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ACTIVIN ENHANCES OSTEOCLAST-LIKE CELL FORMATION IN VITRO

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SUMMARY: The effect of activin (activin A/ EDF) on osteoclast formation was investigated. In mouse bone marrow cell cultures, activin enhanced the formation of tartrate-resistant acid phosphatase (TRACP)-positive multinucleated cells (MNC) in a dose-dependent manner, either in the presence or absence of 1,25-$(OH)_2D_3$ or PTH. In organ cultures of neonatal mouse calvaria, activin also enhanced the generation of TRACP-positive giant cells in the endosteal periosteum and increased the TRACP staining of whole calvaria, but did not exhibit bone resorbing activity. These results indicate that activin stimulates the formation of osteoclasts, but not osteoclast activation. Activin is produced by bone marrow cells and might be involved in the local process of osteoclast differentiation.

Activin (activin A/ EDF) is a member of the transforming growth factor (TGF) ß superfamily which was originally detected by its stimulation of follicle-stimulating hormone secretion (1, 2) and by its induction of the differentiation of Friend erythroleukemia (3). More recently, it has been shown that activin stimulates the proliferation of osteoblastic cells and the synthesis of collagen by these cells (4). Activin has also been shown to promote the *in vivo* induction of ectopic bone formation by bone morphogenic protein (5). These findings suggest the involvement of activin in bone formation. In the case of TGF-ß, there is extensive literature concerning about its activity on bone formation and on bone resorption (6-9).

However, little is known about the role of activin in the process of bone resorption. Since activin is produced by bone marrow cells (10, 11) and has the

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ability to induce the monocytic differentiation of HL-60 cells (12), it might be assumed that activin plays a role in the differentiation of osteoclasts which are believed to generate from the monocytic lineage (13). The present study was, therefore, undertaken to investigate the effects of activin on osteoclast formation in mouse bone marrow cell cultures and neonatal mouse calvarial organ cultures.

MATERIALS AND METHODS

Activin: Recombinant human activin was purified from the culture supernatant of Chinese hamster ovarian cells bearing an expression vector for inhibin β_A subunit cDNA (14)

Bone Marrow Cell Culture: Male ddy mice aged 7-9 weeks were purchased from Funabashi Nohjou (Japan). Bone marrow cells were collected and cultured as described previously(15). In brief, the mice were sacrificed by cervical dislocation under light ether anesthesia, and the femurs and tibias were dissected free from the surrounding tissues. The medial and distal portions of these bones were cut off with scissors and the marrow cavities were flushed out with 1 ml of α-minimal essential medium (α-MEM). The cells were suspended by repeated aspiration with a 26-gauge needle and were washed twice with α-MEM. Then they were cultured for 8 days in α-MEM containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin) at a density of 1 x 10^6 cells /ml in 24-well culture plates (0.75 ml /well). Cultures were fed every 3 days by replacing 0.5 ml of the old medium with fresh one. Activin, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$; Teijin Co., Inc, Japan) and parathormone (PTH; Protein Research Laboratories, Japan) were added at the beginning of culture and at each medium change. Cultures were maintained at 37 °C with a humidified atmosphere of 5% CO_2 in air. At the end of culture, the cells were fixed with 10% formaldehyde containing 1% $CaCl_2$. After washing with ethanol-acetone (50:50, vol/vol), the cells were stained for tartrate-resistant acid phosphatase (TRACP) using a modification of the method of Burstone (16). TRACP-positive cells containing 3 or more nuclei were counted as multinucleated cells (MNC).

Mouse Calvarial Culture: The organ culture of neonatal mouse calvaria has previously been described in detail (17). Briefly, the calvaria (frontal and parietal bones) were removed from 4- to 6-day-old ddy mice and were cultured for 72 h while floating freely in roller tubes in 2 ml of Dulbecco's modified Eagle medium supplemented with 15% horse serum, 10 units/ml of heparin and 100 units/ml of penicillin. The culture medium was changed after 24 h. Activin and PTH were added at the start of culture and at the time of medium change. Bone resorption was quantified by determining the calcium concentration in the medium after 72 h of culture by the OCPC method (18). The cultured calvaria were fixed in 10% formaldehyde containing $CaCl_2$ and stained for TRACP as described above. Then the stained calvaria were mounted in glycerin jelly and TRACP-positive cells were observed under a microscope. Furthermore, the intensity of TRACP staining for each whole calvarium was quantified by densitometry with an image analyzer (ASPECT, Mitani-shoji, Tokyo). The image was fed directly to a TV monitor at a magnification of 20 times using a video camera, and 2-dimensional densitometry was performed to obtain the mean density of staining of the whole calvarium.

Statistics: Differences between groups were analyzed by Student's *t*-test. All results are given as the mean ± SEM.

RESULTS

In bone marrow cell cultures, activin enhanced the formation of TRACP-positive MNC in a dose-dependent manner (Fig. 1). The maximal increase was 4 fold greater than in control cultures at 100 ng/ml of activin, which was less than the effect of 10 nM 1,25-$(OH)_2D_3$ or 10 nM PTH. The microscopic appearance of the TRACP-positive MNC formed in the presence of activin was similar to that of cells generated by 1,25-$(OH)_2D_3$ or PTH (Fig. 2). Activin also increased the number of TRACP-positive MNC in a dose-dependent manner in the presence of 10 nM 1,25-$(OH)_2D_3$ or 10 nM PTH.

The effect of activin on the formation of osteoclast-like cells was also examined in calvarial organ cultures. As shown in Fig. 3, activin treatment increased the number of TRACP-positive giant cells in the endosteal periosteum. The intensity of TRACP staining of the whole calvarium also increased when culture was performed in the presence of activin (Fig. 4A). When 10 nM PTH was added to the culture as a positive control, numerous TRACP-positive giant cells and bone resorption lacunae with TRACP-positive margins appeared in the endosteal periosteum (Fig. 3), and the intensity of whole calvarial TRACP staining was increased (Fig. 4A). Although the calcium concentration of the culture medium

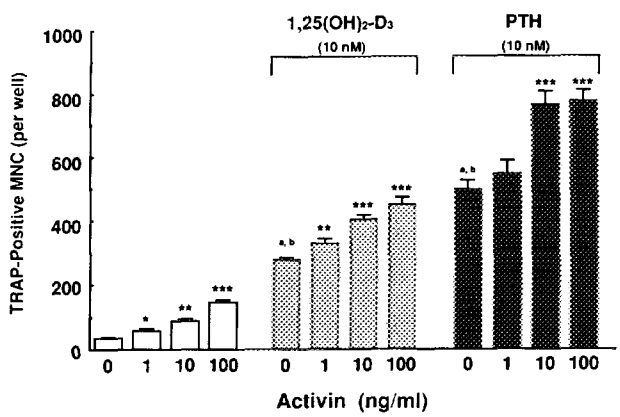

Fig. 1. Effect of activin on the formation of TRACP-positive MNC. Mouse bone marrow cells (7.5 x 10^5 cells per well) were cultured in the presence or absence of 10 nM 1,25-$(OH)_2D_3$ or 10 nM PTH for 8 days, and activin (0-100 ng/ml) was added to each culture. The number of TRACP-positive MNC was counted in each well.
*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. the control without activin in each group.
a: $p<0.001$ vs. the control without 1,25-$(OH)_2D_3$, PTH, or activin.
b: $p<0.001$ vs. 100 ng/ml of activin alone.

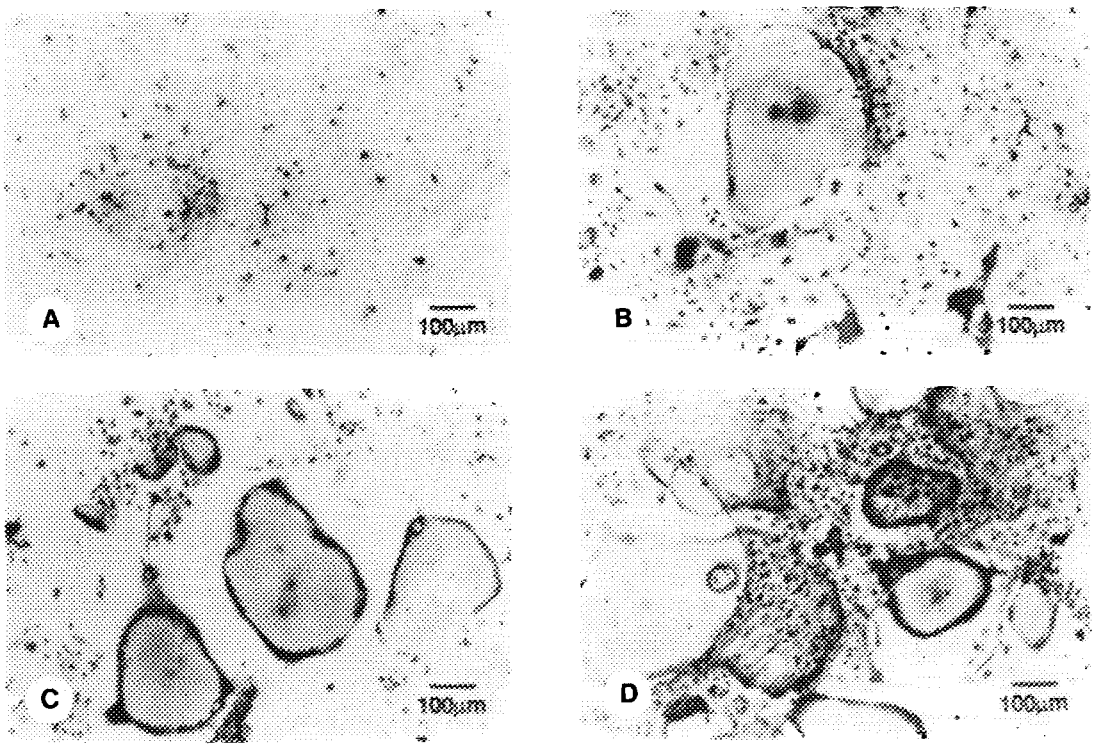

Fig. 2. Microscopic appearance of TRACP-positive MNC in bone marrow cell cultures. Mouse bone marrow cells were cultured in the presence or absence of activin (100 ng/ml), 1,25-$(OH)_2D_3$ (10 nM), or PTH (10 nM) for 8 days. TRACP-positive cells are the darkly stained cells. (A) TRACP-positive cells were rare in control cultures. (B) TRACP-positive mononuclear and multinucleated cells were generated by activin treatment. Numerous TRACP-positive mononuclear and multinucleated cells were seen in cultures with 1,25-$(OH)_2D_3$ (C) and PTH (D).

was doubled by PTH treatment, activin did not change the calcium concentration (Fig. 4B).

DISCUSSION

This study demonstrated that activin stimulated the formation of osteoclast-like cells. It is well known that osteoclast-like cells are formed when mouse bone marrow cells are cultured in the presence of 1,25-$(OH)_2D_3$ or PTH and that these cells have many of the characteristics of osteoclast, including multiple nuclei, calcitonin-binding capacity, enzymatic activity (carbonic anhydrase and TRACP), and the ability to resorb bone when cultured on dentin slices (15, 19). Although the stimulatory effect of activin on osteoclast-like cell formation was less than that of 1,25-$(OH)_2D_3$ or PTH, microscopic appearance of TRACP-positive MNC was similar in all cases. Activin synergistically enhanced the formation of osteoclast-like cells in the presence of 1,25-$(OH)_2D_3$ or PTH, suggesting that it acts through

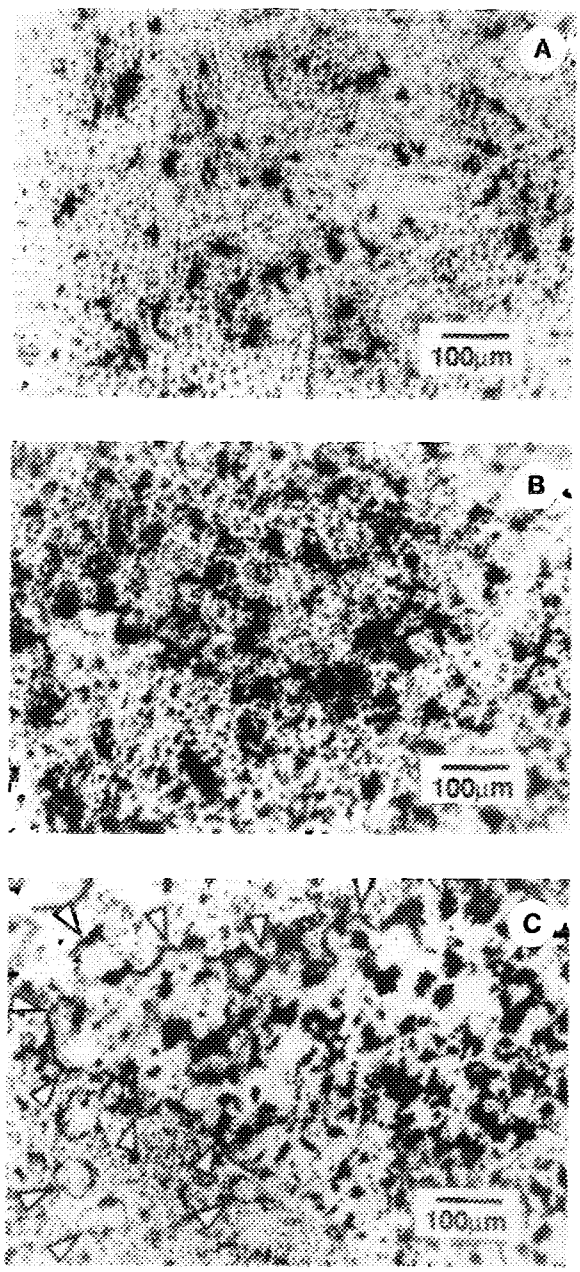

Fig. 3. TRACP staining of cultured calvaria. Neonatal mouse calvaria were cultured for 3 days in the presence or absence of activin (100 ng/ml) or PTH (10 nM), and each calvarium was stained for TRACP. Many TRACP-positive giant cells were seen in the endosteal periosteum of calvaria cultured in the presence of activin (B) and PTH (C), while TRACP staining was weak in control calvaria (A). Bone resorption lacunae margined by TRACP activity (arrows) appeared in the calvaria cultured with PTH, but not in the calvaria cultured with activin.

different mechanisms from those of 1,25-$(OH)_2D_3$ or PTH. In calvarial cultures, activin stimulated the formation of TRACP-positive giant cells and increased the

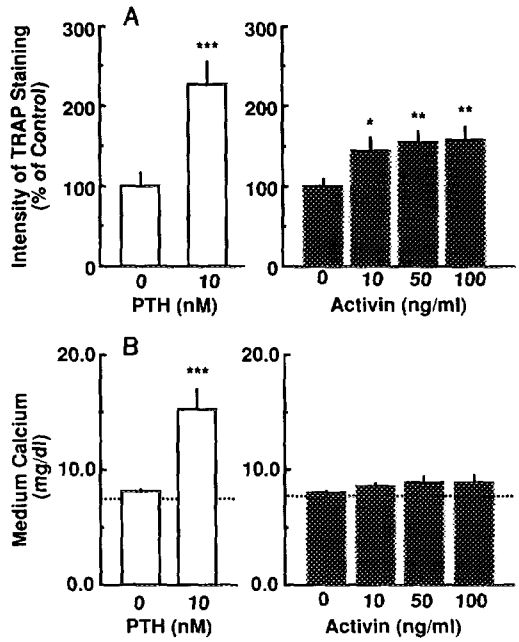

Fig. 4. Effects of activin on the intensity of TRACP staining and bone resorption. Mouse calvaria, containing approximately 600 μg of calcium, were cultured for 3 days in the presence or absence of activin (10-100 ng/ml) or PTH (10 nM). The calvaria were stained for TRACP and the culture medium was collected. (A) The intensity of TRACP staining for the whole calvarium was determined by image analysis. (B) The calcium concentration of each culture medium (24-72 h) was measured. The calcium concentration of the medium before the culture was 7.7 mg/dl (dotted lines).
*: $p<0.05$, **: $p<0.01$, ***: $p<0.005$.

intensity of TRACP staining of the whole calvarium, however, did not affect bone resorption. These results indicate that activin enhances the earlier stages of osteoclast differentiation but does not activate already formed osteoclasts. Other cytokines, such as macrophage colony-stimulating factor (M-CSF) and interleukin-6 (IL-6), are also known to stimulate osteoclast-like cell formation but not bone resorption (20, 21).

Activin may enhance osteoclast-like cell formation both directly and indirectly. Many reports have suggested that osteoclasts are derived from the monocytic lineage. Shavit et al. (13) have demonstrated that 1,25-$(OH)_2D_3$ induces the monocytic differentiation and multinucleation of the human promyelocytic leukemia cell line (HL-60), which is accompanied by the development of bone-resorbing ability. Activin is also known to induce the monocytic differentiation of HL-60 cells (12). Thus, activin might act directly on osteoclast progenitors. Osteoblasts are recognized to be essential for osteoclast differentiation. Takahashi et al. (15) have shown that most of TRACP-positive MNC formed in bone marrow cell culture are adjacent to colonies of alkaline phosphatase-positive

osteoblastic cells. They have also shown that co-culture of calvarial osteoblastic cells enhances osteoclast-like cell formation from spleen cells.(22). Since a specific receptor for activin has been detected on osteoblastic cells (23), the action of activin might be mediated by osteoblastic cells.

The findings presented here suggest that activin may act as a local factor promoting osteoclast differentiation. We have previously reported the production of activin by bone marrow cells (10), and Yamashita et al. have also found that a bone marrow stromal cell line expressed activin mRNA (11). Recently, Ogawa et al. (5) reported the presence of activin in bone matrix and we found the release of activin activity from cultured calvaria accompanied with bone resorption (R. Sakai and H. Shinoda, unpublished). Many studies have focused on the involvement of various cytokines, such as IL-1 (24, 25), IL-6 (20), M-CSF (21) and TGF-ß (7-9), in osteoclast differentiation. These cytokines are normally present in the bone microenviroment. Further studies are needed to determine the individual roles of these various factors in osteoclast differentiation.

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