22], we used live, intact bone rudiments of fetal mice to assess the bone-resorbing capacity of the cell cultures, to exclude macrophage-mediated bone resorption [2,6]. The observed correlation of cell growth and subsequent osteoclast formation, positive for OCP-like cells and negative for mononuclear phagocytes, argues for OCP-like cells, but not mononuclear phagocytes to be osteoclast precursors.

In contrast to studies with cat [21,22] primate [23] or rabbit bone marrow [24], only very few multinucleated cells were formed in the primary mouse bone marrow cultures. This is probably related to the rather short (7 day) culture time used, as multinucleation increased significantly after 14 and 21 days in other studies [22–24]. In addition, it may reflect the importance of bone tissue for osteoclast precursor cell fusion, since in vivo as well as in the cocultures, multinucleation was pre-
dominantly observed in the direct vicinity of mineralized bone tissue.

In view of the presumed relationship between osteoclasts and mononuclear phagocytes, the effect of EMFCM on the growth of OCP-like cells is of interest. EMFCM, which has been shown to contain high concentrations of CSF-1 activity [20], did not stimulate OCP-like cell growth but, rather, inhibited it. This result is surprising in the light of earlier studies [2,6] in which we demonstrated that CSF-stimulated bone marrow cultures formed osteoclasts when cocultured with periosteofree bone rudiments. This seemed to indicate that CSF-1 stimulated the growth of mononuclear phagocytes as well as osteoclast precursors. Others [25,26] have reached a similar conclusion, based on studies with osteopetrotic mice [25] or vitamin D-deficient rats [26]. In both studies the experimental animals showed decreased numbers of osteoclasts and macrophages, which was attributed to a defect in CSF production by the bone marrow stroma. The conditioned medium used in the present study was not purified, and likely contained other factors beside CSF-1, which may have interfered with the response of OCP-like cells. MacDonald et al. [23] have reported that CSF stimulated the proliferation of precursors of osteoclast-like polykaryons in baboon bone marrow cultures. However, CSF-GM, which stimulates the committed progenitor of both the monocyte-macrophage and granulocyte series, stimulated polykaryon formation more consistently than CSF-1, and the effect of CSF on macrophage-excluding bone resorption by the polykaryons was not reported [23]. Recent observations by Horton et al. [24] cast some doubt on the osteoclastic nature of the polykaryons formed in some primary bone marrow cultures and underscore the importance of macrophage-excluding bone resorption assays for osteoclast characterization. The present study shows that by omitting CSF-1 activity from the culture medium we could increase the number of osteoclast precursor-like cells as well as the capacity of the cultures to form osteoclasts, although the proliferation of mononuclear phagocytes was much reduced. As a result, it seems unlikely that, in the mouse, CSF-1 is a growth factor for osteoclast precursors. This conclusion is important for the relationship between osteoclasts and macrophages. CSF-1 is considered a lineage-specific growth factor, restricted to the monocyte-macrophage family [27]. Osteoclasts and macrophages share specific surface receptors [4,5] and are usually considered related cells although this contention has been challenged [28]. Based on the present data we propose that osteoclasts and their precursors are a separate cell family, which is related to but diverges from the mononuclear phagocyte lineage. CSF-1 selectively stimulates monocyte-macrophage but not osteoclast precursor growth and differentiation. The inhibitory effect of CSF-1 on the osteoclast subpopulation may then be explained by exhaustion of the common early progenitor pool, which is selectively directed towards monocyte-macrophages.

A significant increase in the number of OCP-like cells was observed when bone marrow (but not dermal) fibroblasts were present in the cultures. In such cultures OCP-like cells were often observed in small colonies which also contained granulocytes and macrophages. The importance of bone marrow fibroblasts for the maintenance and regulation of hemopoiesis in vitro is well known [29,30]. We speculate that the bone marrow fibroblasts created the appropriate micro-environment for
OCP-like cell growth by providing the necessary growth factors in addition to growth factors for macrophages and granulocytes. The identity of a possible osteoclast growth factor needs further study.

In sum, we demonstrated growth of mononuclear cells showing the enzyme characteristic of osteoclasts and their precursors, from freshly isolated whole mouse bone marrow. Growth conditions of these osteoclast precursor-resembling cells strongly differed from those of mononuclear phagocytes, as CSF-1 acted as an inhibitor rather than a stimulator. We assume that OCP-like cells are a separate cell population, different from, but closely related to, mononuclear phagocytes. A study comparing the antigenic and functional properties of the two cell populations [31] lends further support to this hypothesis.

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References

1 Scheven BAA, Visser JWM, Nijweide PJ. In vitro osteoclast generation from different bone marrow fractions, including a highly enriched haematopoietic stem cell population. Nature (London) 1986;321:79-81.


