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Cell Biology

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 D_3/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 D_3 and dexamethasone. In this study, we developed a new coculture system to determine the origin of osteoclasts. When relatively small numbers of mononuclear cells (10^3–10^5 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 D_3 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 ^{125}I-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 chimera 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 (TRAPase), abundant calcitonin receptors, and the ability to form resorption lacunae on dentine slices (6). Then we reported that the two marrow-derived stromal cell lines, MC3T3-G2/PA6 and ST2, could be substituted for primary osteoblast-rich cell populations in inducing osteoclast-like cells in cocultures with spleen cells in the presence of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and dexamethasone (7).

In this study, we developed a new culture system using ST2 cells to determine the nature of osteoclast precursors and their differentiation into osteoclasts. We report here that osteoclasts are derived not only from immature cells but also from mature cells of the monocyte–macrophage lineage when a suitable microenvironment is provided by bone marrow-derived stromal cells.

MATERIALS AND METHODS

Preparation of Hemopoietic Mononuclear Cells and Alveolar Macrophages. Seven- to 9-week-old male mice, ddy strain, were obtained from the Shizuoka Laboratories Animal Center (Shizuoka, Japan). Bone marrow mononuclear cells were isolated from tibiae of mice as described (8). Splenic tissues and thymus aseptically removed from mice were washed and minced in α-minimal essential medium (α-MEM, Flow Laboratories). Erythrocytes contaminating in the mononuclear cell fractions prepared from marrow, spleen, and thymus were eliminated by adding 10 mM Tris·HCl (pH 7.4) containing 0.83% ammonium chloride to the cell pellets. The cells were washed twice with α-MEM and then suspended in α-MEM containing 10% (vol/vol) fetal calf serum (GIBCO). Peripheral blood was collected from the mice by heart puncture, and blood mononuclear cells were isolated by centrifugation at 300 × *g* for 30 min on mononuclear/polynuclear cell–resolving medium (Flow Laboratories). The monocyte-depleted fraction was prepared by passing the peripheral blood mononuclear cells through a Sephadex G-10 column (9). In some experiments, the monocytes were enriched by allowing the peripheral blood mononuclear cells to adhere to the glass surface and collecting the adherent cells with 0.2% EDTA/5% fetal calf serum. More than 90% of the cells in this fraction were positively stained for nonspecific esterase (NSEase). Alveolar macrophages were collected by the tracheobronchial lavage method as reported (10). More than 99% of the lavaged cells were positive for NSEase staining.

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Abbreviations: TRAPase, tartrate-resistant acid phosphatase; 1α, 25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; α-MEM, α 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 (4×10^4 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. $1\alpha,25(OH)_2D_3$ (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 $1\alpha,25(OH)_2D_3$ 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 (4×10^4 cells per well) were added to the collagen-coated wells and precultured for 24 hr. A limited number of peripheral blood mononuclear cells (4×10^4 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 ^{125}I-labeled calcitonin (^{125}I-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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ and dexamethasone, spleen cell-derived clusters first appeared on the ST2 cell layer on day 5 (Fig. 1*A*). Well-defined colonies grown from the clusters appeared on day 10, and TRAPase-positive mononuclear cells began to appear in some colonies (Fig. 1*B*). 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. 1*C*). TRAPase-positive multinucleated cells also appeared mainly in the peripheral region of the colony on day 12 (Fig. 1*C*). 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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

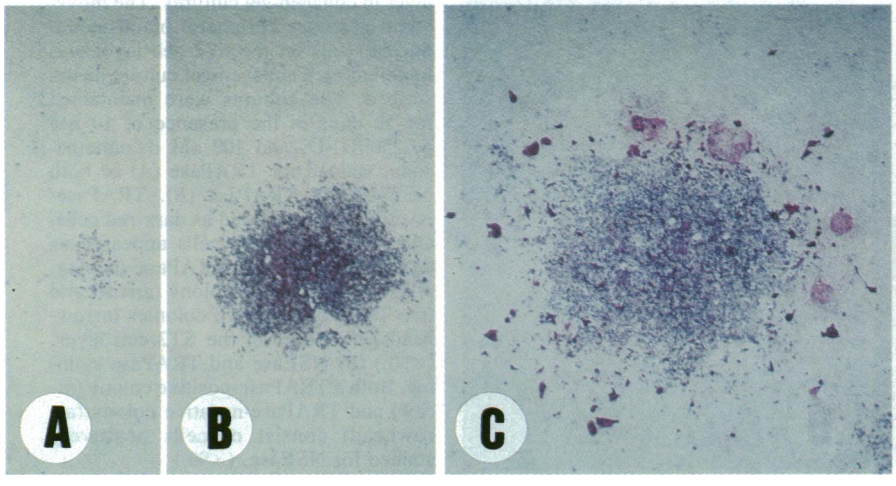

FIG. 1. A spleen cell-derived cluster and a colony formed on the ST2 cell layer. ST2 cells (4×10^4 cells per well) were first cultured for 24 hr. Mouse spleen cells (10^4 cells per well) were added to the ST2 cell layer and cocultured in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 100 nM dexamethasone. 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.)

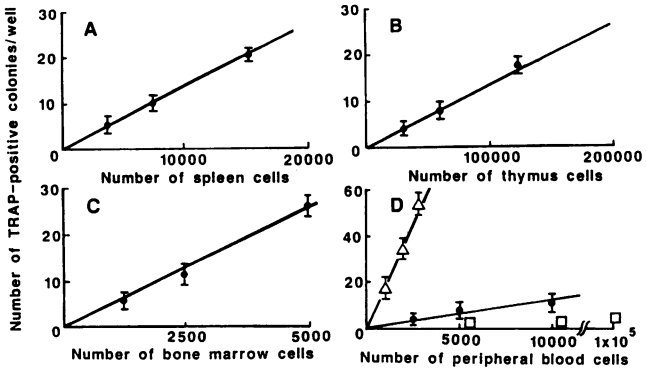

FIG. 2. The relationship between the number of TRAPase (TRAP)-positive colonies formed on the ST2 cell layers and the number of hemopoietic mononuclear cells inoculated. Increasing numbers of mononuclear cells obtained from spleen (*A*), thymus (*B*), bone marrow (*C*), and peripheral blood populations (*D*) were cultured on the ST2 cell layers in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 100 nM dexamethasone. In *D*, peripheral blood mononuclear cells were cultured before (●) and after fractionation into the monocyte-enriched population (△) and the monocyte-depleted population (□). TRAPase-positive colonies were counted after being cultured for 12 days. The results are expressed as the means ± SEM of four cultures.

blood mononuclear cells were fractionated, the monocyte-depleted population failed to form colonies on the ST2 cell layers (only 2 cells per 10^5), whereas the monocyte-enriched population produced a large number of TRAPase-positive colonies (1600 cells per 10^5 cells) (Fig. 2*D*). The ratio of TRAPase-positive colonies to total colonies was roughly 30% regardless of the cell types inoculated.

To further determine whether TRAPase-positive colonies are of single-cell origin, we used a collagen gel culture with peripheral blood mononuclear cells. This procedure allowed peripheral blood mononuclear cells to adhere to the ST2 cell layer but prevented their movement. After being cultured for 12 days in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone, similar TRAPase-positive colonies were formed on the ST2 cell layer (Fig. 3*A*). All of the colonies consisted of NSEase-positive cells, and some of them contained TRAPase-positive cells (Fig. 3*B*). The colonies formed on the ST2 cell layers from mononuclear cells of other hemopoietic tissues were similarly positive for NSEase (data not shown). These results 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 layers. When a small number of alveolar macrophages (8–32 cells) were cultured on the ST2 cell layers in the presence of $1\alpha,25(OH)_2D_3$ 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 ^{125}I-calcitonin to TRAPase-positive cells in the cocultures of alveolar macrophages and ST2 cells in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone. Numerous dense grains due to the binding of ^{125}I-calcitonin appeared on the TRAPase-positive cells (Fig. 5*A*). 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. 5*B*). TRAPase-negative multinucleated cells were formed from alveolar macrophages in response to $1\alpha,25(OH)_2D_3$ and dexamethasone in the absence of ST2 cells, but they accumulated no grains, indicating no detectable calcitonin binding (Fig. 5*C*). 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 ^{125}I-calcitonin binding (Table 1). A similar distribution of the ^{125}I-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 $1\alpha,25(OH)_2D_3$ and dexamethasone, numerous resorption lacunae were formed on their surfaces (Fig. 6*A*). No resorption lacunae were detected on the dentine slice on which alveolar macrophages were cultured without ST2 cells in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone (Fig. 6*B*).

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-

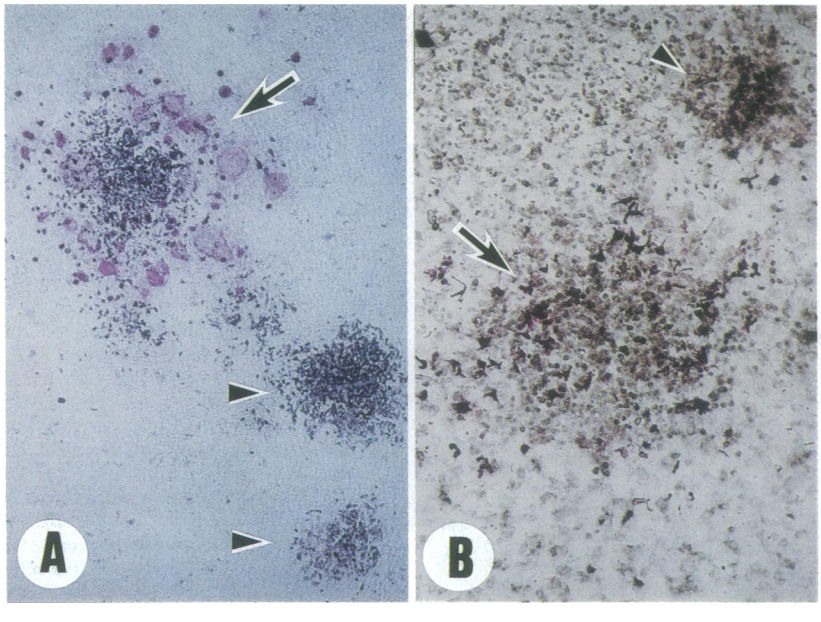

FIG. 3. Enzyme histochemistry for the TRAPase and NSEase activity of peripheral blood mononuclear cell-derived colonies formed on the ST2 cell layer in collagen-gel cultures. The movement of mouse peripheral blood mononuclear cells on the ST2 cell layer was inhibited by a collagen-gel culture as described. The cultures were maintained for 12 days in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 100 nM dexamethasone, stained for TRAPase (*A*) or both NSEase and TRAPase (*B*). TRAPase-positive cells appeared as dark-red cells, and NSEase-positive cells appeared as dark-brown cells. (*A*) TRAPase staining. A TRAPase-positive colony (arrow) and two TRAPase-negative colonies (arrowheads) are seen on the ST2 cell layer. (×90.) (*B*) NSEase and TRAPase staining. Both a TRAPase-positive colony (arrow) and TRAPase-negative colony (arrowhead) consist of cells positively stained for NSEase. (×90.)

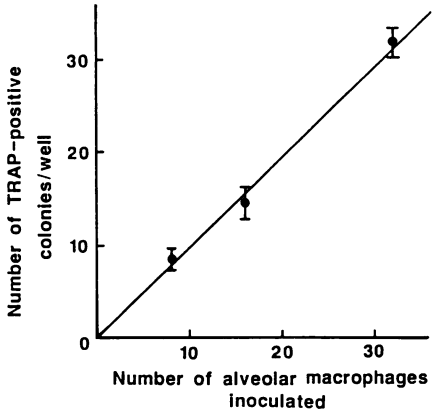

FIG. 4. Relationship between the number of TRAPase (TRAP)-positive colonies formed on the ST2 cell layers and the number of alveolar macrophages inoculated. After a limited number of mouse alveolar macrophages (8–32 cells) were cultured on the ST2 cell layers in the presence of 10 nM $1\alpha,25(OH)_2D_3$ 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.

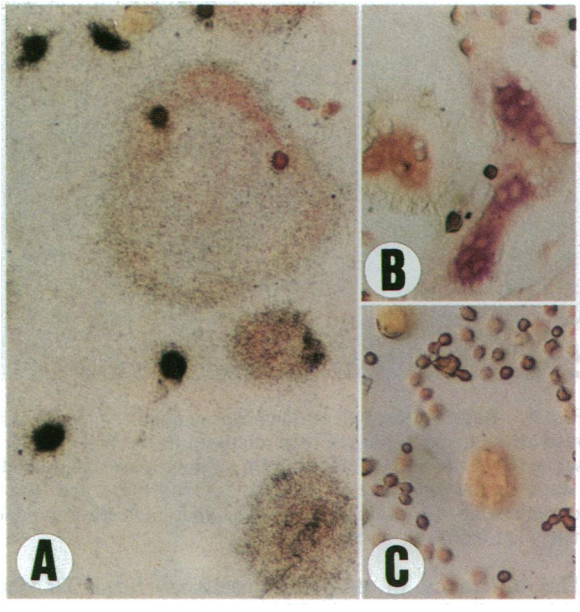

FIG. 5. Autoradiography of salmon ^{125}I-calcitonin binding in the cultures of mouse alveolar macrophages in the presence or absence of ST2 cells. Alveolar macrophages were cultured for 15 days on the ST2 cell layer prepared on Lux coverslips (*A* and *B*). Alveolar macrophages were directly seeded in the center of the coverslips and cultured for 5 days (*C*). Both cultures were maintained in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 100 nM dexamethasone. After culture for the indicated times, cells were incubated with salmon ^{125}I-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 ^{125}I-calcitonin binding appear only on TRAPase-positive cells in *A*.

lated 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 $1\alpha,25(OH)_2D_3$ 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 (Figs. 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 5×10^5 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 10^4 alveolar macrophages per well with ST2 cells (data not shown). Apparently macrophages produce an inhibitory factor(s) for osteoclast differentiation. Peritoneal cells induced by thioglycolate (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 mature tissue macrophages can also differentiate into osteoclasts in the presence of marrow-derived stromal cells.

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

Cells cultured on the ST2 cell layer	$TRAPase^+$ mononuclear cells			$TRAPase^+$ multinucleated cells		
		$CalR^+$ cells			$CalR^+$ cells	
	Total no.	No.	%	Total no.	No.	%
Peripheral blood mononuclear cells	236	221	94	32	29	91
Alveolar macrophages	435	409	94	71	66	93

Peripheral blood mononuclear cells (10^4 cells) or alveolar macrophages (100 cells) were cultured in the presence of 10 nM $1\alpha,25(OH)_2D_3$ 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 ^{125}I-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 μm^2 were counted as $CalR^+$ cells. Specific accumulation of grains was not detected on $TRAPase^-$ cells. In each assay, all cells appearing on three coverslips were counted.

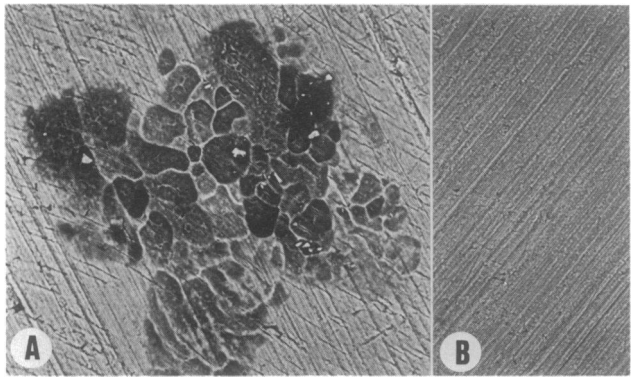

FIG. 6. Backscattered electron images of dentine slices on which mouse alveolar macrophages were cultured in the presence (*A*) or absence (*B*) of the ST2 cell layer. Both cultures were maintained for 15 days in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 100 nM dexamethasone. The dark resorption lacunae indicate low mineral density. (*A*, ×155; *B*, ×95.)

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. There were no differences between the TRAPase-positive and -negative colonies in the size of the colonies or the cell number in each colony. Both TRAPase-negative and -positive colonies were similarly positive for NSEase, indicating that they are of the same lineage of the mononuclear phagocyte family.

One of the growth factors possibly involved in the formation of NSEase-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-forming 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 $1\alpha,25(OH)_2D_3$. 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 10^4 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).

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