Origin of osteoclasts: Mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells (1α,25-dihydroxyvitamin D3/bone resorption/alveolar macrophages/ST2 cells/cell-to-cell contact)

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ABSTRACT We previously reported that osteoclast-like cells were formed in cocultures of a mouse marrow-derived stromal cell line (ST2) with mouse spleen cells in the presence of 1α,25-dihydroxyvitamin D3 and dexamethasone. In this study, we developed a new coculture system to determine the origin of osteoclasts. When relatively small numbers of mononuclear cells (10^4–10^5 cells per well) obtained from mouse bone marrow, spleen, thymus, or peripheral blood were cultured for 12 days on the ST2 cell layers, they formed colonies with a linear relationship between the number of colonies formed and the number of hemopoietic cells inoculated. Tartrate-resistant acid phosphatase (TRAPase)-positive mononuclear and multinucleated cells appeared in the colonies (TRAPase-positive colonies) in response to 1α,25-dihydroxyvitamin D3 and dexamethasone. When hemopoietic cells suspended in a collagen-gel solution were cultured on the ST2 cell layers to prevent their movement, TRAPase-positive colonies were similarly formed, indicating that each colony originated from a single cell. All of the colonies consisted of nonspecific esterase-positive cells. The monocyte-depleted population prepared from peripheral blood failed to form colonies, whereas the monocyte-enriched population produced a large number of TRAPase-positive colonies. In addition, alveolar macrophages formed TRAPase-positive colonies most efficiently on the ST2 cell layers in the presence of the two hormones. Salmon 125I-labeled calcitonin specifically bound to the TRAPase-positive cells. Resorption lacunae were formed on dentine slices on which cocultures were performed. When direct contact between the peripheral blood cells and the ST2 cells was inhibited by a collagen-gel sheet, no TRAPase-positive cells were formed. These results indicate that osteoclasts are also derived from the mature monocytes and macrophages when a suitable microenvironment is provided by bone marrow-derived stromal cells.

Osteoclasts are multinucleated cells responsible for bone resorption. It is evident from chicken–quail chimeric experiments (1), parabiosis experiments (2, 3), and marrow transplantation studies in osteopetrotic animals (4, 5) that osteoclasts are derived from circulating mononuclear precursors in hemopoietic tissues. However, the nature and the differentiation process of osteoclast precursors are still not known.

We recently reported that osteoclast-like multinucleated cells were formed in response to osteotropic hormones in cocultures of mouse spleen cells with osteoblast-rich cell populations freshly isolated from fetal mouse calvaria (6). These multinucleated cells had the typical characteristics of osteoclasts such as tartrate-resistant acid phosphatase (TRA-

**Abbreviations:** TRAPase, tartrate-resistant acid phosphatase; 1α, 25(OH)2D3, 1α,25-dihydroxyvitamin D3; α-MEM, a minimal essential medium; NSEase, nonspecific esterase; M-CSF, macrophage colony stimulating factor.

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**Coculture Systems.** A stock of mouse bone marrow-derived stromal cell line ST2 (11, 12) was obtained from the RIKEN Cell Bank (Tsukuba, Japan). ST2 cells (4 x 10^4 cells per well) were precultured for 24 hr in 0.4 ml of α-MEM supplemented with 10% fetal calf serum in 24-well plates (Corning). A limited number of mononuclear cells from hemopoietic tissues or alveolar macrophages suspended in 0.1 ml of α-MEM with 10% fetal calf serum were then seeded on the ST2 cell layers and cultured for the indicated periods (usually 12 days) at 37°C in a humidified 5% CO_2/95% air. All cultures were fed every 3 days by replacing 0.4 ml of old medium with fresh medium. 1α,25(OH)_2D_3 (Philips-Duphar, Amsterdam) and dexamethasone (Sigma) were added at the beginning of the coculture and at each change of the medium. The final concentrations of 1α,25(OH)_2D_3 and dexamethasone were 10 nM and 100 nM, respectively, when the cultures were treated with the two hormones.

In some experiments, the movement of hemopoietic cells on the ST2 cell layers was inhibited by using a collagen-gel culture. In short, a type I collagen solution (pig acid soluble form, Cellmatrix type I-A) was obtained from Nitta Gelatin (Osaka). The culture wells of 24-well plates were coated with 0.2 ml of collagen gel (0.2%). ST2 cells (4 x 10^4 cells per well) were added to the collagen-coated wells and precultured for 24 hr. A limited number of peripheral blood mononuclear cells (4 x 10^4 cells per ml) were suspended in 0.08% collagen solution at 4°C. A portion (0.25 ml) of the cell suspension was put on the ST2 cell layers, which had been cooled on ice. The plates were left for 1 hr at 4°C to allow the mononuclear cells to adhere to the ST2 cell layer and then put into a CO_2 incubator at 37°C for 1 hr to make the aqueous type I collagen solution gelatinous. Finally the cultures were supplemented with 0.5 ml of α-MEM containing 10% fetal calf serum and maintained for 12 days in the presence of the two hormones.

**Determination of Osteoclast Characteristics.** After being cultured for the indicated times, the adherent cells were fixed and stained for TRAPase in the presence of 50 mM sodium tartrate as described (7). TRAPase-positive cells appeared as dark red cells within 15 min of incubation. Cell clusters were formed on the ST2 cell layer, and those larger than 200 μm in diameter were scored as colonies with a microscope. Colonies containing three or more TRAPase-positive cells were counted as TRAPase-positive colonies. The results were expressed as the means ± SEM of four cultures. Some cultures were first stained for NSEase (α-naphthyl acetate esterase kit, Sigma) and subsequently for TRAPase as described (8). NSEase-positive cells appeared dark brown.

Occurrence of calcitonin receptors was assessed by autoradiography using salmon 125I-labeled calcitonin (125I-calcitonin) as described (13).

The bone resorbing activity of alveolar macrophages was examined in the presence or absence of the ST2 cell layers. Alveolar macrophages (100 cells per well) and ST2 cells were cocultured for 15 days on sperm whale dentine slices (provided by A. Boyde, University College London) in the presence of 1α,25(OH)_2D_3 and dexamethasone. Alveolar macrophages (10^5 cells per 50 μl) were also plated in the center of dentine slices without ST2 cells. The adherent macrophages were cultured in α-MEM containing 10% fetal calf serum for 15 days in the presence of the two hormones. The slices were then fixed, treated with 0.1% trypsin (type I, Sigma) to remove attached cells, washed, and processed for backscattered electron images as described (7).

**RESULTS**

When a small number (10^4 cells per well) of spleen cells were cultured on the ST2 cell layer in the presence of 1α,25(OH)_2D_3 and dexamethasone, spleen cell-derived clusters first appeared on the ST2 cell layer on day 5 (Fig. 1A). Well-defined colonies grown from the clusters appeared on day 10, and TRAPase-positive mononuclear cells began to appear in some colonies (Fig. 1B). On day 12, the number of TRAPase-positive mononuclear cells increased in the colony, and some of them often spread out from the colony (Fig. 1C). TRAPase-positive multinucleated cells also appeared in the peripheral region of the colony on day 12 (Fig. 1C). When cultures were continued for more than 12 days, colonies became larger and began to fuse with each other. The numbers of TRAPase-positive mononuclear cells and multinucleated cells on day 12 were respectively 85.3 ± 50.8 and 18.7 ± 16.5 (means ± SEM of 20 TRAPase-positive colonies scored). Similar colonies were also formed in the absence of 1α,25(OH)_2D_3 and dexamethasone, but no TRAPase-positive cells appeared.

When increasing numbers of mononuclear cells prepared from bone marrow, spleen, thymus, and peripheral blood were cultured for 12 days on the ST2 cell layers in the presence of 1α,25(OH)_2D_3 and dexamethasone, TRAPase-positive colonies were formed in all of the cultures of hemopoietic tissues examined. In each tissue there was a linear relationship between the number of TRAPase-positive colonies formed and the hemopoietic cells inoculated (Fig. 2).

All of the linear plots ran through the origin, indicating that each TRAPase-positive colony is derived from a single cell and that the ST2 cells produce some growth factor(s) for the cells. It is also evident from these linear plots that the bone marrow mononuclear cell fraction contains the largest number of the TRAPase-positive colony-forming cells (500 cells per 10^5 cells), followed by the peripheral blood (140), spleen (130), and thymus (13) in that order (Fig. 2). When peripheral

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**Fig. 1.** A spleen cell-derived cluster and a colony formed on the ST2 cell layer. ST2 cells (4 x 10^4 cells per well) were first cultured for 24 hr. Mouse spleen cells (4 x 10^4 cells per well) were added to the ST2 cell layer and cocultured in the presence of 10 nM 1α,25(OH)_2D_3 and 100 nM dexamethasone. After culture for 5 days (A), 10 days (B), and 12 days (C), the cells were fixed and stained for TRAPase. TRAPase-positive cells appeared as dark red cells. (×55.)
show that TRAPase-positive cells are derived from the cells of the monocyte-macrophage lineage.

We next examined the possibility that fully differentiated macrophages can differentiate into osteoclast-like cells on the ST2 cell layer. When a small number of alveolar macrophages (8–32 cells) were cultured on the ST2 cell layers in the presence of 1α,25(OH)2D3 and dexamethasone, TRAPase-positive colonies were formed most efficiently (Fig. 4). More than 90% of alveolar macrophages formed colonies, and most of them contained TRAPase-positive cells.

Fig. 5 shows autoradiographs of the binding of salmon 125I-calcitonin to TRAPase-positive cells in the cocultures of alveolar macrophages and ST2 cells in the presence of 1α,25(OH)2D3 and dexamethasone. Numerous dense grains due to the binding of 125I-calcitonin appeared on the TRAPase-positive cells (Fig. 5A). There was no radioactivity on TRAPase-negative cells. Simultaneous addition of an excess of unlabeled calcitonin completely removed the accumulation of dense grains from the TRAPase-positive cells (Fig. 5B). TRAPase-negative multinucleated cells were formed from alveolar macrophages in response to 1α,25(OH)2D3 and dexamethasone in the absence of ST2 cells, but they accumulated no grains, indicating no detectable calcitonin binding (Fig. 5C).

More than 90% of the TRAPase-positive mononuclear and multinucleated cells formed in the cocultures with peripheral blood mononuclear cells or alveolar macrophages accumulated dense grains because of the 125I-calcitonin binding (Table 1). A similar distribution of the 125I-calcitonin binding also occurred in the cocultures with mononuclear cells of marrow, spleen, thymus, and peripheral blood (data not shown).

When alveolar macrophages and ST2 cells were cocultured on dentine slices in the presence of 1α,25(OH)2D3 and dexamethasone, numerous resorption lacunae were formed on their surfaces (Fig. 6A). No resorption lacunae were detected on the dentine slice on which alveolar macrophages were cultured without ST2 cells in the presence of 1α,25(OH)2D3 and dexamethasone (Fig. 6B).

**DISCUSSION**

At first the mononuclear phagocytes were considered to be osteoclast precursors on the basis of their morphologic and enzymatic properties as well as their capacity to form multinucleated giant cells (14). But it was concluded later that terminally differentiated macrophages are only distantly re-
related to osteoclasts, as evidenced by the inability of macrophages to form ruffled borders and to resorb bone (15, 16), the absence of calcitonin receptors and responsiveness in macrophages (17, 18), and differences in membrane phenotypic expression between macrophages and osteoclasts (19, 20). These observations obtained over the last decade indicate either that osteoclasts develop independently of the monocyte–macrophage lineage from a specialized progenitor, or that the two arise from the same monocyte–macrophage population but diverge early in the differentiation pathways to produce the characteristic mature cells.

The present study clearly demonstrates that mononuclear cells prepared from various hemopoietic tissues form NSEase-positive colonies on the ST2 cell layers, in which TRAPase-positive cells appear in response to 1α,25(OH)2D3 and dexamethasone. It is highly likely from the following evidence that the TRAPase-positive colonies are of single-cell origin. First, when all of the linear plots of the TRAPase-positive colonies formed were plotted against the mononuclear cells inoculated, the line ran through the origin (Fig. 2). Second, TRAPase-positive colonies were similarly formed in the collagen-gel cultures, which allowed mononuclear cells to adhere to the ST2 cell layers but inhibited their movement. The monocyte-depleted fraction of peripheral blood mononuclear cells failed to form colonies and the monocyte-enriched fraction produced a large number of TRAPase-positive colonies. These results show that each TRAPase-positive colony is derived from a single cell that belongs to the mononuclear phagocyte family.

It is striking that alveolar macrophages also formed TRAPase-positive colonies on the ST2 cell layers, since alveolar macrophages have been considered to be terminally differentiated cells. TRAPase-positive cells formed from alveolar macrophages on the ST2 cell layers had calcitonin receptors and were able to produce classical resorption pits on dentine slices (Fig. 5 and 6), thus satisfying the major criteria for osteoclasts. Our previous work (6, 8, 13) and that of others (21, 22) in mouse marrow cultures have established the validity of these criteria. We previously reported that no TRAPase-positive cells appeared when 5 × 104 alveolar macrophages per well were cocultured with primary osteoblastic cell populations (6). Indeed, the formation of TRAPase-positive cells was markedly suppressed in cocultures of more than 104 alveolar macrophages per well with ST2 cells (data not shown). Apparently macrophages produce an inhibitory factor(s) for osteoclast differentiation. Peritoneal cells induced by thyglocinate (mainly mature macrophages) also differentiated into osteoclast-like cells with very high efficiency on the ST2 cell layers (data not included). These results show that macrophage populations can also differentiate into osteoclasts in the presence of marrow-derived stromal cells.

![Figure 4](image_url)

**Fig. 4.** Relationship between the number of TRAPase (TRAP)-positive colonies formed on the ST2 cell layers and the number of alveolar macrophages inoculated. After a limited number of mouse alveolar macrophages (8–32 cells) were cultured on the ST2 cell layers in the presence of 10 nM 1α,25(OH)2D3 and 100 nM dexamethasone, the TRAPase-positive colonies formed were counted on day 12. The results are expressed as the means ± SEM of four cultures.

![Figure 5](image_url)

**Fig. 5.** Autoradiography of salmon 125I-calcitonin binding in the cultures of mouse alveolar macrophages in the presence or absence of ST2 cells. Alveolar macrophages were cultured for 15 days on the ST2 cell layer prepared on Lux coverslips (A and B). Alveolar macrophages were directly seeded in the center of the coverslips and cultured for 5 days (C). Both cultures were maintained in the presence of 10 nM 1α,25(OH)2D3 and 100 nM dexamethasone. After culture for the indicated times, cells were incubated with salmon 125I-calcitonin (0.2 nM) in the absence (A and C) or presence (B) of an excess amount (200 nM) of unlabeled salmon calcitonin, stained for TRAPase, and were processed for autoradiography. (× 290.) Note that the dense grains due to 125I-calcitonin binding appear only on TRAPase-positive cells in A.

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**Table 1.** The quantitative relation between the calcitonin receptor-positive (CaIR+) cells and the TRAPase+ cells formed from peripheral blood mononuclear cells or alveolar macrophages on the ST2 cell layers

<table>
<thead>
<tr>
<th>Cells cultured on the ST2 cell layer</th>
<th>TRAPase+ mononuclear cells</th>
<th>TRAPase+ multinucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>CaIR+ cells</td>
</tr>
<tr>
<td>Peripheral blood mononuclear cells</td>
<td>236</td>
<td>221</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>435</td>
<td>409</td>
</tr>
</tbody>
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Peripheral blood mononuclear cells (10⁴ cells) or alveolar macrophages (100 cells) were cultured in the presence of 10 nM 1α,25(OH)2D3 and 100 nM dexamethasone on the ST2 cell layers that had been prepared on Lux coverslips. After culture for 15 days, cells were incubated with 125I-calcitonin, stained for TRAPase, and processed for autoradiography. The number of TRAPase+ mononuclear cells and that of multinucleated cells that contained more than three nuclei were separately scored. Cells accumulating more than 20 grains per 100 μm² were counted as CaIR+ cells. Specific accumulation of grains was not detected on TRAPase+ cells. In each assay, all cells appearing on three coverslips were counted.
The efficiency of progenitor cells to form TRAPase-positive colonies was the highest in the cocultures with alveolar macrophages. Most (>95%) of the alveolar macrophage-derived colonies contained TRAPase-positive cells. In contrast, only 30% of the colonies in the cocultures with the other tissue-derived mononuclear cells, including monocyte-enriched populations, were TRAPase-positive colonies.

One of the growth factors possibly involved in the formation of TRAPase-positive colonies on the ST2 cell layers is macrophage colony stimulating factor (M-CSF), which induces the proliferation and differentiation of committed cells into macrophages. It is also reported that M-CSF stimulates the proliferation of alveolar macrophages (23). ST2 cells have been reported to produce M-CSF spontaneously but not interleukin 3 and granulocyte-macrophage colony stimulating factor (11, 12).

Preliminary experiments showed that the marrow cells isolated from methylcellulose cultures in the presence of M-CSF specifically produced a large number of osteoclast-like cells in cocultures with primary osteoblastic cell populations (N.T., unpublished data). Felix et al. (24) also reported that calvaria of osteopetrotic (op/op) mice failed to produce M-CSF. These results suggest that M-CSF produced by stromal cells is an important factor for maintaining growth of osteoclast progenitors, and the deficiency of this cytokine may attribute to osteopetrosis.

Several lines of evidence have suggested that mature macrophages do not have the capacity to form osteoclasts in vitro. Burger et al. (25) have shown that no osteoclasts were formed in coculture of osteoclast-free fetal mouse bone rudiments with mature macrophages. Enriched populations of macrophage colony-stimulating cells were also ineffective in forming osteoclasts in cocultures with fetal rat metatarsal bones (26). In our system, TRAPase-positive cells did not appear in the absence of 1α,25(OH)2D3. In contrast, in the coculture system using fetal bone rudiments, no osteotropic hormones were used to induce osteoclasts from immature precursor cells (25, 26). This indicates that the osteoclast differentiation of mature macrophages may be more strictly regulated by osteotropic hormones than that of immature cells. Alternatively, the number of macrophages cocultured with bone rudiments may be attributable to this discrepancy.

since osteoclast differentiation of alveolar macrophages was strikingly inhibited when more than 104 alveolar macrophages were cultured on the ST2 cell layers. Although further studies are necessary to elucidate this point, our findings are in agreement with the classical pioneering studies (27-29) which identified cells of monocyte–macrophage lineage as the precursor for osteoclasts.

In conclusion, osteoclasts can be formed not only from immature cells of the monocyte–macrophage lineage but also from mature tissue macrophages when a suitable microenvironment is provided by bone marrow-derived stromal cells. This has significant implications for the study of osteoclast development. Further studies are needed to elucidate the precise interaction between marrow-derived stromal cells and monocyte–macrophage populations.

Note Added in Proof. Very recently, Yoshida et al. reported that op mutation is due to a single-base-pair insertion in the coding region of the M-CSF gene (30).