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# SYNTHESIS OF COLONY-STIMULATING FACTOR (CSF) AND DIFFERENTIATION-INDUCING FACTOR (D-FACTOR) BY OSTEOBLASTIC CELLS, CLONE MC3T3-E1

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SUMMARY: The role of osteoblasts in inducing the proliferation and differentiation of bone marrow cells was examined. Conditioned medium obtained from mouse osteoblastic cell (MC3T3-E1) cultures stimulated colony formation of mouse bone marrow cells (CSF) and differentiation of mouse myeloid leukemia cells (M1) into macrophage-like cells (D-factor). The CSF activity increased time dependently in parallel with the increase of alkaline phosphatase activity during the culturing of the MC3T3-E1 cells. The activity of the D-factor attained a maximum on days 12 - 15 and decreased thereafter. Both the CSF and the D-factor were eluted in a range of 25,000 to 67,000 daltons on gel filtration. The fraction containing both factors exhibited bone-resorbing activity. These results suggest that osteoblasts are involved in bone resorption at least in part by enhancing the proliferation and differentiation of osteoclast progenitors. 

The hypothesis that cells belonging to the osteoblastic lineage regulate bone resorption is based primarily on the observation that the osteoblastic cells possess receptors to bone-resorbing hormones such as parathyroid hormone, 1α,25-dihydroxyvitamin $D_3$ and prostaglandins (1). Rodan and Martin (2) proposed that such bone-resorbing agents induce a marked morphological change in osteoblasts. This change uncovers the bone matrix, exposing it to osteoclasts or osteoclast progenitors (2). The resulting bone matrix digestion further increases bone resorption (2). Subsequently, Malone et al. (3) showed that osteocalcin derived from bone matrix or produced by osteoblasts is chemotactic for osteoclast precursors. Chambers et al. (4) demonstrated that PTH had no effect on the spreading of isolated osteoclasts, but it caused a marked

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Abbreviations used: MC3T3-E1, a mouse osteoblastic cell line; M1, a mouse myeloid leukemia cell line; CSF, colony-stimulating factor; D-factor, differentiation-inducing factor.

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increase in spreading of osteoclasts when they were co-cultured with osteoblasts. These results suggest that osteoblasts are somehow involved in the osteoclastic bone resorption. However, the role of osteoblasts in inducing osteoclastic bone resorption is not known.

It is believed that osteoclasts are derived from immature bone marrow cells (5). There are two schools of thought about the origin of osteoclasts. One school assumes that the origin of osteoclasts is the cells of the monocyte-macrophage lineage (6). The other claims the existence of a specific unidentified osteoclast progenitor cell (7). In either case, multinucleated osteoclasts are formed by proliferation, differentiation and fusion of immature bone marrow cells. Therefore, it is interesting to determine whether osteoblasts have any effect on the proliferation and differentiation of immature bone marrow cells.

We report here that the clonal mouse osteoblastic cell line, MC3T3-E1, originally established by Kodama et al. (8), produces factors which stimulate colony formation of mouse bone marrow cells (colony-stimulating factor, CSF) and also induce differentiation of mouse myeloid leukemia cells (M1) into macrophage-like cells (differentiation-inducing factor, D-factor).

## MATERIALS AND METHODS

Cells and cell culture: Clonal MC3T3-E1 cells were isolated from the MC3T3-E cell line derived from newborn C57/BL/6 mouse calvaria (8). These cells were generously provided by Dr. H. Kodama (Tohoku Dental College, Koriyama, Japan). Cells were cultured in α-MEM (Flow Laboratories, Va.) supplemented with 10% fetal calf serum (M. A. Bioproducts, Md.) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. For the experiments, $1.5 \times 10^5$ cells were plated on 25 $cm^2$ plastic bottles and cultured for up to 21 days in 5 ml of medium supplemented with 10% fetal calf serum. Medium was changed every 3 days. At days 6, 12 and 18, cells were transferred to serum-free medium with 0.1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Mo), and cultured for 3 more days. Cell number and alkaline phosphatase activity in the cells were determined, and conditioned media were collected to determine the activities of the CSF and the D-factor. The cell number was determined after treating the cells for 30 min with 0.2% collagenase (Wako Pure Chemicals, Osaka, Japan) and for 10 min with 0.6% Dispase (Sanko Pure Chemicals, Tokyo).

Measurement of alkaline phosphatase activity: After being cultured for the indicated number of days, the cells were washed, suspended in 0.05% Triton X-100 containing 1 mM $MgCl_2$, and homogenized. The homogenates were centrifuged, and the supernatant was used to assay the alkaline phosphatase activity with p-nitrophenyl phosphate as substrate by the method of Lowry et al. (9). One unit of the enzyme activity was defined as the activity hydrolyzing 1 nmol of the substrate per min.

Measurement of CSF: Bone marrow cells were flushed from femurs of 6 - 8-weeks-old mice of the ddy strain. Routinely, 1 x $10^5$ nucleated cells were cultured at 37°C in 1 ml of McCoy's modified 5A medium (GIBCO, Grand Island, NY) containing 0.3% agar, 20% fetal calf serum (GIBCO) and 25% conditioned medium from the MC3T3-E1 cells. After incubation for 7 days, colonies consisting of 50 or more cells were scored with a microscope (CSF) as described previously (10).

Measurement of D-factor: Mouse myeloid leukemia cells (M1) were generously provided by Dr. M. Hozumi (Saitama Cancer Center, Oomiya, Japan). The cells (5 x $10^4$) were cultured for 3 days with conditioned medium from the MC3T3-E1 cells at a concentration of 25% (v/v) at 37°C under 5% $CO_2$ in air. Then, the cells were washed and further incubated for 4 h with polystyrene particles (2 µl/ml, Difco) at 37°C. After incubation, the cells were washed and the number of phagocytic cells was counted under a microscope as described previously (11). At least 200 viable cells were counted.

Measurement of bone-resorbing activity: To examine bone-resorbing activity of the conditioned medium from osteoblast cultures, 24 ml of the conditioned medium obtained from the MC3T3-E1 cells cultured on days 12 - 15 in the presence of 0.1% BSA was concentrated 50-fold by ultrafiltration with a membrane YM-10 (Amicon Corp., Cambridge, Mass.) and applied to a Toyopearl HW-50 column (Toyo Soda, Tokyo). The column was eluted with Earle's balanced salt solution. The elution position was calibrated with bovine serum albumin (M.W. 67,000), chymotrypsinogen A (25,000), and egg white lysozyme (14,000) as marker proteins. Fractions containing CSF and D-factor were pooled, concentrated 8-fold, and used to assay bone-resorbing activity. The procedure of bone-resorbing activity has been described in a previous paper (12). To assay the bone-resorbing activity, at least 3 paired calvaria were used. Results were expressed as the treated/control ratio (T/C ratio) by the following formula:

$$^{45}\text{Ca release (\%)} = \frac{^{45}\text{Ca in medium}}{^{45}\text{Ca in medium} + ^{45}\text{Ca in bone}} \times 100$$

$$\text{T/C ratio} = \frac{^{45}\text{Ca release (\%) from the treated calvaria}}{^{45}\text{Ca release (\%) from the control calvaria}}$$

## RESULTS AND DISCUSSION

The MC3T3-E1 cells are naturally destined to differentiate into osteoblasts to form calcified bone tissues *in vitro* (13). Figure 1 shows the growth curve and the change in alkaline phosphatase activity of the MC3T3-E1 cells. The cells continued to grow after becoming confluent on day 3, and the cell number reached a maximum on day 15. Alkaline phosphatase activity, a marker for osteoblasts, began to increase when the cells became confluent on day 3, and it attained a level of 280 units/mg protein on day 21.

It is known that bone marrow progenitor cells called CFU-C (colony-forming unit in culture) can be grown and induced to differentiate into granulocytes and macrophages *in vitro* in the presence of CSF (14). The conditioned medium obtained from the MC3T3-E1 cells cultured for the first 3 days (days

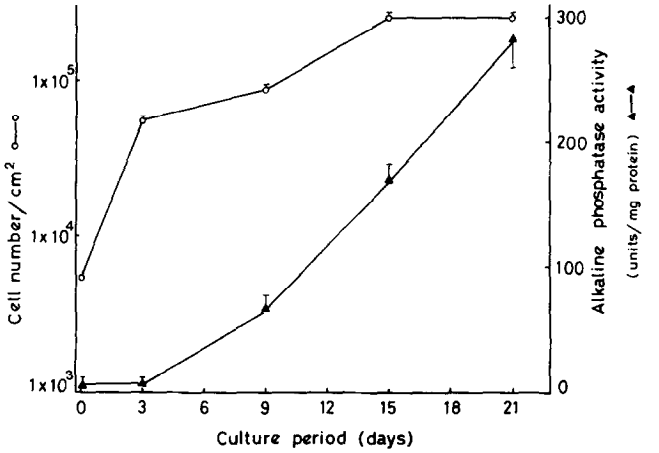


Fig. 1 Growth curve ( ○ ) and change in the alkaline phosphatase activity ( ▲ ) of the MC3T3-E1 cells. Points and bars are means ± S.E.M. of 6 replicates.

0 - 3), at a concentration of 25% (v/v), significantly stimulated the colony formation of mouse bone marrow cells (Fig. 2). No colony was formed in the absence of the conditioned medium (data not shown). The CSF activity increased time dependently in parallel with the increase of alkaline phosphatase activity.

The conditioned medium obtained from later stage cultures of the MC3T3-E1 cells induced differentiation of mouse myeloid leukemia cells (M1) into macrophage-like cells (Fig. 2). The activity of the D-factor attained a maximum on days 12 - 15 and decreased thereafter. As much as 50% of the cells exhibited phagocytic activity, when the conditioned medium obtained from the

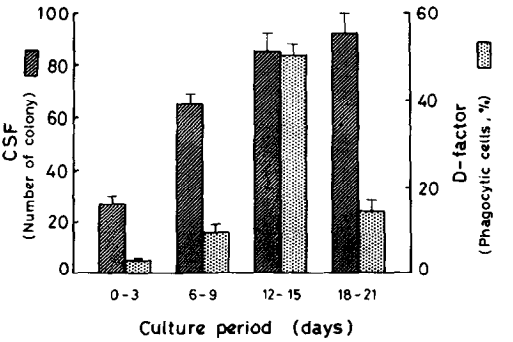


Fig. 2 Time course of change in CSF ( ▨ ) and D-factor ( ▩ ) in the conditioned medium of the MC3T3-E1 cells. No colony was formed without conditioned medium. The control value of phagocytic activity of M1 cells was 6.6 ± 0.5%. Data and bars are means ± S.E.M. of 6 replicates.

MC3T3-E1 cell cultures on days 12 - 15 was added at a concentration of 25% (v/v). These results suggest that the differentiated osteoblasts with the high alkaline phosphatase activity not only stimulate colony formation of immature bone marrow cells, but also induce differentiation of these cells to the monocyte-macrophage lineage cells. It is likely that the CSF and the D-factor in the conditioned medium from the MC3T3-E1 cells are different compounds, since time course of change in these factors was different. Tomida et al. have reported that the antiserum to the D-factor purified from conditioned medium of L929 cell cultures did not cross-react with any CSFs (15).

To confirm that osteoblasts are in fact involved in bone resorption, the conditioned medium from the MC3T3-E1 cells was added directly to the assay of bone-resorbing activity. The medium, however, did not exhibit bone-resorbing activity at any concentrations. This appeared to be due to the fact that the concentration of the conditioned medium was too low to stimulate bone resorption. Therefore, 24 ml of the conditioned medium from the MC3T3-E1 cells cultured on days 12 - 15 in the presence of 0.1% BSA was pooled, concentrated 50-fold by ultracentrifugation, and subjected to gel filtration with a Toyopearl HW-50 column. Both the CSF and the D-factor were eluted at similar po-

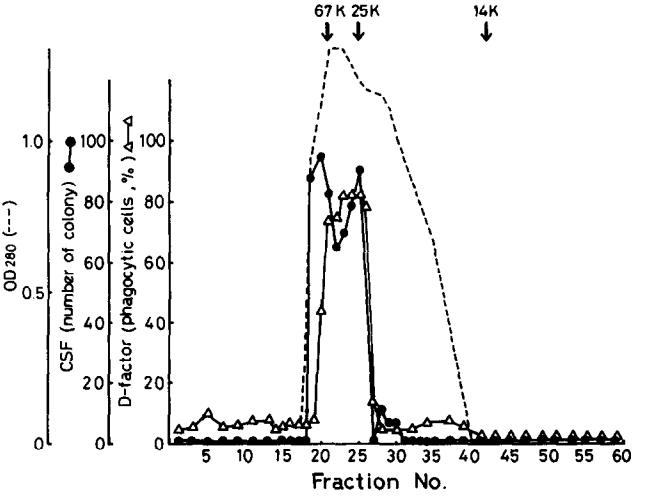


Fig. 3 Gel filtration of conditioned medium from MC3T3-E1 cells on Toyopearl HW-50. Cells were cultured in medium containing 0.1% BSA on days 12 - 15, and the conditioned medium was pooled and concentrated 50-fold. Each 1 ml-fraction was assayed for the CSF ( ● ) and the D-factor ( △ ) at a concentration of 15%.

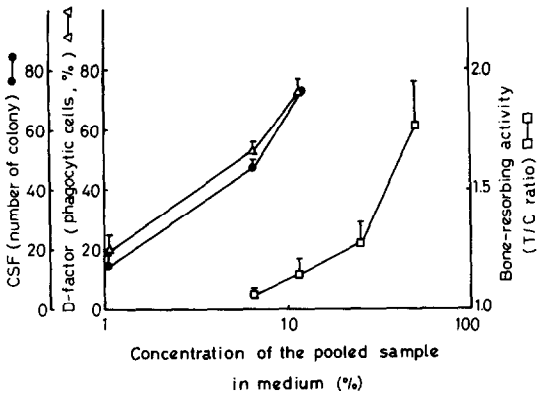


Fig. 4 Dose-response curves of the concentrated fractions from Toyopearl HW-50 gel filtration on the CSF ( ● ), D-factor ( △ ) and bone-resorbing activity ( □ ). Fractions (No. 19-26) on gel filtration were pooled, concentrated 8-fold, and used to assay each activity.

sitions on gel filtration (Fig. 3). The activity which induces fusion of macrophages (macrophage fusion factor, MFF) (16) was not detected in any of the fractions.

Fractions (tubes 19-26) containing the CSF and the D-factor on gel filtration were pooled, further concentrated 8-fold by ultrafiltration, and used to assay the CSF, the D-factor and bone-resorbing activity (Fig. 4). Dose-response curves were recognized in all three assays. Bone resorption was stimulated by adding the concentrated fraction at a concentration of 50% to the assay system of bone-resorbing activity, whereas the CSF and the D-factor were detected at a concentration of 6.25%. Preliminary experiments showed that highly purified D-factors from L929 cells and Ehrlich ascitic tumor cells stimulated bone resorption (unpublished results). These results suggest that osteoblasts are involved in bone resorption at least in part by enhancing proliferation and differentiation of osteoclast progenitors. Further purification of the CSF and the D-factor obtained from the MC3T3-E1 cells is in progress in our laboratory.

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