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LACK OF BONE RESORPTION IN OSTEOSCLEROTIC (*oc/oc*) MICE IS DUE TO A DEFECT IN OSTEOCLAST PROGENITORS RATHER THAN THE LOCAL MICROENVIRONMENT PROVIDED BY OSTEOBLASTIC CELLS

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SUMMARY: In a co-culture system of mouse spleen cells and osteoblastic cells, we have demonstrated that a suitable microenvironment must be provided by osteoblastic cells in order for osteoclast-like multinucleated cell (MNC) formation. Using this co-culture system, we examined the pathogenetic mechanism underlying the lack of bone resorption in osteosclerotic *oc/oc* mice. Numerous tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme)-positive MNCs were formed in response to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] both in co-cultures of *oc/oc* spleen cells and normal osteoblastic cells and in those of normal spleen cells and *oc/oc* osteoblastic cells. TRAP-positive MNCs derived from normal spleen cells tended to spread out on culture dishes, whereas those from *oc/oc* spleen cells remained as small, compact MNCs. When TRAP-positive MNCs enriched from co-cultures of normal spleen cells and *oc/oc* osteoblastic cells were cultured on dentine slices, they formed numerous resorption pits with ruffled borders and clear zones. In contrast, none of the TRAP-positive MNCs derived from *oc/oc* spleen cells formed either ruffled borders or resorption pits. These results indicate that the lack of bone resorption in *oc/oc* mice is due to a defect in osteoclast progenitors rather than the local microenvironment provided by osteoblastic cells. © 1992 Academic Press, Inc.

Osteopetrosis is an inherited metabolic bone syndrome caused by several different developmental and functional abnormalities of osteoclasts (1). An osteopetrotic *op/op* strain of mice lacks osteoclasts, the cause of which has been considered to be a defect in the local microenvironment within the bone. This conclusion is based on the observation that bone marrow transplantation cannot cure the osteopetrotic disorders in this strain of mice. Recently, the osteoclast deficiency in *op/op* mice was reported to be due to a mutation in the coding region of the macrophage colony-stimulating factor (M-CSF) gene (2,3). The defect of osteoclast formation and bone resorption in *op/op* mice was restored by the administration of recombinant M-CSF (4,5). We have developed a co-culture system of mouse spleen and osteoblastic cells, in which osteoclast-like multinucleated cells (MNCs) are formed in response to Iα,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (6-8). Using this co-culture system, we found that osteoblastic cells obtained from *op/op* mice could not support osteoclast development from normal spleen cells (9). Addition of M-CSF together with 1α,25$(OH)_2D_3$ to co-cultures with *op/op* osteoblastic cells completely reversed osteoclast-like MNC formation. This indicates that M-CSF from osteoblastic cells is essential for osteoclast development (9).

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The osteosclerotic *oc/oc* mutation, another osteopetrotic disorder found in mice, is lethal typically causing death within 3 weeks of birth (1). It has been reported that transplantation of hemopoietic cells into *oc/oc* mice failed to cure the bone disorders in this strain of mice as well. This suggests that the lack of bone resorption in *oc/oc* mice is due to a defect in the local microenvironment within the bone, as in *op/op* mice (10). In the present study, we examined the pathogenesis of osteosclerosis in *oc/oc* mice using our co-culture system. We report here that the lack of bone resorption in *oc/oc* mice is due to a defect in osteoclast progenitors rather than in the local microenvironment provided by osteoblastic cells.

MATERIALS AND METHODS

Preparation of Osteoblastic Cells and Spleen Cells

(C57BL/6J x C3HeB/FeJ) F1-*oc*/+ parent mice were obtained from the Jackson Laboratory (Bar Harbor, ME). A quarter of their littermates are expected to be osteosclerotic (*oc/oc*). The *oc/oc* homozygotes were radiologically distinguished at birth from phenotypically normal, +/? siblings. The calvaria, spleen and tibia were individually removed from 3-day-old newborn littermates. Tibiae were subjected to enzyme histochemistry for TRAP activity. Osteoblastic cells were prepared from each calvaria which had been cultured in type I collagen gels as described previously (9). Spleen cells were also individually obtained from the splenic tissues of littermates. Normal osteoblastic cells and spleen cells were prepared from 3-day-old ddy mice and 7-to 9-week old ddy mice, respectively, as described previously (9).

Co-culture system

Osteoblastic cells (10^4 cells/well) obtained from *oc/oc* or +/? littermates were co-cultured with normal ddy spleen cells (5×10^5 cells/well) in α-Minimum Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY) in 24-well Corning plates (Corning, NY) . Similarly, spleen cells from either *oc/oc* or +/? littermates were co-cultured with normal osteoblastic cells from ddy mice. $1\alpha,25(OH)_2D_3$ (Philips Duphar, Amsterdam, The Netherlands) was added to the co-cultures at 10 nM. In order to obtain a TRAP-positive MNC-enriched population, co-cultures were performed for 7 days on collagen gel-coated dishes in the presence of $1\alpha,25(OH)_2D_3$ as described previously (11). Cultures were then treated with 0.2% bacterial collagenase (11). The resulting cell suspension was gently layered on 35% Percoll in Tyrode's solution and centrifuged at 250 g for 20 min. The cells accumulated at the interface layer were collected, suspended in α-MEM containing 10% FBS, and used for determining bone-resorbing activity (pit formation on dentine slices).

Determination of Osteoclast Characteristics

After being cultured for 7 days, adherent cells were fixed and stained for TRAP, and the number of TRAP-positive MNCs was scored as described previously (3). For autoradiography using [^{125}I]-salmon calcitonin, co-cultures were performed for 7 days on coverslips placed in 24-well plates. Cultures were then incubated with 0.2 nM [^{125}I]-calcitonin in the presence or absence of an excess amount (200 nM) of unlabeled salmon calcitonin, stained for TRAP and processed for autoradiography as described previously (9,12). To determine bone-resorbing activity, TRAP-positive MNC-enriched populations were put on dentine slices (200 to 300 TRAP-positive MNCs/12.6 mm^2 slice). After they had been cultured for 48 to 72 h, resorption pits were detected using a scanning electron microscope or backscattered electron imaging as described previously (8). Ultrastructural features of MNCs on dentine slices were also observed by means of transmission electron microscopy.

RESULTS

Many TRAP-positive osteoclasts were detected in bone sections prepared from *oc/oc* littermates (data not shown). This was in sharp contrast to the bone sections from *op/op* mice, in which TRAP-positive osteoclasts were barely detectable. Similarly, numerous TRAP-positive MNCs were formed both in co-cultures of *oc/oc* spleen cells and normal osteoblastic cells and in those of normal spleen cells and *oc/oc* osteoblastic cells, when the cells were treated with 10 nM $1\alpha,25(OH)_2D_3$ for 7 days. Interestingly, TRAP-positive MNCs derived from *oc/oc* spleen cells were not capable of spreading out on the dish surface, and their size was somewhat smaller than that of TRAP-positive MNCs derived from spleen cells of +/? littermates or ddy mice (Fig. 1 and Table I). The mean numbers of nuclei per TRAP-positive MNC, however, were not significantly different between TRAP-positive MNCs formed in co-cultures of four combinations of osteoblastic cells and spleen cells either from *oc/oc* or +/? littermates, or from normal ddy mice (Table I). No morphological differences were recognized between osteoblastic cells obtained from *oc/oc* mutants and those from +/? littermates (data not shown). [^{125}I]-salmon calcitonin specifically bound to 95% of the TRAP-positive MNCs formed both in co-cultures of normal spleen cells and *oc/oc* osteoblastic cells and in those of *oc/oc* spleen cells and normal osteoblastic cells (Fig. 1, insets).

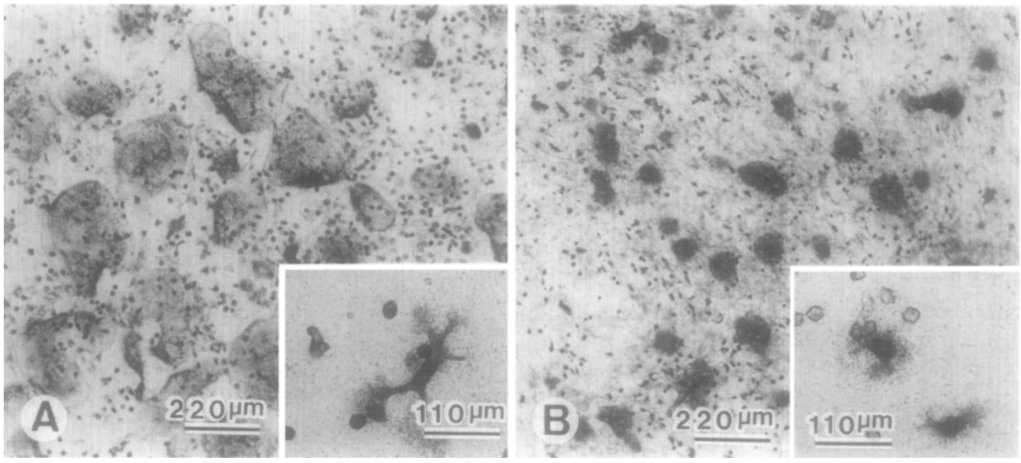

Fig. 1. Localization of TRAP activity and [^{125}I]-calcitonin binding in co-cultures of spleen cells or osteoblastic cells obtained from *oc/oc* mice and their counterparts from normal ddy mice. Co-cultures were performed for 7 days in the presence of 10 nM 1α,25$(OH)_2D_3$. (A) A co-culture of normal ddy spleen cells and *oc/oc* osteoblastic cells. (B) A co-culture of *oc/oc* spleen cells and normal ddy osteoblastic cells. Note that TRAP-positive MNCs formed from normal spleen cells tended to spread out on the dish surface (A), but those from *oc/oc* spleen cells remained as small, compact MNCs (B). [^{125}I]-calcitonin specifically bound to TRAP-positive cells formed in both co-cultures in a similar manner (insets).

In order to determine their bone-resorbing activity, TRAP-positive MNC-enriched populations were cultured on dentine slices. TRAP-positive MNC-enriched populations prepared from co-cultures of normal spleen cells and *oc/oc* osteoblastic cells (Fig. 2A), normal spleen cells and +/? osteoblastic cells (data not shown), and +/? spleen cells and normal osteoblastic cells (data not shown) similarly formed many resorption pits within 48 h on dentine slices. In contrast, TRAP-positive MNC-enriched populations individually prepared from 23 co-cultures of *oc/oc* spleen cells and normal osteoblastic cells failed to form resorption pits

Table I. Characteristics of TRAP-positive MNCs formed in co-cultures ot spleen cells or osteoblastic cells obtained from +/? and *oc/oc* littermates and their counterparts from normal ddy mice

Co-culture Systems		TRAP-positive MNCs			
Spleen Cells	Osteoblasts	Cells/well[1)]	Nuclei/cell[2)]	Plan Area[3)]	Pit formation[4)]
		(number/well)	(number/cell)	($mm^2 \times 10^{-3}$/cell)	
Normal[5)]	+/?	150 ± 16 (4)[6)]	10.3 ± 2.5	38.1 ± 4.3	+ (15)[6)]
Normal	*oc/oc*	167 ± 20 (4)	11.5 ± 1.9	40.6 ± 4.5	+ (6)
+/?	Normal	177 ± 22 (6)	10.7 ± 3.0	37.6 ± 4.3	+ (20)
oc/oc	Normal	184 ± 26 (5)	11.7 ± 3.2	7.0 ± 2.6*	− (23)

Co-cultures were performed in 24-well plates or on collagen-gel coated plates in the presence of 10 nM 1α,25(OH)2D3 for 8 days. The results are expressed as means ± SEM.

*, Significantly different from the other co-cultures, $p < 0.01$.

1) TRAP-positive cells containing three or more nuclei were counted as TRAP-positive MNCs.

2) Numbers of nuclei were scored at 35 TRAP-positive MNCs in a typical experiment.

3) Plan areas covered with each TRAP-positive MNC containing five or more nuclei were individually measured at 35 randomly selected TRAP-positive MNCs in a typical experiment.

4) TRAP-positive MNCs enriched from individual co-cultures were incubated on dentine slices for 48 to 72 h. Bone-resorbing ability is shown by + or −.

5) Normal spleen cells and osteoblastic cells were obtained from ddy mice.

6) Figures in parentheses are the numbers of independent sets of experiments.

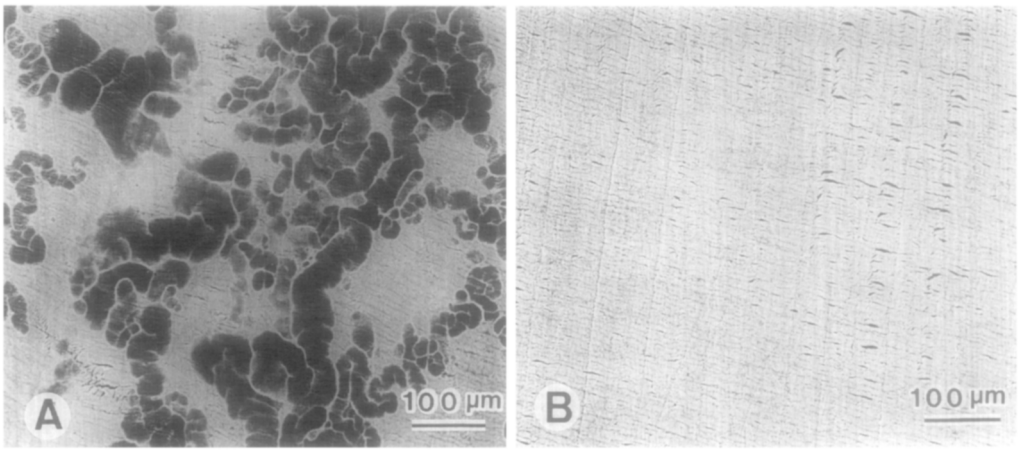

Fig. 2. Backscattered electron images of dentine slices on which TRAP-positive MNC-enriched populations prepared from co-cultures of normal ddy spleen cells and *oc/oc* osteoblastic cells (A) and those from co-cultures of *oc/oc* spleen cells and normal ddy osteoblastic cells (B) had been cultured for 72 h. The dark resorption lacunae indicate low mineral density. Note that no resorption pits are detecteable on the slices on which *oc/oc* spleen cell-derived TRAP-positive MNCs had been cultured (B).

(Fig. 2B and Table I). The TRAP-positive MNCs obtained from co-cultures of normal spleen cells and oc/oc osteoblastic cells developed characteristic ruffled borders and clear zones on dentine slices (Fig. 3A). In contrast, no MNCs derived from *oc/oc* spleen cells formed ruffled borders, though they showed other cytological features of osteoclasts such as abundant pleomorphic mitochondria and large numbers of lysosomes (Fig. 3B). Clear zones were also detected in peripheral regions of the MNCs (Fig. 3B).

DISCUSSION

Osteoblastic cells isolated from *oc/oc* mice supported the formation of functionally active osteoclast-like cells in co-cultures with normal spleen cells. In contrast, *oc/oc* spleen cells differentiated into small, compact TRAP-positive MNCs in co-cultures with normal osteoblastic cells, which failed to form ruffled borders and resorption pits on dentine slices. These abnormalities of the osteoclast-like MNCs formed *in vitro* were very similar to those of osteoclasts found in bone tissues of *oc/oc* mice (13). TRAP-positive MNCs derived from *oc/oc* spleen cells did not appear to produce a soluble inhibitor(s) of bone resorption, since pit formation by normal TRAP-positive MNCs was unaffected by simultaneous addition of *oc/oc* spleen cell-derived TRAP-positive MNCs (data not shown). These results suggest that the lack of bone resorption in *oc/oc* mice is due to a defect in osteoclast progenitors rather than the local microenvironment provided by osteoblastic cells. The inability of marrow transplantation to cure osteosclerotic disorders in *oc/oc* mice may be related to the fact that *oc/oc* mutants die within 3 weeks of birth.

The MNCs derived from *oc/oc* spleen cells in co-cultures with normal osteoblastic cells had several characteristics of osteoclasts such as TRAP activity, calcitonin receptors, and large numbers of mitochondria and lysosomes. Clear zones were also recognized. In support of these observations, we found that F-actin visualized by rhodamine-phalloidin staining was distributed as a ringed structure in peripheral regions of *oc/oc* spleen cell-derived osteoclast-like MNCs cultured on dentine slices (Udagawa N, Kanehisa J, Suda T, unpublished observation). Furthermore, in immunological studies using antibodies for carbonic anhydrase II (kindly provided by Dr. Väänänen, University of Oulu), vacuolar type proton-ATPase (kindly provided by Drs. Futai and Moriyama, Osaka University), and c-*src*, we found that *oc/oc* spleen cell-derived

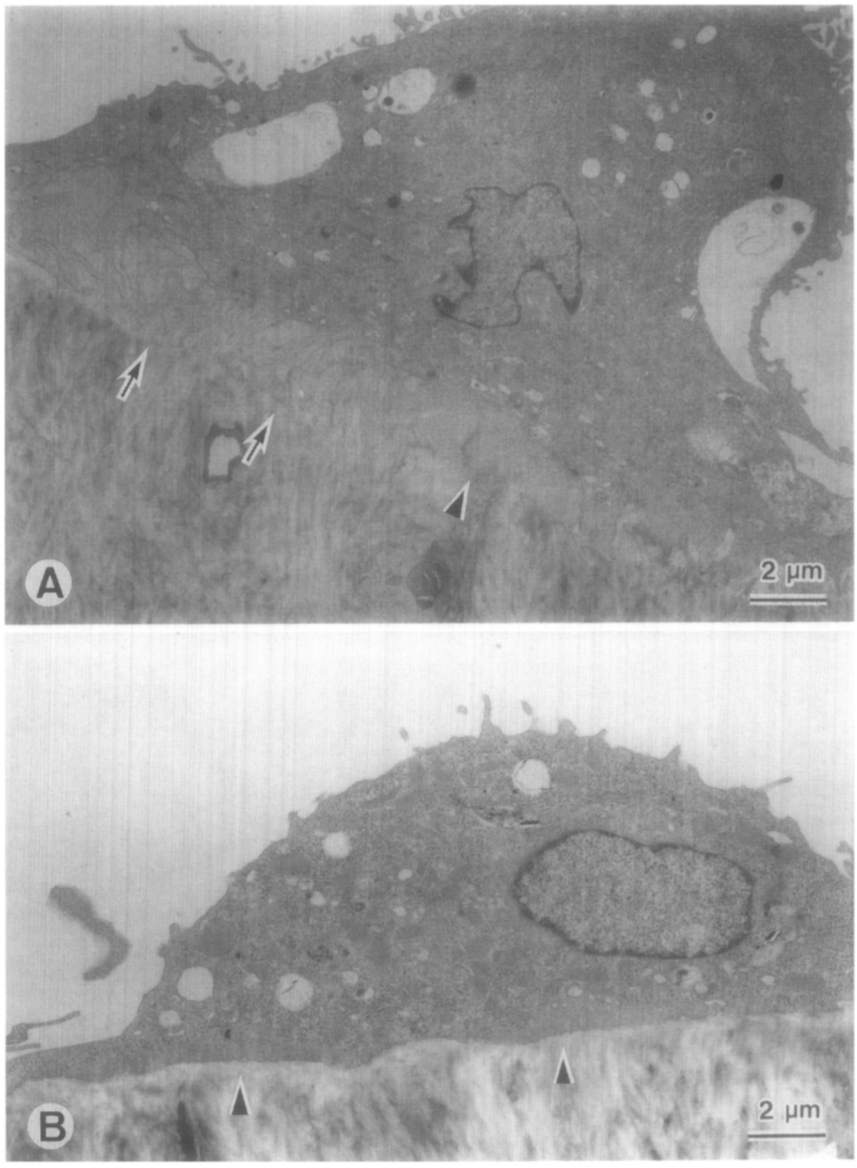

Fig. 3. Ultrastructural features of MNCs cultured on dentine slices. (A) An MNC derived from co-cultures of normal ddy spleen cells and *oc/oc* osteoblastic cells. Characteristic ruffled borders (arrows) and clear zones (arrow heads) are seen in the MNC. (B) An MNC derived from co-cultures of *oc/oc* spleen cells and normal ddy osteoblastic cells. No ruffled borders are detectable. Other characteristics of osteoclasts such as clear zones (arrow heads), abundant pleomorphic mitochondria, and large numbers of lysosomes are seen in the *oc/oc* spleen cell-derived MNC.

osteoclast-like MNCs strongly expressed these proteins (data not shown), though it is not known whether such proteins are functionally active in *oc/oc* osteoclasts.

In summary, the lack of bone resorption in *oc/oc* mice is due to a defect in osteoclast progenitors rather than the local microenvironment provided by osteoblastic cells. The cause of this inability of *oc/oc* osteoclasts to form ruffled borders is not known. Elucidation of the mechanism underlying the defect will shed light on unsolved problems of osteoclast function.

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