Brief Definitive Report

Essential Role of Macrophage Colony-Stimulating Factor in the Osteoclast Differentiation Supported by Stromal Cells

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Summary

Severe deficiency of osteoclasts, monocytes, and peritoneal macrophages in osteopetrotic (*op/op*) mutant mice is caused by the absence of functional macrophage colony-stimulating factor (M-CSF). To clarify the role of M-CSF in the osteoclast differentiation, we established a clonal stromal cell line OP6L7 capable of supporting hemopoiesis from newborn *op/op* mouse calvaria. Although very few macrophages appeared in the cocultures of bone marrow cells and OP6L7 cells, a 50-fold larger number of macrophages was detected in the day 7 cocultures when purified recombinant human M-CSF (rhM-CSF) was exogenously supplied. Tartrate-resistant acid phosphatase (TRACP; a marker enzyme of osteoclasts)-positive cells appeared only when bone marrow cells were cultured in contact with OP6L7 cells and both rhM-CSF and 1α, 25 $(OH)_2D_3$ were added. The TRACP-positive cells became multinucleated with increasing time in culture and expressed the *c-fms*/M-CSF receptor. These results indicate that both contact with stromal cells and M-CSF are requisite for osteoclast differentiation under physiological conditions.

Macrophage colony-stimulating factor (M-CSF) has been identified in vitro by its ability to support the clonal growth and differentiation of precursor cells of monocyte-macrophage lineage. Mice homozygous for the recessive osteopetrosis mutation (*op/op*), having a severe deficiency of osteoclasts, monocytes, and peritoneal macrophages (1–3), have recently been found to be defective in the production of functional M-CSF (4–6) as a result of a one base-pair insertion within the coding region of the M-CSF gene on chromosome 3 (4). Wiktor-Jedrzejczak et al. (5) reported that the macrophage deficiency in the peritoneal cavity of the mutants is partially corrected by implantation of diffusion chambers containing M-CSF–secreting L-929 cells. Felix et al. (7) and Kodama et al. (8) have demonstrated that the deficiencies of osteoclasts and monocytes can be completely cured by daily injections of purified recombinant human M-CSF (rhM-CSF). These findings indicate that M-CSF is essential for the differentiation not only of monocyte/macrophages but also of osteoclasts under physiological conditions.

However, the precise role of M-CSF in osteoclast differentiation has not yet been made clear. Kurihara et al. (9) reported that M-CSF, unlike interleukin 3 and granulocyte/macrophage colony-stimulating factor, cannot support in vitro osteoclast formation. Udagawa et al. (10) developed another in vitro culture system for osteoclast differentiation, one in which spleen cells are cocultured with stromal cell line MC3T3-G2/PA6 or ST2. MC3T3-G2/PA6 cells support differentiation of osteoclasts (10), as well as proliferation of hemopoietic stem cells defined as colony-forming units in spleen (CFU-S) (11), only through short range cell-to-cell interaction; whereas the stromal cells per se produce M-CSF (Sudo, T., personal communication).

In this study, we established a clonal stromal cell line OP6L7 from newborn *op/op* mouse calvaria capable of supporting hemopoiesis but not differentiation of macrophages and osteoclasts, and demonstrated that both contact with stromal cells and M-CSF are requisite for the osteoclast differentiation.

Materials and Methods

Mice. F_2 hybrid mice of +/+, *op*/+, and *op/op* genotypes were raised in our laboratory from breeding pairs of B6C3F1-*a/a*, *op*/+ mice obtained from The Jackson Laboratory (Bar Harbor, ME). Female C57BL/6CrSlc mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Establishment of Cell Lines. Calvariae were aseptically removed from a litter of nine newborn F_2 mice and minced in 35-mm plastic dishes (Sumitomo Bakelite, Tokyo, Japan). The bone fragments were suspended in medium consisting of α-MEM (Irvine Scientific, Santa Ana, CA), 20% fetal bovine serum (FBS; Irvine Scientific), and 0.08% type I collagen (Nitta Gelatin, Osaka, Japan). After the collagen had gelled, the cultures were incubated at 37°C in 5% CO_2 for 4 d. The cells from two mice (3 and 6) showing

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an osteopetrotic phenotype, i.e., an excessive amount of bone trabeculae and very few osteoclasts in their femurs, were subcultured on 3T3 schedule using α-MEM supplemented with 10% FBS (12, 13). Three of 15 cell lines established could support hemopoiesis for more than 4 wk. One clone, designated OP6L7, was used for the following experiments.

Coculture of Bone Marrow Cells with OP6L7 Cells. Bone marrow cells of femurs from 6–10-wk-old C57BL/6CrSlc mice were inoculated at 10^5 cells/dish onto confluent OP6L7 cell layers established in collagen-coated 35-mm dishes and cultured with 1.5 ml of α-MEM supplemented with 20% horse serum (Hyclone Laboratories, Logan, UT) and 10^{-8} M dexamethasone (DEX) in the absence or presence of 0.5 μg/ml of purified rhM-CSF (a gift of Morinaga Milk Industry, Kanagawa, Japan) for 7 d. Cells were harvested by treatment with 0.1% collagenase (Nitta Gelatin). Total number of the cells was determined using a hemocytometer. For differential cell counts, cytospun preparations were stained with May-Grünwald Giemsa solution or treated with F4/80 mAb specific for macrophages (14) (American Type Culture Collection, Rockville, MD) and subsequently with the reagents of a Vectastain ABC-GO kit (Vector Laboratories, Inc., Burlingame, CA). CFU-S assay was performed as described previously (15).

Osteoclast Formation. Bone marrow cells and OP6L7 cells were cocultured as described above in 1.5 ml of α-MEM supplemented with 10% FBS, 10^{-8} M 1α, 25 $(OH)_2D_3$ (a gift of Teijin Institute for Bio-Medical Research, Tokyo, Japan), and 10^{-8} M DEX in the absence or presence of 0.5 μg/ml rhM-CSF, and the medium was changed twice a week. To examine the necessity of cell-to-cell contact with stromal cells for osteoclast differentiation, we inoculated bone marrow cells onto OP6L7 cell layers covered with 1 ml of collagen gel and cultured them as described above. At indicated times, the cultures were fixed and stained for tartrate-resistant acid phosphatase (TRACP) activity as described previously (10) or immunocytochemically stained with monoclonal anti-c-*fms*/M-CSF receptor (Ab-1) antibody (Oncogene Science, Manhasset, NY). To confirm the osteoclastic activity of the multinucleated cells formed in the cocultures, we seeded bone marrow cells onto OP6L7 cell layers established on ivory slices (provided by Dr. M. Kumegawa, Meikai University School of Dentistry, Sakado, Japan) placed in 35-mm dishes and cultured as described above for 21 d. The slices were fixed and trypsinized to remove attached cells. After conductive staining of the slices, their secondary electron images were examined with a Hitachi S-450 scanning electron microscope (Hitachi, Tokyo, Japan).

Results and Discussion

By coculturing bone marrow cells with the stromal cell lines established from newborn *op/op* mouse calvariae, we found that very few macrophages appeared within the cocultures and that three of these cell lines could support hemopoiesis for more than 4 wk and differentiate into adipocytes in response to glucocorticoid, similarly to MC3T3-G2/PA6 cells (11, 13). Then we isolated a clone, designated OP6L7, from one of these three lines, the OP6L line.

When bone marrow cells were cocultured with OP6L7 cells for 7 d, the number of CFU-S increased about threefold, since 10^5 normal bone marrow cells contained 23 ± 2.0 CFU-S (Table 1). Very few F4/80-positive macrophages were detected in the cocultures. When rhM-CSF (0.5 μg/ml) was added to the cocultures, a 50-fold larger number of macrophages appeared, while the number of neutrophils and blasts increased only 2.4- and 1.4-fold, respectively. The CFU-S number did not change significantly, showing that rhM-CSF does not affect CFU-S growth in our coculture system. These results demonstrate that OP6L7 cells can support the proliferation of hemopoietic stem cells and their differentiation into neutrophils, but not into macrophages, because of functional M-CSF.

Table 1. *Effect of rhM-CSF on the Hemopoiesis Supported by OP6L7 Cells**

	No. of cells/dish			
Addition	Macrophages	Neutrophils	Blasts	CFU-S (mean ± S.D.)
None	0.7×10^4	2.0×10^5	3.8×10^4	75 ± 17
M-CSF	3.5×10^5	4.8×10^5	5.2×10^4	80 ± 20

* Bone marrow cells were inoculated onto confluent OP6L7 cell layers at 10^5 cells/dish and cocultured in the absence or presence of 0.5 μg/ml rhM-CSF for 7 d.

To explore the conditions required for the osteoclast differen-

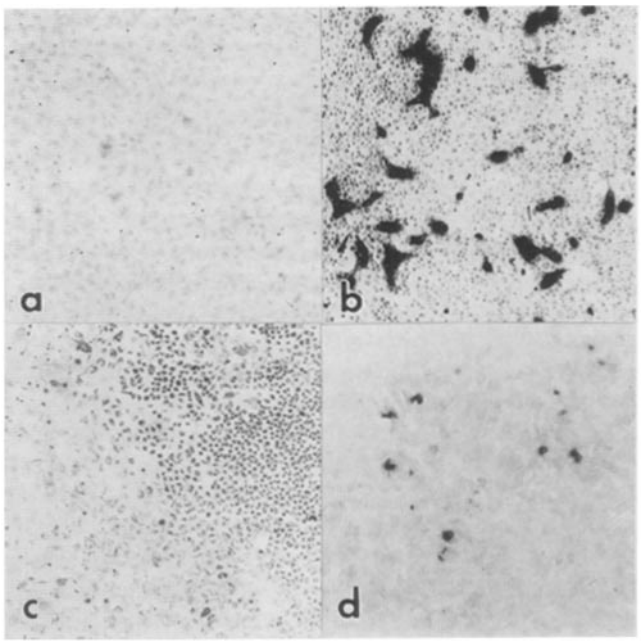

Figure 1. Culture conditions required for the osteoclast differentiation supported by OP6L7 cells. Bone marrow cells were cocultured with OP6L7 cells in the presence of 10^{-8} M 1 α, 25 $(OH)_2D_3$ for 12 d under the following culture conditions, and the cultures were then stained for TRACP activity and counterstained with hematoxylin: (*a*) in contact with the stromal cells and without addition of rhM-CSF; (*b*) in contact with the stromal cells and in the presence of 0.5 μg/ml rhM-CSF; (*c*) without contact with the stromal cells and in the presence of rhM-CSF. 40×. (*d*) Micrograph shows a pure population of TRACP-positive cells that developed after coculturing of a single precursor cell with OP6L7 cells in the presence of rhM-CSF and 1 α, 25 $(OH)_2D_3$ for 7 d. 50×.

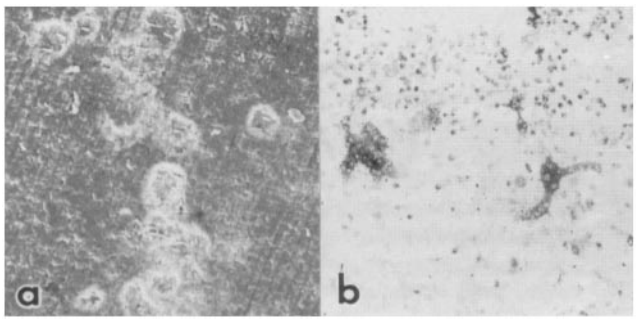

Figure 2. Characteristics of multinucleated cells formed in cocultures of bone marrow cells and OP6L7 cells. (*a*) Secondary electron image of an ivory slice on which bone marrow cells and OP6L7 cells were cocultured in the presence of 10^{-8} M 1 α, 25 $(OH)_2D_3$ and 0.5 μg/ml rhM-CSF for 21 d. 260×. (*b*) Immunocytochemical demonstration of the presence of c-*fms*/M-CSF receptor on the cells in the day 12 culture. Multinucleated cells and macrophages are positively stained, but OP6L7 cells are negative. 50×.

tiation in the cocultures with OP6L7 cells, we added 10^{-8} M 1 α, 25 $(OH)_2D_3$ to cultures as described previously (10). In the absence of rhM-CSF, TRACP-positive cells, as well as macrophages and neutrophils, were hardly detectable (Fig. 1 *a*). 10^{-8} M 1 α, 25 $(OH)_2D_3$ was inhibitory for the neutrophil differentiation. When rhM-CSF was added to the cocultures, both TRACP-positive cells and macrophages appeared (Fig. 1 *b*). In contrast, when bone marrow cells and OP6L7 cells were cocultured under the condition where they were prevented from coming into contact with each other, TRACP-positive cells failed to appear even in the presence of rhM-CSF, while macrophage differentiation was still observed (Fig. 1 *c*). In addition, when bone marrow cells alone were cultured in the presence of rhM-CSF and 1 α, 25 $(OH)_2D_3$, only macrophage differentiation was observed (data not shown). Most of the TRACP-positive cells became multinucleated (up to 20 nuclei) by day 21 of culture and displayed calcitonin receptors (data not shown). Resorption lacunae were formed on ivory slices only when bone marrow cells were cocultured with OP6L7 cells under the condition where TRACP-positive multinucleated cells were formed (Fig. 2 *a*). The giant cells and macrophages, but not OP6L7 cells, were positively stained with anti-c-*fms*/M-CSF receptor mAb (Fig. 2 *b*). These results demonstrate that OP6L7 cells can support osteoclast differentiation through direct cell-to-cell interaction only when M-CSF is exogenously supplied.

To examine whether other cells derived from bone marrow are involved in the osteoclast differentiation in our coculture system, we isolated single precursor cells from the colonies that appeared in the day 3 cocultures of bone marrow cells and OP6L7 cells and cocultured them with OP6L7 cells again. Using a micromanipulator, we inoculated 379 single precursors onto OP6L7 cell layers established in 48-well multiplates and cocultured them for 7 d. Hemopoietic cell growth was observed in 157 wells (41%). In 93 wells (59%), a pure macrophage population was detected, and a mixed population of macrophages and TRACP-positive cells was detected in 59 wells (38%). A pure population of TRACP-positive cells was observed in 5 wells (3%) (Fig. 1 *d*). These results demonstrate that OP6L7 cells can support osteoclast differentiation without any help from other types of cells.

In the present study, we successfully reproduced a severe deficiency of osteoclasts, monocytes, and peritoneal macrophages in *op/op* mice in the coculture system of bone marrow cells and OP6L7 cells derived from the mutant mouse, and confirmed the finding that M-CSF is requisite for the differentiation not only of macrophages but also of osteoclasts (4–8). Since the c-*fms*/M-CSF receptor was detected on osteoclasts and macrophages, but not on OP6L7 cells, M-CSF probably act directly on osteoclast precursors. Furthermore, we confirmed the findings of Udagawa et al. (10) that stromal cells can exert their effect on osteoclast differentiation only through short range cell-to-cell interaction and of Kurihara et al. (9) that M-CSF by itself cannot support osteoclast differentiation. Therefore, we conclude that both contact with stromal cells and the presence of M-CSF, normally produced by the stromal cells, are required for osteoclast differentiation under physiological conditions. In addition, our results indicate that the primary role of stromal cells in macrophage differentiation is the production of M-CSF.

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