Osteoclast differentiation antigen, distinct from receptor activator of nuclear factor kappa B, is involved in osteoclastogenesis under calcitonin-regulated conditions

T Kukita, A Kukita¹, T Watanabe and T Iijima²

Oral Cellular and Molecular Biology, Faculty of Dentistry, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan

¹Department of Microbiology, Saga Medical School, Saga, Saga 849-0937, Japan

²Oral and Maxillofacial Morphology, Faculty of Dentistry, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan

(Requests for offprints should be addressed to T Kukita, Oral Cellular and Molecular Biology, Division of Oral Biological Sciences, Faculty of Dentistry, Kyushu University, Maidashi 3-1-1, Fukuoka, Fukuoka 812-8582, Japan; Email: kukitat@dent.kyushu-u.ac.jp)

Abstract

Although calcitonin has been clinically utilized as a primary treatment for several metabolic bone diseases, its inhibitory effects against osteoclastic function diminish after several days owing to the calcitonin 'escape phenomenon'. We have previously found a unique cell-surface antigen (Kat1-antigen) expressed on rat osteoclasts. Here we show evidence that, in the presence of calcitonin, the Kat1-antigen is involved in osteoclastogenesis. Treatment of bone marrow cultures for forming osteoclast-like cells with anti-Kat1-antigen monoclonal antibody (mAb Kat1) provoked a marked stimulation of osteoclast-like cell formation only in the presence of calcitonin but not in its absence. Osteoclastogenesis stimulated by the receptor activator of nuclear factor kappa B (NF- κ B) ligand/

osteoclast differentiation factor was further augmented by mAb Kat1 in the presence of calcitonin. Furthermore, even in the presence of the osteoprotegerin/osteoclast inhibitory factor, mAb Kat1 induced osteoclast-like cell formation. Our current data suggest that the Kat1-antigen is a molecule that is distinct from receptor activator of NF- κ B. The presence of the unique Kat1-antigen on cells in the osteoclast lineage appears to contribute to the fine regulation of osteoclastogenesis *in vivo*. Expression of this cell-surface molecule in cells in the osteoclast lineage may partly explain the mechanism responsible for the escape phenomenon.

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Introduction

Calcitonin, first described by Copp *et al.* (1962), is a peptide hormone that decreases the blood calcium level. The main target tissues of this hormone are thought to be the kidney and bone tissue, although a high level of expression of the calcitonin receptor (CTR) is observed in the central nervous system (Lin *et al.* 1991). In bone, osteoclasts express a high level of CTR and administration of calcitonin causes a dramatic decrease in the bone-resorbing activity of these cells (Minkin *et al.* 1971). Morphological observations clearly showed that ruffled borders of bone-resorbing osteoclasts disappeared when the osteoclasts were treated with calcitonin (Kallio *et al.* 1972). Therefore this peptide hormone is thought to be an ideal reagents for suppressing osteoclast-mediated bone resorption. However, calcitonin's inhibitory effect on bone resorption disappears after continuous administration of this hormone (Wener *et al.* 1972, Heersche 1992). This phenomenon, known as the 'escape phenomenon', is not well understood although several lines of evidence have

suggested that it is to some extent related to the regulation of CTR expressed in osteoclasts. It has been shown that the accumulation of calcitonin in the Golgi apparatus of osteoclasts is related to the down-regulation of CTR (Ikegame *et al.* 1994). It has also been demonstrated that the synthesis of CTR is suppressed at the transcription level when osteoclast-like multinucleated cells (MNCs) are treated with calcitonin (Takahashi *et al.* 1995, Findlay & Martin 1997). Such prolonged down-regulation of the CTR appears to be an important molecular aspect of the escape phenomenon. However, alternative events are also thought to occur *in vivo*. The escape phenomenon does not occur in irradiated animals (Krieger *et al.* 1982, Nakamura *et al.* 1985), suggesting that it requires proliferation of osteoclast progenitors. These studies have hypothesized that newly formed osteoclasts share an important role in the escape phenomenon.

Osteoclasts are multinucleated giant cells that resorb bone. These cells are formed by the cell fusion process among mononuclear precursors of the osteoclasts, i.e. preosteoclasts. Recently, a membrane-bound tumor

necrosis factor (TNF) family protein, osteoclast differentiation factor (ODF), also called receptor activator of nuclear factor kappa B (NF- κ B) ligand (RANKL), was found to be an essential cytokine for osteoclastogenesis (Anderson *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998, Kong *et al.* 1999). Before these findings, osteoclast inhibitory factor (OCIF), also called osteoprotegerin (OPG), had been discovered (Simonet *et al.* 1997). OPG/OCIF was shown to act as the decoy receptor for RANKL/ODF, causing an inhibition of osteoclastogenesis *in vivo* as well as *in vitro* (Lacey *et al.* 1998, Yasuda *et al.* 1998).

Previously we found a unique cell-surface antigen distinct from CTR that regulated the function of CTR expressed on rat osteoclasts (Kukita *et al.* 1994). This antigen, designated Kat1-antigen (Kat1-Ag), is expressed on the cell surface of osteoclasts in a highly specific manner. Morphological studies demonstrated that a high level of Kat1-Ag was expressed on the surface of actively resorbing osteoclasts and newly formed osteoclasts (Kukita *et al.* 1998). In the present study, we investigated whether Kat1-Ag has a regulatory role in osteoclastogenesis in the presence or the absence of calcitonin. Treatment of bone marrow cultures with anti-Kat1-Ag monoclonal antibody (mAb Kat1) resulted in a significant stimulation of osteoclastogenesis only in the presence of calcitonin. These results suggest that Kat1-Ag is involved in osteoclast recruitment in the presence of calcitonin. We have obtained lines of evidence that Kat1-Ag is a molecule distinct from the receptor activator of NF- κ B (RANK), the receptor of RANKL/ODF.

Materials and Methods

Materials

Sprague–Dawley (SD) rats were obtained from SEAC Yoshitomi (Fukuoka, Japan). Molecular mass standards for SDS-PAGE were from Daiichi Pure Chemical (Tokyo, Japan). Glutaraldehyde was from Nakalai Tesque (Kyoto, Japan) and paraformaldehyde was from Merck (Darmstadt, Germany). RANKL/ODF (human) and OPG/OCIF (human) were obtained from Pepro Tech Inc. (Rocky Hill, NJ, USA).

Animal treatment

All experimental animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals in Kyushu University.

Bone marrow cultures

Bone marrow cells for forming osteoclast-like MNCs and preosteoclast-like cells were obtained from tibiae and femora of SD rats and cultured as described previously

(Kukita *et al.* 1993). Briefly, bone marrow cells were obtained from tibiae and femora of SD rats. For forming osteoclast-like MNCs, 10^6 cells were cultured in wells of 24-multiwell culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in α MEM supplemented with 15% fetal bovine serum (FBS), 10% heat-treated rat osteosarcoma ROS 17/2.8 cell-conditioned medium (htROSCM), and 10^{-8} M 1 α ,25 dihydroxy vitamin D₃ for 4 days. For forming mononuclear preosteoclasts, stromal cell deprived bone marrow cells of 10^6 cells were cultured in α MEM supplemented with 15% FBS, 10% htROSCM, and 10^{-8} M 1 α ,25 dihydroxy vitamin D₃ for 4 days. After fixation with acetone–citrate, the cells were stained for tartrate-resistant acid phosphatase (TRAP) activity by use of an acid phosphatase kit (Sigma Chemical Co., St Louis, MO, USA). TRAP-positive cells having more than three nuclei were counted as osteoclast-like MNCs.

Western blotting analysis

Rat bone marrow cells were cultured in 100 mm dishes in α MEM containing 15% FBS in the presence of 10% htROSCM, 10^{-8} M 1 α ,25 dihydroxy vitamin D₃ and 10 ng/ml RANKL/ODF for 4 days. ROS 17/2.8 cells were cultured in DME containing 10% FBS. Cells were fixed in ice-chilled 10% (w/v) trichloroacetic acid for 60 min, scraped off the plates, and centrifuged at 10 000 *g* at 4 °C. The pellets were immediately dissolved in 80 μ l 9 M urea, 2% (v/v) Triton X-100, 1% (w/v) dithiothreitol, mixed with 20 μ l of 10% (w/v) lithium dodecyl sulfate, completely dissociated by vigorous pipetting and frozen in aliquots at -80 °C. The protein samples were subjected to SDS-PAGE (13% polyacrylamide gel, 10 μ g/lane). Proteins were transferred onto a nitrocellulose membrane. The membrane was immersed in Iscove's Modified Dulbecco's Medium containing 10% FBS and 1 mM CaCl₂ at room temperature for 30 min and reacted with mAb Kat1 or control antibodies at room temperature for 60 min. After rinsing in TBS (pH 7.5) containing 1 mM CaCl₂, the membrane was fixed in ice-chilled 2% glutaraldehyde prepared in TBS containing 1 mM CaCl₂ for 6 min. The membrane was rinsed in TBS containing 1 mM CaCl₂ and then immersed overnight in PBS (pH 7.5) containing 10% (w/v) BSA and 3% goat serum at 4 °C to block non-specific binding. Immunoreactive bands were detected by use of biotinylated anti-mouse IgM (μ chain specific) and an ABC-AP kit (Vector Laboratories, Bucks, UK).

Statistical analysis

The number of TRAP-positive cells was counted and each value was represented as the mean $\pm$ s.e. of quadruplicate cultures in 24-well culture plates. All data obtained from bone marrow cultures were analyzed by use of Student's *t*-test and a post-ANOVA test.

Figure 1 Marked stimulation of osteoclast-like MNC formation by mAb Kat1 only when cells are treated with calcitonin. Bone marrow cells were cultured to form osteoclast-like MNCs in the presence of various concentrations of mAb Kat1 with (B–D) or without (A) calcitonin as described in Materials and Methods. Cells were stained for TRAP on day 4 of the culture and the number of TRAP-positive MNCs counted. (A) Formation of osteoclast-like MNCs was not affected by any concentration of mAb Kat1, in the absence of calcitonin. (B–D) Significant stimulation of osteoclast-like MNC formation was observed in the presence of calcitonin (B 0.01, C 0.1, D 10 ng/ml calcitonin). Data show the mean number of TRAP-positive MNCs $\pm$ S.E. from quadruplicate cultures in 24-well culture plates. Three independent experiments were performed and similar results obtained. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Stimulation of osteoclastogenesis by treatment of bone marrow cultures with mAb Kat1 in the presence of calcitonin

Figure 1 shows the dosage effect of mAb Kat1 on the formation of osteoclast-like MNCs in the presence of

Table 1 Absence of stimulatory effects of mAb Kat1 on preosteoclast formation in both the presence and absence of calcitonin. Bone marrow cells were depleted of stromal cells and cultured for forming mononuclear preosteoclast-like cells as described in Materials and Methods in the presence of various concentrations of mAb Kat1 with or without calcitonin. After 4 days of culture, cells were stained for TRAP and the number of TRAP-positive mononuclear cells counted. Data represent means $\pm$ S.E. from quadruplicate cultures. Significant effects of mAb Kat1 were not observed on the formation of preosteoclast-like mononuclear cells in either the presence or absence of calcitonin

	mAb Kat1 (µg/ml)	TRAP-positive mononuclear cells per culture
Calcitonin 0 ng/ml	0	171.5 $\pm$ 11.7
	0.01	187.2 $\pm$ 29.0
	0.1	160.5 $\pm$ 20.2
	1	144.0 $\pm$ 12.7
	10	140.0 $\pm$ 11.1
	100	203.7 $\pm$ 20.0
Calcitonin 1 ng/ml	0	161.2 $\pm$ 13.4
	0.01	173.0 $\pm$ 14.0
	0.1	140.7 $\pm$ 24.4
	1	150.5 $\pm$ 27.9
	10	171.0 $\pm$ 18.8
	100	220.5 $\pm$ 20.1

various concentrations of calcitonin. In the absence of calcitonin, no significant effect was observed when mAb Kat1 was added to cultures for forming osteoclast-like MNCs (Fig. 1A). However, in the presence of calcitonin, mAb Kat1 markedly stimulated osteoclast-like MNC formation (Fig. 1B–D). The peak of the stimulation was around 1 µg/ml mAb Kat1. Higher concentrations of mAb Kat1 tended to suppress formation of osteoclast-like MNCs. Control mAbs having the same immunoglobulin class as mAb Kat1 (IgM) showed no significant effect on MNC formation at any concentrations and at any dose of calcitonin (data not shown).

These data strongly suggest that osteoclastogenesis is finely regulated by the cell-surface Kat1-Ag only in the presence of calcitonin. We confirmed that osteoclast-like MNCs formed in the presence of calcitonin and mAb Kat1 resorbed dentin (data not shown). To determine whether mAb Kat1 stimulates the formation of preosteoclasts in the presence and absence of calcitonin, we examined the dosage effect of mAb Kat1 on formation of mononuclear preosteoclast-like cells from bone marrow cells depleted of stromal cells (Table 1). Formation of preosteoclast-like cells was not significantly affected by treatment with mAb Kat1, in either the presence or absence of calcitonin (1 ng/ml). These data suggest that the multinucleation step was enhanced by the presence of mAb Kat1 under the suppressed conditions mediated by calcitonin.

Kat1-Ag is a molecule distinct from RANK

The findings that Kat1-Ag is expressed on cells in the osteoclast lineage and is involved in the formation of

Figure 2 Effect of RANKL/ODF on osteoclast-like MNC formation, in the presence of various concentrations of calcitonin. Bone marrow cells were cultured to form osteoclast-like MNCs in the presence of various concentrations of RANKL/ODF with (B–D) (B 0.01, C 0.1, D 1 ng/ml) or without (A) calcitonin. (A) Osteoclast-like MNC formation was markedly stimulated by the addition of RANKL/ODF, in the absence of calcitonin. (B–D) Suppressed level of stimulation was observed in the presence of calcitonin. Data show the mean number of TRAP-positive MNCs $\pm$ S.E. from quadruplicate cultures in 24-well culture plates. Three independent experiments were performed and almost the same results obtained. * $P < 0.05$, ** $P < 0.01$.

osteoclast-like MNCs raise the possibility that the ligand of Kat1-Ag is RANKL/ODF. In other words, Kat1-Ag might be RANK. To address this question, we have examined the effect of RANKL/ODF on MNC formation in the presence of various concentrations of calci-

Figure 3 RANKL/ODF-induced osteoclastogenesis was further stimulated by mAb Kat1 only in the presence of calcitonin. Bone marrow cells were cultured in the presence or absence of 10 ng/ml RANKL/ODF with or without mAb Kat1 in the presence (B) or in the absence (A) of 1 ng/ml calcitonin. After culture for 4 days, cells were stained for TRAP and the number of TRAP-positive MNCs counted. Data show the mean number of TRAP-positive MNCs $\pm$ S.E. from quadruplicate cultures in 24-well culture plates. Three independent experiments were performed and almost the same results obtained. (*) $P < 0.05$, (**) $P < 0.01$, based on data of the control cultures obtained in the absence of both RANKL/ODF and mAb Kat1. * $P < 0.05$, based on data obtained in the presence of 10 ng/ml RANKL/ODF but without mAb Kat1.

tonin (Fig. 2). RANKL/ODF markedly stimulated the formation of osteoclast-like MNCs in the absence of calcitonin (Fig. 2A). This result is in contrast to the results shown in Fig. 1A, in which no stimulating effect was observed in the osteoclast-like MNC formation in the absence of calcitonin by treatment with mAb Kat1. The stimulatory effect mediated by RANKL/ODF was to some extent suppressed in the presence of calcitonin (Fig. 2B–D). No additional stimulatory effect was observed by treatment with various concentrations of RANKL/ODF, in the presence of calcitonin. We further examined the dosage effect of mAb Kat1 on RANKL/ODF-mediated osteoclastogenesis (Fig. 3). In the absence of calcitonin, RANKL/ODF-induced osteoclast-like MNC formation was not affected by the addition of mAb Kat1 (Fig. 3A and Fig. 4A and B). However, in the presence of 1 ng/ml

Figure 4 Photomicrographic demonstration of osteoclast-like MNCs induced by RANKL/ODF with or without mAb Kat1. Bone marrow cells were cultured in the presence of 10 ng/ml RANKL/ODF with (B, D) or without (A, C) mAb Kat1 in the presence (C, D) or absence (A, B) of 1 ng/ml calcitonin. After culture for 4 days, cells were stained for TRAP and observed under the microscope. Scale bar 200 μ m.

calcitonin, formation of TRAP-positive MNCs was significantly stimulated by treatment with mAb Kat1 (Fig. 3B and Fig. 4C and D). In comparison with the data in Fig. 1, it required higher concentrations of mAb Kat1 (10–100 μ g/ml) for stimulation of osteoclastogenesis in the presence of RANKL/ODF and calcitonin. Morphology of osteoclast-like MNCs stimulated with mAb Kat1 was similar to that of osteoclast-like MNCs unstimulated with this antibody (Fig. 4).

These data demonstrate that Kat1-Ag-mediated osteoclastogenesis still remained even if cultures were stimulated with RANKL/ODF. These data also indicate that interactions between RANKL/ODF and RANK were not interfered with by mAb Kat1 and that mAb Kat1 showed a unique activity in osteoclastogenesis only in the presence of calcitonin.

We further examined the stimulatory effect of mAb Kat1 on osteoclastogenesis in the presence of OPG/OCIF,

Figure 5 Marked stimulation of osteoclastogenesis by mAb Kat1 in the presence of OPG/OCIF, in cultures stimulated with RANKL/ODF. Bone marrow cells were cultured to form osteoclast-like MNCs as described in Materials and Methods in the presence of 1 ng/ml calcitonin with or without 10 ng/ml RANKL/ODF. These cultures were treated with various concentrations of mAb Kat1. After 1 day of culture, OPG/OCIF (100 ng/ml) was added and the cells cultured for up to 4 days. Cells were stained for TRAP and the number of TRAP-positive MNCs per culture counted. The mAb Kat1 markedly stimulated formation of osteoclast-like MNCs even in the presence of OPG/OCIF. Data show the mean number of TRAP-positive MNCs $\pm$ S.E. from quadruplicate cultures in 24-well culture plates. Three independent experiments were performed and almost the same results obtained. ** $P < 0.01$, based on data obtained in the presence of 10 ng/ml RANKL/ODF and 100 ng/ml OPG/OCIF but without mAb Kat1 (cont.).

a specific inhibitor of the interaction between RANKL/ODF and RANK (Fig. 5). Osteoclastogenesis stimulated by RANKL/ODF was suppressed in the presence of OPG/OCIF. However, even in the presence of OPG/OCIF, mAb Kat1 markedly stimulated the formation of osteoclast-like MNCs when cultures were treated with 1 ng/ml calcitonin. These osteoclast-like MNCs efficiently resorbed dentin (data not shown). This mAb Kat1-induced osteoclastogenesis was not inhibited by OPG/OCIF (Table 2).

A stimulatory effect of mAb Kat1 was also observed in bone marrow cultures treated with 100 ng/ml OPG/OCIF in the absence of RANKL/ODF only when cultures were treated with calcitonin (1 ng/ml) (Fig. 6). Furthermore, treatment of osteoclast-like MNCs with a high concentration of RANKL/ODF (500 ng/ml) did not cause a significant change in the level of Kat1-Ag expression, as detected by immunocytochemical staining with mAb Kat1 (data not shown).

To determine the molecular size of the Kat1-Ag, we performed a Western blotting analysis under reducing

Table 2 Effect of OPG/OCIF on Kat1-Ag-mediated osteoclastogenesis. Bone marrow cells were cultured for forming osteoclast-like MNCs as described in Materials and Methods in the presence of 1 ng/ml calcitonin with or without 100 μ g/ml mAb Kat1. After 1 day of culture, cells were treated with OPG/OCIF and culture continued up to 4 days. Cells were stained for TRAP and the number of TRAP-positive MNCs counted. The number of TRAP-positive MNCs induced by mAb Kat1 was not decreased even in the presence of higher concentrations of OPG/OCIF

OPG/OCIF (ng/ml)	Number of TRAP-positive MNCs per culture ($\pm$ S.E.)		Number of mAb Kat1 induced TRAP-positive MNCs per culture*
	with mAb Kat1	without mAb Kat1	
0	547.5 $\pm$ 44.3	376.7 $\pm$ 9.7	170.7
50	457.0 $\pm$ 37.9	247.7 $\pm$ 17.8	209.2
100	426.7 $\pm$ 33.8	211.5 $\pm$ 12.4	215.2

*Number of mAb Kat1-induced TRAP-positive cells calculated as difference between the numbers with and without mAb Kat1 in the preceding columns.

conditions (Fig. 7). The cell lysate from osteoclast-like MNCs showed a single band of 32 kDa that was specifically detected by mAb Kat1. In contrast, no band was detected in the cell lysate from ROS 17/2.8 cells, a rat osteoblastic cell line. These data demonstrate that the 32 kDa protein is the molecular size of the Kat1-Ag in a reduced condition. These data strongly suggest that Kat1-Ag is a unique cell-surface antigen distinct from RANK.

Discussion

In the present study, we had expected some regulatory role of Kat1-Ag in osteoclastogenesis because we observed that osteoclast-like MNCs frequently adhered to mononuclear cells expressing Kat1-Ag and that mononuclear cells expressing Kat1-Ag frequently interacted with each other. In the absence of calcitonin, treatment of cultures with mAb Kat1 had no effect on MNC formation. However, in the presence of calcitonin, mAb Kat1 treatment caused a marked increase in the formation of osteoclast-like MNCs. Such a stimulatory effect was observed even at lower concentrations (0.01–1 ng/ml) of calcitonin. Calcitonin is known to inhibit osteoclast formation in various cultures that are used for assessing osteoclastogenesis (MacDonald *et al.* 1986, Takahashi *et al.* 1988). Higher concentrations of calcitonin (more than 10 ng/ml) are required to express an inhibitory effect on osteoclast formation. In the current study, it was surprising to find that mAb Kat1 stimulated formation of osteoclast-like MNCs only in the presence of calcitonin. Few reports have investigated osteoclastogenesis in the presence of calcitonin. There might be some

Figure 6 Stimulation of osteoclastogenesis by mAb Kat1 in the presence of OPG/OCIF, in cultures not stimulated with RANKL/ODF. Bone marrow cells were cultured to form osteoclast-like MNCs as described in Materials and Methods in the presence of 100 ng/ml OPG/OCIF with (B) or without (A) 1 ng/ml calcitonin. Cultures were treated (+) or untreated (–) with mAb Kat1 and cultured for 4 days. Cells were stained for TRAP and the number of TRAP-positive MNCs counted. The mAb Kat1 significantly stimulated osteoclast-like MNC formation only in the presence of calcitonin even in the presence of 100 ng/ml OPG/OCIF. Data show the mean number of TRAP-positive MNCs $\pm$ s.e. from quadruplicate cultures in 24-well culture plates. Three independent experiments were performed and almost the same results obtained. * $P < 0.05$ compared with cells cultured without mAb Kat1.

interactions between CTR, Kat1-Ag and the putative fusion machinery required for osteoclastogenesis, which are expressed on the surface of cells in the osteoclast lineage. However, such stimulatory effects were not observed when cells were treated with higher concentrations of mAb Kat1. Previously, we have demonstrated that in the presence of 10 ng/ml mAb Kat1, even lower concentrations (0.1–1 ng/ml) of calcitonin inhibited formation of osteoclast-like MNCs. In our current experiments, similar suppressive effects of calcitonin on osteoclastogenesis were observed when cells were treated with 100 ng/ml mAb Kat1. Such a difference in the effective concentrations of mAb Kat1 from our previous report is supposed to be attributed to a difference in the immunological titer of each antibody preparations. As the formation of mononuclear preosteoclasts was not significantly

Figure 7 Western blotting analysis of the Kat1-Ag under reducing conditions. Cells were cultured and treated with 10% trichloroacetic acid, followed by preparation of samples for SDS-PAGE under reducing conditions as described in Materials and Methods. After immunoblotting the proteins onto the nitrocellulose, they were reacted with mAb Kat1 (lanes 1 and 2) or with control mAb (lanes 3 and 4) followed by detection with an ABC-AP kit. Lane 1 and lane 3, cell lysates from osteoclast-like MNCs; lanes 2 and 4, cell lysates from ROS 17/2-8 cells. mAb Kat1 specifically detected a clear single band having a molecular mass of 32 kDa in cell lysates from osteoclast-like MNCs (lane 1) but not in lysates of ROS 17/2-8 cells (lane 2). No band was detected with the control antibody (lanes 3 and 4).

affected by mAb Kat1, in either the presence or absence of calcitonin, the following fusion process of preosteoclasts was supposed to be a target step in Kat1-Ag-mediated regulation of osteoclastogenesis in the presence of calcitonin. The fusion efficiency of the preosteoclasts was supposed to be augmented by treatment with mAb Kat1 and calcitonin. Treatment of human peripheral blood cultures with anti-CD98 mAb induces the formation of osteoclast-like MNCs (Higuchi *et al.* 1998). Anti-macrophage-fusion-receptor mAb inhibits the fusion of peritoneal macrophages when these cells are cultured to form macrophage polykaryons (Saginario *et al.* 1998).

Figure 8 Regulation of osteoclastogenesis through Kat1-Ag – a hypothesis. A putative natural ligand of the Kat1-Ag binds to Kat1-Ag expressed on the surface of cells in the osteoclast (OC) lineage and this ligand stimulates osteoclastogenesis in the presence of calcitonin.

However, to our knowledge, no one has yet reported cell-surface molecules being involved in osteoclast formation only under suppressed conditions mediated by calcitonin. Such a mechanism for regulating osteoclastogenesis needs to be further investigated to understand the cell biology of osteoclasts.

The escape phenomenon is a clinically defined event, in which calcitonin treatments become ineffective after bone cells are exposed to pharmacological concentrations of calcitonin. Our data may provide cell-biological evidence suggesting that the escape phenomenon involves the formation of osteoclasts. An increase in the number of newly formed osteoclasts could diminish the inhibitory effect of administered calcitonin and it could participate in the occurrence of the calcitonin-mediated escape phenomenon. Although our experiments were only performed *in vitro* with mAb Kat1, we assume that stimulation of Kat1-Ag by the natural ligand for this antigen would lead to a result similar to that when cells were treated with mAb Kat1 (Fig. 8). We expect that our culture conditions containing low concentrations of calcitonin (0.01 ng/ml)

could partly reflect the hormonal environments observed *in vivo*, as the upper normal level of serum calcitonin is approximately 0.03 ng/ml (Mallette & Gagel 1993). When osteoclastic bone resorption is directly inhibited by calcitonin, formation of osteoclasts may be provoked through the Kat1-Ag-mediated signal in osteoclast precursors *in vivo*. These newly formed osteoclasts are thought to diminish the inhibitory effect of administered calcitonin on osteoclastic bone resorption.

RANKL/ODF is a membrane-bound cytokine that induces osteoclastogenesis as well as osteoclast activation (Burgess 1999, Jimi *et al.* 1999). Hsu *et al.* (1999) recently reported about the induction of osteoclastogenesis when cell-surface RANK molecules expressed on osteoclast progenitors were cross-linked by anti-RANK antibody, even in the absence of RANKL/ODF. Therefore we examined the possibility that Kat1-Ag is RANK. However, our current data indicate that there is a clear difference between Kat1-Ag and RANK. Even in the presence of inhibitory concentrations of OPG/OCIF, mAb Kat1 markedly stimulated the formation of osteoclast-like MNCs in the presence of calcitonin. A Western blotting analysis revealed that the Kat1-Ag has a molecular mass around 32 kDa under reducing conditions. The molecular mass of RANK, which is around 110 kDa, strongly suggests that Kat1-Ag is a unique cell-surface antigen completely distinct from RANK. Recently, lines of evidence showed that TNF- α induces osteoclastogenesis in a mechanism independent from the RANKL/ODF-RANK system (Azuma *et al.* 2000, Kobayashi *et al.* 2000, Komine *et al.* 2001). The current study also showed a possible mechanism of osteoclastogenesis independent from the RANKL/ODF-RANK system.

Further studies are needed to characterize the Kat1-Ag as well as its natural ligand. Our observations will open the way to develop new strategies to suppress the calcitonin-mediated escape phenomenon. As calcitonin is widely utilized and calcitonin treatments of metabolic bone diseases are considered to be among the safest therapies, development of a method for regulating Kat1-Ag may lead to calcitonin-based novel therapies against metabolic bone diseases.

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