BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 199–204 (1998)
ARTICLE NO. RC988586

Osteoclast Differentiation Factor (ODF) Induces Osteoclast-like Cell Formation in Human Peripheral Blood Mononuclear Cell Cultures

Kenichiro Matsuzaki,*,‡ Nobuyuki Udagawa,* Naoyuki Takahashi,* Kyoji Yamaguchi,† Hisataka Yasuda,† Nobuyuki Shima,† Tomonori Morinaga,† Yoshiaki Toyama,‡ Yutaka Yabe,‡ Kanji Higashio,† and Tatsuo Suda*,[1]

**Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan; †Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., 519 Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan; and ‡Department of Orthopaedic Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan*

Received April 1, 1998

We have reported that osteoclast differentiation factor (ODF) expressed on the plasma membrane of osteoblasts/stromal cells is a ligand for osteoclastogenesis inhibitory factor (OCIF). A genetically engineered soluble form of ODF (sODF) induced osteoclast-like multinucleated cells (OCLs) in the presence of M-CSF in mouse spleen cell cultures. Osteoblasts/stromal cells were not required in this process. To elucidate the mechanism of human osteoclastogenesis, human peripheral blood mononuclear cells (PBMCs) were cultured for 7 days with sODF and human M-CSF in the presence or absence of dexamethasone. Treatment of human PBMCs with sODF together with M-CSF induced OCLs, which expressed tartrate-resistant acid phosphatase and vitronectin receptors, produced cAMP in response to calcitonin, and formed resorption pits on dentine slices. OCLs were also formed from the adherent cell population of human PBMCs. Dexamethasone was required for human OCL formation in culture of whole PBMCs but not in culture of the adherent cell population. OCL formation was strongly inhibited by OCIF simultaneously added. These results clearly indicate that like in mouse osteoclastogenesis, ODF is a critical factor for human osteoclastogenesis. The present study also indicates that OCIF acts as a naturally occurring decoy receptor for ODF in inhibiting signal transduction in human osteoclast formation.

Osteoclasts are multinucleated cells responsible for bone resorption (1-3). It is well recognized that osteoclasts are derived from hematopoietic progenitor cells (1-3). We have established a co-culture system of mouse hematopoietic cells and osteoblasts/stromal cells, in which osteoclast-like multinucleated cells (OCLs) were formed in response to several bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], parathyroid hormone (PTH) and interleukin-11 (IL-11) (1,2). Using this co-culture system, it has been shown that osteoblasts/stromal cells play a crucial role in osteoclast formation through a mechanism of cell-to-cell interaction with osteoclast progenitors (1-5). We also reported that osteoclasts can be formed not only from mouse spleen or bone marrow cells but also from some mature macrophages or peripheral blood monocytes in co-cultures with osteoblasts/stromal cells (6). We hypothesized that a membrane bound factor designated "osteoclast differentiation factor (ODF)" is expressed on osteoblasts/stromal cells in response to bone-resorbing factors, and that it induces osteoclastogenesis by signaling to osteoclast progenitors (1,2).

Recently, we cloned cDNA of osteoclastogenesis-inhibitory factor (OCIF) (7-9), which was found to be identical to osteoprotegerin (OPG) (10). OCIF was a secretory member of the tumor necrosis factor (TNF) receptor family and inhibited osteoclastogenesis by interrupting the cell-to-cell interaction between osteoblasts/stromal cells and osteoclast progenitors (7-9). More recently, we succeeded in the molecular cloning of a ligand for OCIF, which was a member of the membrane-associated TNF ligand family (11). We showed that this molecule was indeed ODF, the long-sought ligand mediating an essential signal to osteoclast progenitors for their differentiation into osteoclasts (11). The cloned molecule was also identical to TRANCE (12) and RANKL (13), which were independently cloned

[1] Correspondence address: Tatsuo Suda, Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan. Fax: (813) 3784-5555.

0006-291X/98 $25.00

from mouse T cell hybridomas and mouse thymoma cells, respectively. Expression of ODF mRNA by osteoblasts/stromal cells was up-regulated by osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH and IL-11. Macrophage colony stimulating factor (M-CSF) produced by osteoblasts/stromal cells has been shown to be also an essential factor in mouse osteoclast formation (14-16). A genetically engineered soluble form of ODF (sODF) together with M-CSF induced OCLs from mouse spleen cells in the absence of osteoblasts/stromal cells (11).

The generation of human osteoclasts in vitro, as assessed by bone resorption, has been shown to be difficult to achieve (17). Fujikawa et al. (18) first reported that the rat osteosarcoma cell line UMR 106 and the mouse bone marrow-derived stromal cell line ST2 supported human OCL formation in co-culture with human peripheral blood mononuclear cells (PBMCs) in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone. In their experiments, addition of human M-CSF to the co-culture was essential to induce human OCLs, because UMR 106 and ST2 cells produce rat and mouse M-CSF, respectively, which do not act on human M-CSF receptor. Furthermore, Sarma et al. (19 ,20) reported that addition of M-CSF to human bone marrow cultures stimulated human osteoclast formation. These results indicate that both M-CSF and osteoblasts/stromal cells are involved in human osteoclast formation as well. However, there are several reports that human hematopoietic progenitors differentiate into mature osteoclasts in cultures in the absence of M-CSF and osteoblasts/stromal cells (21-23). Thus, the regulation of human osteoclast formation is still controversial with regard to the requirement of M-CSF and osteoblasts/stromal cells.

To elucidate the mechanism of human osteoclast formation, human PBMCs were cultured with sODF and/or human M-CSF. We report here that human OCLs are formed exactly in the same way as mouse OCLs in response to sODF in the presence of human M-CSF without any help of other osteotropic factors and osteoblasts/stromal cells.

MATERIALS AND METHODS

Materials. Recombinant mouse sODF was prepared by fusing the extracellular domain of ODF (Asp^{76}-Asp^{316}) to the C-terminal end of His-Patch thioredoxin (Invitrogen) using His-Patch ThioFusion Expression System (Invitrogen). A DNA fragment encoding the extracellular domain of ODF on pTrx-ODF (11) was inserted between KpnI and SaII sites of pThioHisB to generate pHP-Trx-ODF. sODF was purified from the soluble cytoplasmic fraction of DH5α harboring pHP-Trx-ODF by ProBond (Invitrogen) column chromatography, affinity chromatography on an OCIF-immobilized column and gel filtration chromatography. Recombinant human OCIF was prepared as described previously (8). Recombinant human M-CSF (Leukoprol) was obtained from Green Cross Co. Ltd. (Osaka, Japan). Dexamethasone was purchased from Wako Pure Chemicals Co. (Osaka). Eel calcitonin (Elcatonin) was kindly provided by Asahi Chemical Industry Co. (Tokyo, Japan).

Human peripheral blood mononuclear cell cultures. Human peripheral blood was collected from healthy normal donors in syringes containing 1,000 U/ml of preservative-free heparin. Informed consent was obtained in all cases before blood aspiration. PBMCs were isolated by centrifugation over Histopaque 1077 (Sigma Chemicals Co., St. Louis, MO) density gradients, washed, and resuspended at 1.3×10^6 cells/ml in α-Minimal Essential Medium (αMEM) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). PBMCs were then cultured for 7 days in 48-well plates (4×10^5 cells/0.3 ml/well) in the presence or absence of various concentrations of mouse sODF, human M-CSF, human OCIF and dexamethasone. In some experiments, nonadherent cells were removed by pipetting after culture for 1 hr, and the remaining adherent cells were further cultured for 7 days. Culture medium was replaced every three days with fresh medium supplemented with agents described above.

Determination of characteristics of osteoclasts. After culture for 7 days, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells containing more than 3 nuclei were counted as OCLs as described previously (24). For immunohistochemical detection of vitronectin receptors, cells were fixed and incubated with monoclonal antibody against vitronectin receptors (23C6) (25), and the bound antibodies were visualized as described (26). To determine calcitonin-dependent cAMP production, human PBMCs were cultured in 48-well plates as described above. After culture for 7 days, cells were washed with αMEM and incubated with eel calcitonin (10^{-8} M) for 10 min in the presence of 1 mM isobutylmethylxanthine. Intracellular cAMP was then extracted with 65% ethanol, and the amount of cAMP was determined using a cAMP assay kit (Yamasa Shoyu Co., Chiba, Japan). For a pit formation assay, human PBMCs were cultured in the presence or absence of sODF (100 ng/ml), human M-CSF (200 ng/ml) and dexamethasone (10^{-7} M) in 48-well plates (4×10^5 cells/0.3 ml/well), in which a dentine slice (diameter : 4 mm) had been placed in each well. OCIF (100 ng/ml) was added to some cultures. After culture for 12 days, the resorption pits on dentine slices were visualized by staining with Mayer's hematoxylin as described (24).

RESULTS

Addition of mouse sODF to human PBMC cultures stimulated TRAP-positive OCL formation in a dose-dependent manner in the presence of human M-CSF and dexamethasone (Figs. 1A and 1B). Without dexamethasone, TRAP-positive OCLs were scarcely formed in the PBMC cultures treated with sODF and M-CSF. Without M-CSF, most of the PBMCs disappeared during the 7 day-culture even in the presence of sODF and/or dexamethasone. Addition of human OCIF (10-100 ng/ml) to the culture dose-dependently inhibited TRAP-positive OCL formation induced by a mixture of M-CSF (200 ng/ml), sODF (100 ng/ml) and dexamethasone (10^{-7} M) (data not shown), and the complete inhibition was observed at 100 ng/ml of OCIF (Fig. 1A). A time course study revealed that TRAP-positive OCLs first appeared on day 4 in the culture treated with sODF (100 ng/ml), M-CSF (200 ng/ml) and dexamethasone (10^{-7} M). The number of OCLs attained a maximum on days 6 - 8 and decreased thereafter (data not shown).

Multinucleated cells formed in human PBMC cultures treated simultaneously with the three factors (sODF, M-CSF and dexamethasone) were positively

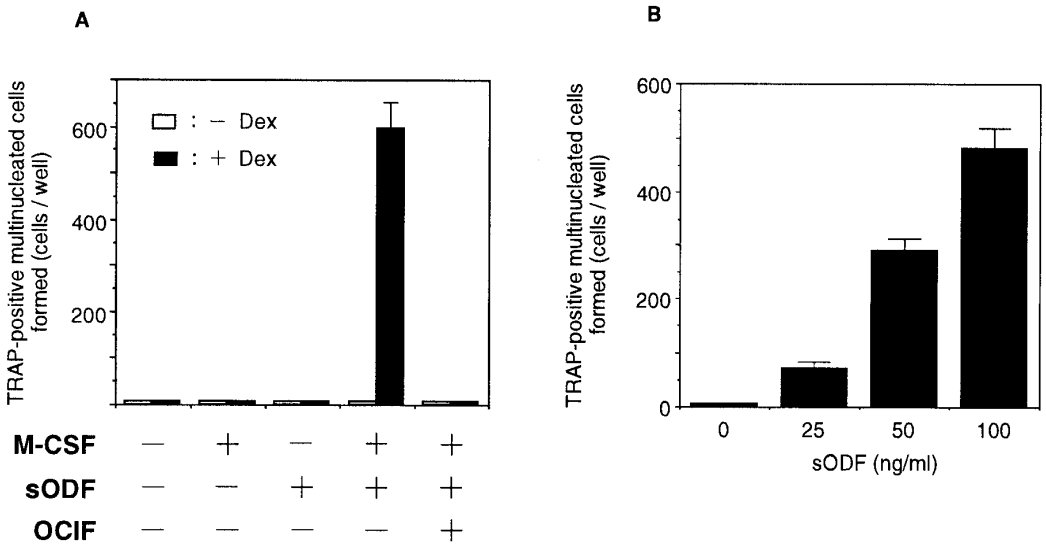

FIG. 1. Effects of sODF, M-CSF, OCIF and dexamethasone on TRAP-positive OCL formation in human PBMC cultures. (A) Human PBMCs were cultured in 48-well plates (4×10^5 cells/0.3 ml/well) in the presence or absence of human M-CSF (200 ng/ml), mouse sODF (100 ng/ml), human OCIF (100 ng/ml) and dexamethasone (Dex) (10^{-7} M). (B) Human PBMCs were cultured in 48-well plates (4×10^5 cells/0.3 ml/well) with various concentrations of mouse sODF in the presence of human M-CSF (200 ng/ml) and dexamethasone (10^{-7} M). After culture for 7 days, cells were stained for TRAP. TRAP-positive multinucleated cells containing 3 or more nuclei were counted as OCLs. Results are expressed as the mean ± SD of six cultures. Similar results were obtained in three additional independent experiments.

stained for TRAP and anti-vitronectin receptor antibody (23C6) (Fig. 2A). 23C6-positive multinucleated cells were scarcely detected on day 7 in the PBMC culture treated without sODF even in the presence of M-CSF and dexamethasone. Addition of OCIF at 100 ng/ml to the culture strongly inhibited the formation of TRAP-, and 23C6-positive OCLs induced by a mixture of sODF, M-CSF and dexamethasone (Fig. 2A). Calcitonin-induced cAMP production was also detected in the PBMC cultures treated simultaneously with sODF, M-CSF and dexamethasone (Fig. 2B). Treatment of PBMCs with OCIF (100 ng/ml) also inhibited the increase in the calcitonin-dependent cAMP accumulation (Fig. 2B).

Numerous resorption pits were formed on dentine slices, on which human PBMCs were cultured with sODF, M-CSF and dexamethasone (Fig. 3). No resorption pits were formed on the slices, when PBMCs were cultured in the absence of sODF. OCIF added to the PBMC cultures markedly inhibited the formation of resorption pits on dentine slices (Fig. 3).

PBMCs consist of the heterogeneous cell population including monocytes, granulocytes, platelets and lymphocytes. It was shown that OCLs are derived from the adherent cell population of mouse PBMCs (3,6). When nonadherent cells were removed from the human PBMC cultures, TRAP-positive OCLs were formed from the remaining adherent cells in response to sODF and M-CSF. Dexamethasone was not required for inducing OCLs from the adherent cell population of PBMCs (Fig. 4A). The multinucleated cells derived from the adherent cells expressed vitronectin receptors (positive for 23C6 antibody) and formed resorption pits on dentine slices (data not shown). Treatment of the adherent cells with OCIF (100 ng/ml) strongly inhibited TRAP-positive OCL formation induced by sODF and M-CSF (Fig. 4B).

DISCUSSION

The present study clearly demonstrates that sODF stimulates OCL formation in the presence of human M-CSF in human PBMC cultures. The multinucleated cells induced by sODF expressed TRAP and vitronectin receptors, produced cAMP in response to calcitonin, and formed resorption pits on dentine slices. Thus, sODF-induced multinucleated cells derived from human PBMCs satisfy major criteria of osteoclasts (1,2). This study also indicates that sODF not only stimulates differentiation of human osteoclast progenitors but also activates human osteoclasts to resorb dentine slices. OCIF inhibited human OCL formation induced by sODF from PBMCs. This suggests that OCIF acts as a naturally occurring decoy receptor for ODF in inhibiting signal transduction in osteoclastogenesis in humans as well as in mice (8,11).

We have established a subclone (SaOS-4/3) of the human osteosarcoma cell line SaOS-2 by transfection with an expression vector of the human PTH/PTH-related protein receptor (27). Human OCLs were formed in response to PTH but not other bone resorbing factors such as $1\alpha,25(OH)_2D_3$, and IL-11 in

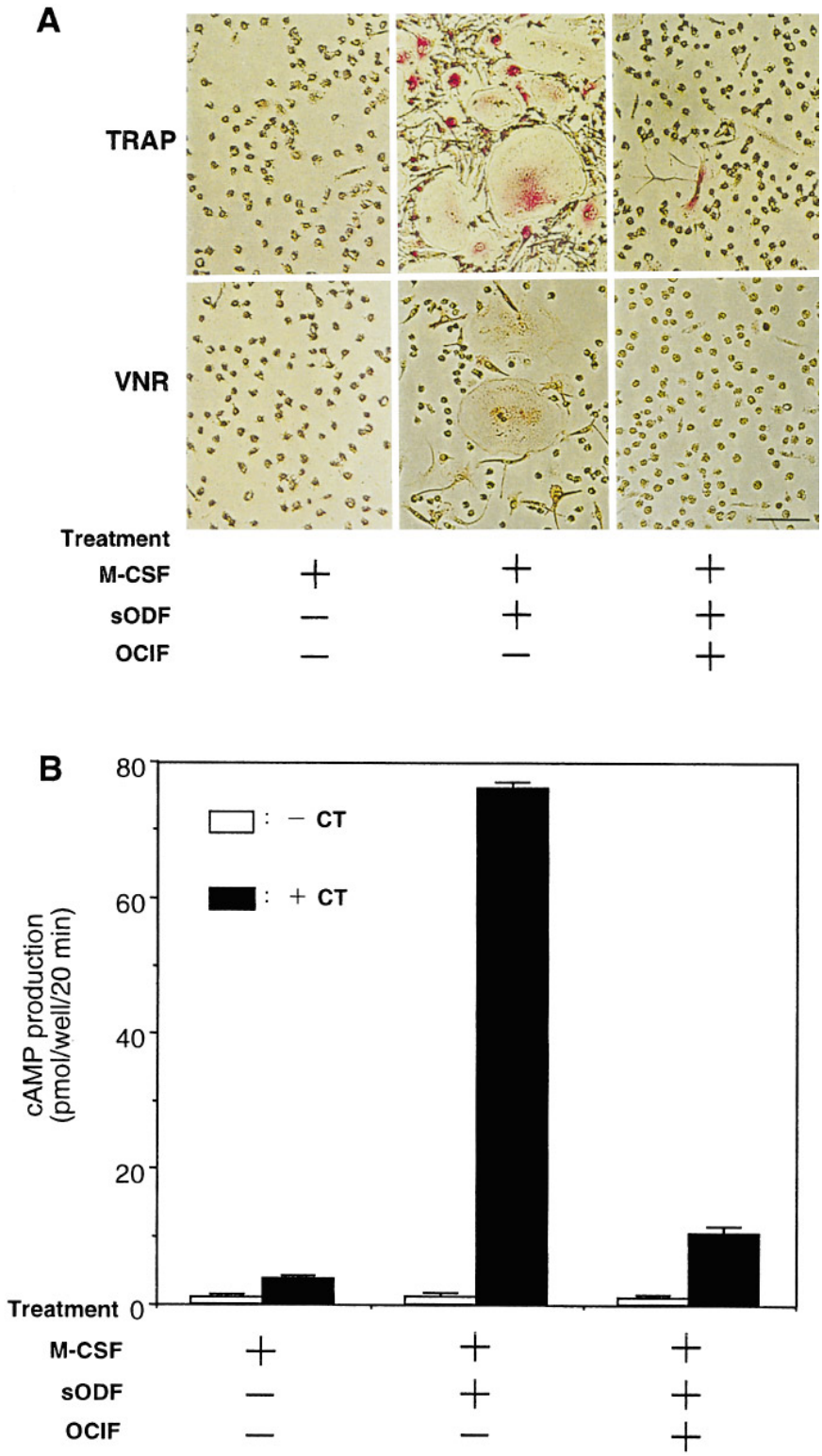

FIG. 2. Osteoclast characteristics of multinucleated cells induced by a combination of sODF and M-CSF. Human PBMCs were cultured in 48-well plates (4×10^5 cells/0.3 ml/well) in the presence of human M-CSF (200 ng/ml) and dexamethasone (10^{-7} M). Some cultures were treated with mouse sODF (100 ng/ml) together with or without human OCIF (100 ng/ml). (A) After culture for 7 days, cells were stained for TRAP and vitronectin receptors (23C6). TRAP-positive cells appeared as red cells, and 23C6-positive cells as brown cells. Bar = 200 μm. (B) After culture for 7 days, cells were incubated for 10 min with eel calcitonin (CT) (10^{-8} M) in the presence of 1 mM isobutylmethylxanthine, and the amount of cAMP accumulated in the PBMC culture was determined. Results are expressed as the mean ± SD of quadruplicate cultures. Similar results were obtained in an additional independent experiment.

co-culture of SaOS-4/3 cells and human PBMCs (Matsuzaki, K., et al., submitted for publication). In our preliminary experiments, expression of ODF mRNA by SaOS 4/3 cells was markedly increased by the treatment with PTH but not with $1\alpha,25(OH)_2D_3$ or IL-11 (Matsuzaki, K., unpublished observation). These results suggest that expression of ODF by osteoblasts/stromal cells is the key step for inducing osteoclasts in humans as well as in mice (11).

Dexamethasone was required for human OCL formation in cultures of whole PBMCs but not in cultures of the adherent cell population. It is generally believed that addition of glucocorticoids such as dexamethasone or hydrocortisone to human culture systems is necessary for inducing human OCLs (3, 18-20). In contrast, glucocorticoids are not necessarily required for mouse OCL formation (24). This suggests that human but not mouse nonadherent cells produce an inhibitory factor(s) against human osteoclastogenesis, production of which appears to be down-regulated by dexamethasone. Further studies are necessary to identify the inhibitory factor(s) produced by nonadherent cells of human PBMCs.

M-CSF is shown to be a crucial factor in mouse osteoclast development (14-16). Here, we showed that that M-CSF was also essential for inducing OCLs from human PBMCs. Without M-CSF, most of the PBMCs disappeared even in the presence of sODF during the 7 day-culture, suggesting that M-CSF is involved in the proliferation and survival of osteoclast progenitors. These findings are also consistent with the previous reports which showed that M-CSF is a critical factor in human OCL formation in vitro (18-20).

The present study shows that ODF and M-CSF are the two important factors required for inducing human osteoclastogenesis. This indicates that a model for osteoclastogenesis in mice (1,2,11) is also applicable to humans. Although both ODF and M-CSF are indispensable for OCL formation, ODF, expression of which is up-regulated by bone-resorbing factors (11), should be the most critical factor for osteoclastogenesis. The culture system of human PBMCs will provide a new tool to investigate human osteoclast formation and function. The present study also raises a possibility that ODF and OCIF may be useful for the treatment of human bone diseases such as osteopetrosis and osteoporosis, respectively.

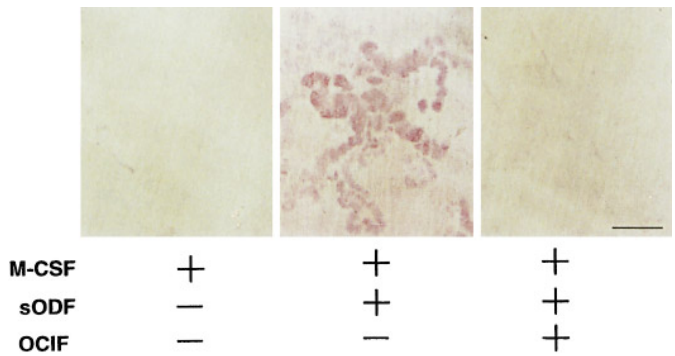

FIG. 3. Pit formation by human OCLs induced by sODF and M-CSF. Human PBMCs were cultured in 48-well plates (4×10^5 cells/0.3 ml/well) in the presence of human M-CSF (200 ng/ml) and dexamethasone (10^{-7} M), in which a dentine slice had been placed in each well. Some cultures were treated with sODF (100 ng/ml) or sODF (100 ng/ml) plus OCIF (100 ng/ml). After culture for 12 days, adherent cells were removed from the slices, and resorption pits formed on the slice were stained with Mayer's hematoxylin. Bar = 200 μm.

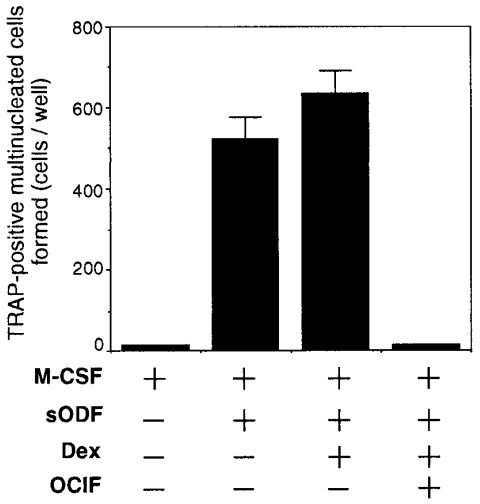

FIG. 4. Effect of the removal of nonadherent cells from human PBMC cultures on TRAP-positive OCL formation induced by sODF and M-CSF. Human PBMCs were cultured in 48-well plates (4×10^5 cells/0.3 ml/well). Nonadherent cells were removed by pipetting after culture for 1 hr, and adherent cells were further cultured in the presence of human M-CSF (200 ng/ml). Cultures were treated with or without mouse sODF (100 ng/ml), dexamethasone (Dex) (10^{-7} M) and human OCIF (100 ng/ml). After culture for 7 days, cells were stained for TRAP, and TRAP-positive OCLs were counted. Results are expressed as the mean ± SD of quadruplicate cultures. Similar results were obtained in two additional independent experiments.

ACKNOWLEDGMENTS

We thank Yota Kato for his technical assistance, and Dr. Michael A. Horton (The Middlesex Hospital, UK) for providing the anti-vitronectin receptor, 23C6. This work was supported in part by the grants in aid (08557101 and 07557118) from the Ministry of Science, Education and Culture of Japan.

REFERENCES

1. Suda, T., Takahashi, N., and Martin, T. J. (1992) *Endocr. Rev.* **13,** 66–80.
2. Suda, T., Udagawa, N., and Takahashi, N. (1996) *in* Principles of Bone Biology (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.), pp. 87–102, Academic Press, San Diego, CA.
3. Athanasou, N. A. (1996) *J. Bone Joint. Surg.* **78-A,** 1096–1112.
4. Chambers, T. J., Owens, J. M., Hattersley, G., Jat, P. S., and Noble, M. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5578–5582.

5. Matsumoto, H. N., Tamura, M., Denhardt, D. T., Obinata, M., and Noda, M. (1995) *Endocrinology* **136,** 4084–4091.
6. Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T. J., and Suda, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7260–7264.
7. Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T., and Higashio, K (1997) *Biochem. Biophys. Res. Commun.* **234,** 137–142.
8. Yasuda, H., Shima, N, Nakagawa, N., Mochizuki, S., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, A., Tsuda, E., Morinaga, T., and Higashio, K. (1998) *Endocrinology* **139,** 1329–1337.
9. Yamaguchi, K., Kinosaki, M., Goto, M., Kobayashi, F., Tsuda, E., Morinaga, T., and Higashio, K. (1998) *J. Biol. Chem.* **273,** 5117–5123.
10. Shimonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M.-S., Lüthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H.-L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R., Amgen EST Program, and Boyle, W. J. (1997) *Cell* **89,** 309–319.
11. Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K, Udagawa, N., Takahashi, N. and Suda, T. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3597–3602.
12. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlet III, F. S., Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) *J. Biol. Chem.* **272,** 25190–25194.
13. Anderson, D. M., Maraskovsky, E., Billingsley, W. L., Dougall, W. C., Tometsko, M. E., Roux, E. R., Teepe, M. C., DuBose, R. F., Cosman, D., and Galibert, L. (1997) *Nature (London)* **390,** 175–179.
14. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D., and Nishikawa, S. (1990) *Nature (London)* **345,** 442–444.
15. Felix, R., Cecchini, M. G., and Fleisch, H. (1990) *Endocrinology* **127,** 2592–2594.
16. Kodama, H., Yamasaki, A., Nose, M., Niida, S., Ohgame, Y., Abe, M., Kumegawa, M., and Suda. T. (1991) *J. Exp. Med.* **173,** 269–272.
17. Flanagan, A. M., Horton, M. A., Dorey, E. L., Collins, D. A., Evely, R. S., Moseley, J. M., Firkin, F. C., Chambers, T. J., Helfrich, M. H., and Martin, T. J. (1992) *Int. J. Exp. Pathol.* **73,** 387–401.
18. Fujikawa, Y., Quinn, J. M., Sabokbar, A., McGee, J. O., and Athanasou, N. A. (1996) *Endocrinology* **137,** 4058–4060.
19. Sarma, U., and Flanagan, A. M. (1996) *Blood* **88,** 2531–2540.
20. Sarma, U., Edwards, M., Motoyoshi, K., and Flanagan, A. M. (1998) *J. Cell. Physiol.* **175,** 99–108.
21. Roodman, G. D. (1996) *Endocr. Rev.* **17,** 308–332.
22. Purton, L. E., Lee, M. Y., and Torok-Strob, B. (1996) *Blood* **87,** 1802–1808.
23. Matayoshi, A., Brown, C., DiPersio, J. F., Haug, J., Abu-Amer, Y., Liapis, H., Kuestner, R., and Pacifici, R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10785–10790.
24. Suda, T., Jimi, E., Nakamura, I., Takahashi, N. (1997) *Methods Enzymol.* **282,** 223–235.
25. Horton, M. A., Lewis, D., McNulty, K., Pringle, J. A. S., and Chambers, T. J. (1985) *Cancer Res.* **45,** 5663–5669.
26. Kotake, S., Sato, K., Kim, K. J., Takahashi, N., Udagawa, N., Nakamura, I., Yamaguchi, A., Kishimoto, T., Suda, T., and Kashiwazaki, S. (1996) *J. Bone Miner. Res.* **11,** 88–95.
27. Matsuzaki, K., Katayama, K., Takahashi, Y., Jimi, E., Nakamura, I., Udagawa, N., Yabe, Y., Noda, T., Takahashi, N., and Suda, T. (1997) *J. Bone Miner. Res.* **12,** S197. (Abstract)