Pathol. Oncol. Res. (2019) 25:409–419
https://doi.org/10.1007/s12253-017-0362-8

ORIGINAL ARTICLE

In Vitro Study of the Effects of Denosumab on Giant Cell Tumor of Bone: Comparison with Zoledronic Acid

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Received: 3 March 2017 / Accepted: 8 November 2017 / Published online: 20 November 2017

Abstract Giant cell tumor of bone (GCTB) is a locally aggressive primary bone tumor that contains numerous osteoclasts formed from marrow-derived precursors through receptor activator of nuclear factor κ-B ligand (RANKL), an osteoclast differentiation factor expressed in neoplastic cells of GCTB. Denosumab, a fully human monoclonal antibody targeting RANKL, has recently been used for the treatment of GCTB, and superior treatment effects have been reported. The aim of this work was to elucidate the mechanism of action of denosumab, and the differences between denosumab and zoledronic acid at the level of GCTB cells. We isolated GCTB cells from 3 patients and separated them into osteoclasts, osteoclast precursors and proliferating spindle-shaped stromal cells (the true neoplastic component), and examined the action of denosumab on differentiation, survival and bone resorption activity of osteoclasts. Denosumab and zoledronic acid inhibited osteoclast differentiation from mononuclear cells containing osteoclast precursors. Zoledronic acid inhibited osteoclast survival, whereas an inhibitory effect of denosumab on osteoclast survival was not observed. The inhibitory effect on bone resorption by both agents was confirmed in culture on dentin slices. Furthermore, zoledronic acid showed dose-dependent inhibition of cell growth of neoplastic cells whereas denosumab had no inhibitory effect on these cells. Denosumab has an inhibitory effect on osteoclast differentiation, but no inhibitory effects on survival of osteoclasts or growth of neoplastic cells in GCTBs.

Keywords Giant cell tumor of bone · Osteoclast · Bisphosphonate · Denosumab · Bone tumor

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Introduction

Giant cell tumor of bone (GCTB) is an intermediate type primary bone tumor appearing commonly in the epiphyseal end of long bones and the pelvic bone in young adults. Histologically, it is characterized by the presence of multinucleated giant cells (from which the name is derived) and mononuclear cells [1–3]. The multinucleated giant cell has both functional and morphological aspects of osteoclasts [4–7]. Moreover, the mononuclear cells consist of proliferating spindle-shaped stromal cells, which are the neoplastic component, and osteoclast precursors, which are part of the monocytic macrophage lineage [8, 9]. GCTB shows locally aggressive osteolysis caused by osteoclasts and often recurs after surgical resection [10, 11]. Among GCTB patients, about 10% present with malignant transformation at recurrence [12–14], and <5% of cases develop histologically benign metastases [15, 16].

The osteoclast, a component of GCTB, is a multinucleated cell that regulates remodeling of bone through bone resorption and blood calcium concentration. Differentiation from monocyte-originated hematopoietic stem cells and the survival of the osteoclasts are known to be regulated by the cytokines receptor activator of nuclear factor κ-B ligand

(RANKL) and macrophage colony-stimulating factor. RANKL is mostly expressed on osteoblasts and osteocytes, and the expression is induced by parathyroid hormone, parathyroid hormone–related peptide, active vitamin D_3 and prostaglandin E_2 [17–19]. After differentiation and activation, osteoclasts attach to the bone surface, and secrete acid and proteinases. The secreted H^- and Cl^-, via V − ATPase and chloride channels, respectively, demineralize the bone matrix, and proteinases including cathepsin K digest bone matrix proteins. These phenomena are observed not only under physiological conditions, but also under conditions of pathological bone resorption such as in rheumatoid arthritis and bone tumors including GCTB. Following cessation of the stimulation of RANKL, osteoclasts undergo apoptosis [20]. As described, RANKL is an essential mediator of osteoclast differentiation and activation resulting in bone resorption, and survival.

Complete surgical excision is the mainstay of management for GCTB; however, repeated operations due to local recurrence after the primary surgery often lead to serious functional morbidity [21–23]. In the case of the pelvic bone or spinal column curative resection can sometimes be difficult to perform. Thus, adjuvant therapy would be useful for the prevention of recurrence in patients for whom complete excision is difficult to perform. However, unlike high-grade sarcoma, there are no suitable anti-tumor agents for GCTB, and irradiation provides a potentially high risk of sarcomatous transformation [24]. From this point of view, zoledronic acid, which is the bisphosphonate currently used for treating metastatic bone tumors, has been tested for the treatment of GCTB. A number of superior effects of zoledronic acid have been reported [25–28].

Denosumab, which has been administered in patients with bone metastasis or osteoporosis with participating osteoclasts, is a bone resorption inhibitor that consists of a fully human monoclonal IgG type antibody that targets RANKL; it has been found to bind RANKL with high affinity and specificity [29, 30]. By binding to both membrane-bound and free RANKL, denosumab inhibits RANKL-induced osteoclast differentiation, activation and survival [30–32]. Mononuclear stromal cells of GCTB express RANKL [33–35], thereby a number of osteoclasts appear in GCTB tissue by endogenous RANKL. Based on this finding, denosumab has been approved as a therapeutic agent for GCTB. In a phase II study, the safety and efficacy of denosumab in patients with recurrent or unresectable GCTB were described [31]. Furthermore, beneficial clinical efficacy has been noted in some case reports [36, 37].

Zoledronic acid, classified as a nitrogen-containing bisphosphonate, induces the apoptosis of osteoclasts by interference with the activation of RAS-related protein, which is essential for the survival of osteoclasts, through blocking the effect of farnesyl-diphosphate synthase on the mevalonate pathway [38]. Although it is expected to have the same effect as denosumab, its pharmacological mechanism of action is different.

Large randomized controlled trials comparing denosumab and zoledronic acid have recently been conducted in patients with bone metastasis [39–41], but no such trials yet exist for GCTB. Furthermore, all reports of histological change caused by denosumab and zoledronic acid have described the whole histological image of GCTB at only the end of administration [31, 42–44], and therefore the detailed mechanism of the effect of these agents on the different cell types involved in GCTB remains unknown. Osteoclast precursors and neoplastic cells, in additional to osteoclasts, have roles in GCTB [33, 45, 46]. It is not yet clear whether denosumab and zoledronic acid act on the differentiation of osteoclast precursor into osteoclast, the activation of osteoclasts, the survival of osteoclasts. To determine the mechanism of action of denosumab in GCTB, and to clarify the differences between denosumab and zoledronic acid, we analyzed the differentiation and survival of osteoclasts, as well as bone resorption by osteoclasts prepared from GCTBs. We divided GCTBs cells from patients into two populations: osteoclasts and mononuclear cells containing osteoclast precursors and neoplastic cells. We found that although both agents inhibited osteoclast differentiation, the inhibitory effect on the survival of osteoclasts could be seen with zoledronic acid but not denosumab. An inhibitory effect on bone resorption was recognized with both agents.

These results suggest that the effects of denosumab on GCTB include inhibition of the differentiation of osteoclasts and bone resorption, but that there is no influence on the survival of osteoclasts. To our knowledge, this is the first study to demonstrate the effects of denosumab on osteoclast differentiation, survival and bone resorption using GCTB-derived cells in vitro.

Materials and Methods

Patients

The tissue samples were obtained from 3 patients (Table 1) who had no comorbidities. The tumors were classified as grade I or II on plain radiographs according to the Campanacci grading system [10]. Based on histological examination at the Teikyo University Mizonokuchi Hospital of specimens that were removed during surgery for therapeutic purposes, each patient was diagnosed with GCTB.

Reagents

Denosumab and zoledronic acid were purchased from Daiichi-Sankyo Co., Ltd. (Tokyo, Japan) and Novartis Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. A stock solution of denosumab was prepared in saline. TRAP

Table 1 Clinical characteristics of three giant cell tumors of bone

Case No.	Age	Sex	Location	Remarks	Grade
GCT 1	25	Male	Proxmal tibia	Primary	I
GCT 2	43	Male	Distal ulna	Primary	II
GCT 3	48	Male	Proxmal tibia	Recurrence	I

GCT, giant cell tumor

stain solution contained 0.01% naphthol AS-MX (Sigma-Aldrich, St. Louis, MO) dissolved in 1% *N*,*N*-dimethylformamide (Wako, Osaka, Japan) and 0.06% FAST RED Violet LB Salt (Sigma-Aldrich) dissolved in TRAP buffer (0.1 M sodium acetate buffer containing 0.1 M sodium tartrate, pH 5.0).

Preparation of Primary Cells, Osteoclasts, Mononuclear Cells and Neoplastic Cells

To prepare the cell culture, tumor specimens were minced in culture dishes, placed in 0.1% collagenase (Wako) and 0.2% dispase (Wako) diluted with αMEM (Wako) at 37 °C for 15 min, and shaken at 200 rpm for 1 h. Cells obtained by this procedure were used as primary cells. To evaluate the differentiation and survival of osteoclasts we isolated primary cells to osteoclasts and mononuclear cells as follow. After 2 h of culture, which is the minimum time necessary for the cells to adhere to the culture dish, primary cells were treated with 0.05% trypsin-EDTA for 5 min. The cells harvested from suspending cells obtained by treating primary cells with trypsin were termed mononuclear cells, and consisted of neoplastic cells and osteoclast precursor cells. The cells that remained on the surface of the culture dish after trypsin-EDTA treatment were used as osteoclasts, consisting of mainly osteoclasts with a few neoplastic cells. After culturing mononuclear cells for 2 weeks, the remaining mononuclear cells were used as neoplastic cells.

Cell Culture System

The harvested cells were cultured at 37 °C under 5% CO_2 in αMEM containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 U/mL penicillin G and amphotericin B 0.25 μg/mL. The culture medium was replaced with fresh medium every 2 days of culture.

TRAP Staining

The cell cultures were stained for TRAP for 30 min after fixation with 10% formaldehyde and acetone/ethanol (1:1) using conventional methods [47].

Osteoclast Differentiation Assay

Mononuclear cells were cultured in 24-well plates (6×10^5 cells/2 mL/well) in the presence or absence of denosumab (30 μg/mL) or zoledronic acid (2 μg/mL). After 2 h of culture, which is considered the minimum time necessary for the cells to adhere to the dish before the occurrence of spontaneous osteoclast formation, the culture medium was replaced with fresh medium in the presence or absence of denosumab or zoledronic acid. At 2 and 4 days of culture, the cells were stained for TRAP. TRAP-positive multinuclear cells containing >3 nuclei were counted as osteoclasts.

Osteoclast Survival Assay

Multinucleated cells were cultured in 24-well plates (150 cells/2 mL/well) in the presence or absence of denosumab (30 μg/mL) or zoledronic acid (2 μg/mL). After 2 h of culture, the culture medium was replaced with fresh medium in the presence or absence of denosumab or zoledronic acid. At 2 and 4 days of culture, the cells were stained for TRAP. TRAP-positive multinucleated cells containing >3 nuclei were counted as osteoclasts.

Pit Formation Assay

The primary cells prepared from GCTB were cultured on dentin slices (4 mm in diameter, 0.3 mm in thickness) in 96-well culture plates at 6×10^4 cells/slice in 0.2 mL/well of αMEM containing 10% FBS with or without denosumab (30 μg/mL) or zoledronic acid (2 μg/mL). The culture medium was replaced every day with fresh medium supplemented with denosumab (30 μg/mL) or zoledronic acid (2 μg/mL). On the fourth day after wiping the cells off the dentin slices with cotton, slices were immersed in toluidine blue (Sigma-Aldrich) to stain lacunar resorption formed by osteoclasts.

Evaluation of Cell Proliferation

Neoplastic cells prepared from GCTB were seeded at 4×10^3 cells/well in 96-well cell culture plates. Cells were cultured in the presence or absence denosumab (0, 15, 30, 60, 120 μg/mL) or zoledronic acid (0, 1, 2, 4, 8 μg/mL). The culture medium was replaced with fresh medium at every 2 days of culture. On the sixth day of culture, proliferation was evaluated using Cell Titer 96 Aqueous One Solution® kit (Promega, Madison, WI).

Statistical Analysis

All data are expressed as means ± SD ($n = 3$ or 4). Student's t test was used for statistical analyses, and p values <0.05 and 0.01 were considered significant.

Results

Radiographical and Histological Characteristics of GCTB

We presented plain X-ray image of a patient, as well as hematoxylin & eosin-stained histological images (GCT2). Plain X-ray examination showed a well-demarcated radiolucent lesion located in the epiphysis of the ulna (Fig. 1a). Hematoxylin & eosin staining showed multinucleated giant cells surrounded by mononuclear cells in the GCTB (Fig. 1b). These results suggest that the GCTB contained numerous multinucleated cells, which were considered osteoclasts.

Isolation of Mononuclear Cells, Osteoclasts and Neoplastic Cells from GCTB

To analyze the actions of denosumab on osteoclast differentiation and the survival of existing osteoclasts, isolation of the existing osteoclasts from other cells containing osteoclast precursor cells was necessary. On trypsin treatment, we isolated non-osteoclast mononuclear cells from primary cells based on the greater adhesion of the osteoclasts to the dish. The cells harvested after suspending the cells obtained by treating primary cells with trypsin were mononuclear cells, which consist of neoplastic cells and osteoclast precursor cells (Fig. 2a). Fig. 2b shows a microscopic TRAP-stained image of primary cells at the start of culturing, with TRAP-positive multinucleated cells (osteoclasts) among mononuclear cells. The isolated cells obtained from suspending cells in trypsin treatment demonstrated mononuclear cells, which are TRAP negative and uniformly small (Fig. 2c), and osteoclasts, which are TRAP positive and unequally large (Fig. 2d). Using this isolation method, a few TRAP-negative mononuclear cells were also identified in the osteoclast fraction. We analyzed osteoclast differentiation using the mononuclear cell fraction, and the survival of osteoclasts using the osteoclast fraction. After 2 weeks of culture of mononuclear cells, no TRAP-positive cells were seen, while TRAP-negative neoplastic cells remained (Fig. 2e). Using mononuclear cells and osteoclasts, we examined the differentiation and survival of osteoclast, respectively. In addition, we examined the cytotoxic effect of denosumab and zoledronic acid on neoplastic cells.

Effects of Denosumab and Zoledronic Acid on Osteoclast Differentiation

To examine the effect of denosumab and zoledronic acid on osteoclast differentiation, mononuclear cells were cultured in the presence or absence denosumab or zoledronic acid. In the control group, TRAP-positive multinucleated osteoclasts appeared on day 2 of culture, and their number increased until day 4 (culture day 0 is shown in Fig. 2c). These findings indicate that the mononuclear cells contained not only neoplastic cells but also osteoclast precursors. After addition of denosumab and zoledronic acid, the number of osteoclasts differentiated from osteoclast precursors did not increase on days 2 and 4 of culture. In the culture of GCT2 cells with zoledronic acid, of the number of osteoclasts increased on day 2 and decreased on day 4 (Fig. 3a, b). These results suggest that denosumab and zoledronic acid inhibited osteoclast differentiation in GCTB.

Effects of Denosumab and Zoledronic Acid on Survival of Osteoclasts

Next, the effects of denosumab and zoledronic acid on the survival of osteoclasts were analyzed by culturing isolated osteoclasts. The number of TRAP-positive multinucleated cells on both days 2 and 4 showed no significant difference between control and denosumab. By contrast, the number decreased significantly compared to control and denosumab on both days 2 and 4 following addition of zoledronic acid. On day 4 of culture, most of the TRAP-positive multinucleated

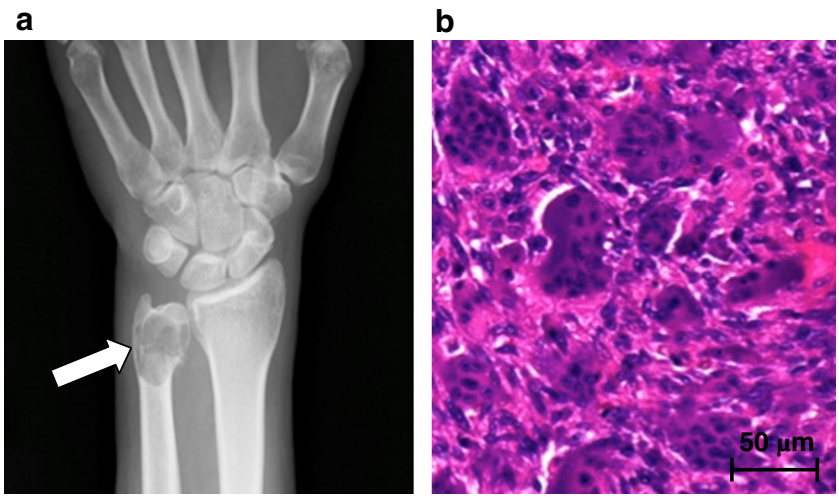

Fig. 1 Radiograph of affected bone and histological appearance in a patient with GCTB. **a** Demarcated lytic lesion in the proximal tibia by anteroposterior plain radiograph (arrow). **b** Multinucleated cells surrounded by abundant mononuclear cells in hematoxylin & eosin-stained section (arrows)

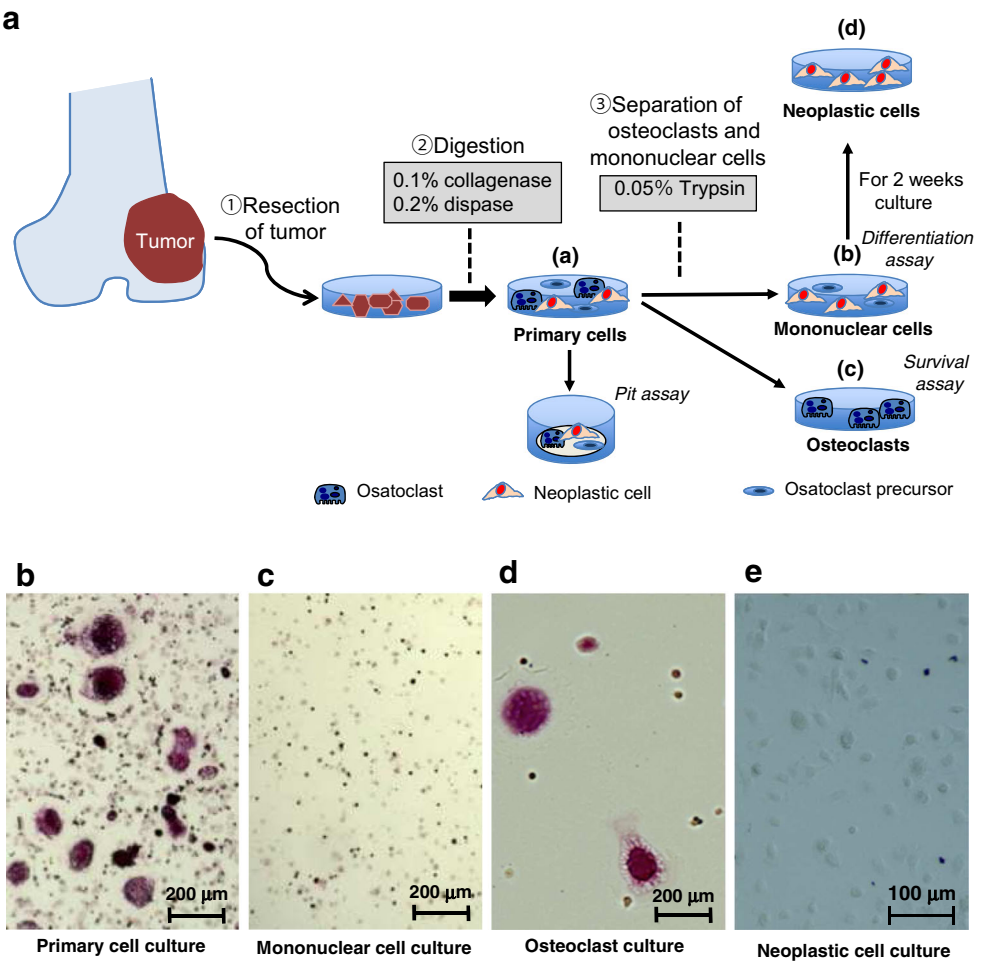

Fig. 2 **a** Schema of the method for purification of mononuclear cells, neoplastic cells and osteoclasts from primary cells of GCTB. Primary cells obtained by digestion of tumor tissue were cultured for 2 h, then treated with 0.05% trypsin-EDTA for 5 min (**b**). Cells removed from the culture dish were collected and used as mononuclear cells (**c**), while cells attached to the culture dish were used as osteoclasts (**d**). The cells obtained by culture for 2 weeks were used as neoplastic cells (**e**). Cells were stained for TRAP. TRAP-positive cells appeared as red cells

cells disappeared following addition of zoledronic acid (Fig. 4a, b). These results suggest that denosumab does not affect survival of osteoclasts prepared from GCTB, whereas zoledronic acid inhibits survival.

Effect of Denosumab and Zoledronic Acid on Bone Resorption

The effects of denosumab and zoledronic acid on bone resorption by osteoclasts in GCTBs were examined. In the cultures of primary cells on dentin slices, the number of resorption pits in the presence of denosumab or zoledronic acid was significantly less than in the control group (Fig. 5a, b). These results suggest that zoledronic acid and denosumab both inhibit bone resorption of osteoclasts prepared from GCTB.

Cytotoxicity of Denosumab and Zoledronic Acid on Neoplastic Cells

Finally, we examined how denosumab and zoledronic acid affect the survival and proliferation of neoplastic cells of GCTB. The cells used for the experiment were spindle-shaped mononuclear cells, in which osteoclasts do not form during culture, unlike primary cells or mononuclear cells used in analysis of osteoclast differentiation. In an MTS assay, denosumab did not show an inhibitory effect on proliferation of neoplastic cells at a dose of 30 μg/mL (Fig. 6). In contrast, zoledronic acid demonstrated an inhibitory effect on proliferation of neopastic cells at 2 μg/mL (a concentration at which an inhibitory effect on osteoclast differentiation was seen). Moreover, as concentration increased further, zoledronic acid showed a dose-dependent inhibitory effect on neoplastic cells. However, the effect was not observed with denosumab at the concentrations examined in the present study. These results suggest that denosumab does not inhibit proliferation of neoplastic cells prepared from GCTB.

Discussion

The excellent clinical effect of denosumab, a widely used antiresorptive agent indicated for GCTB, has been reported [48–50]. Although its inhibitory effect on the action of

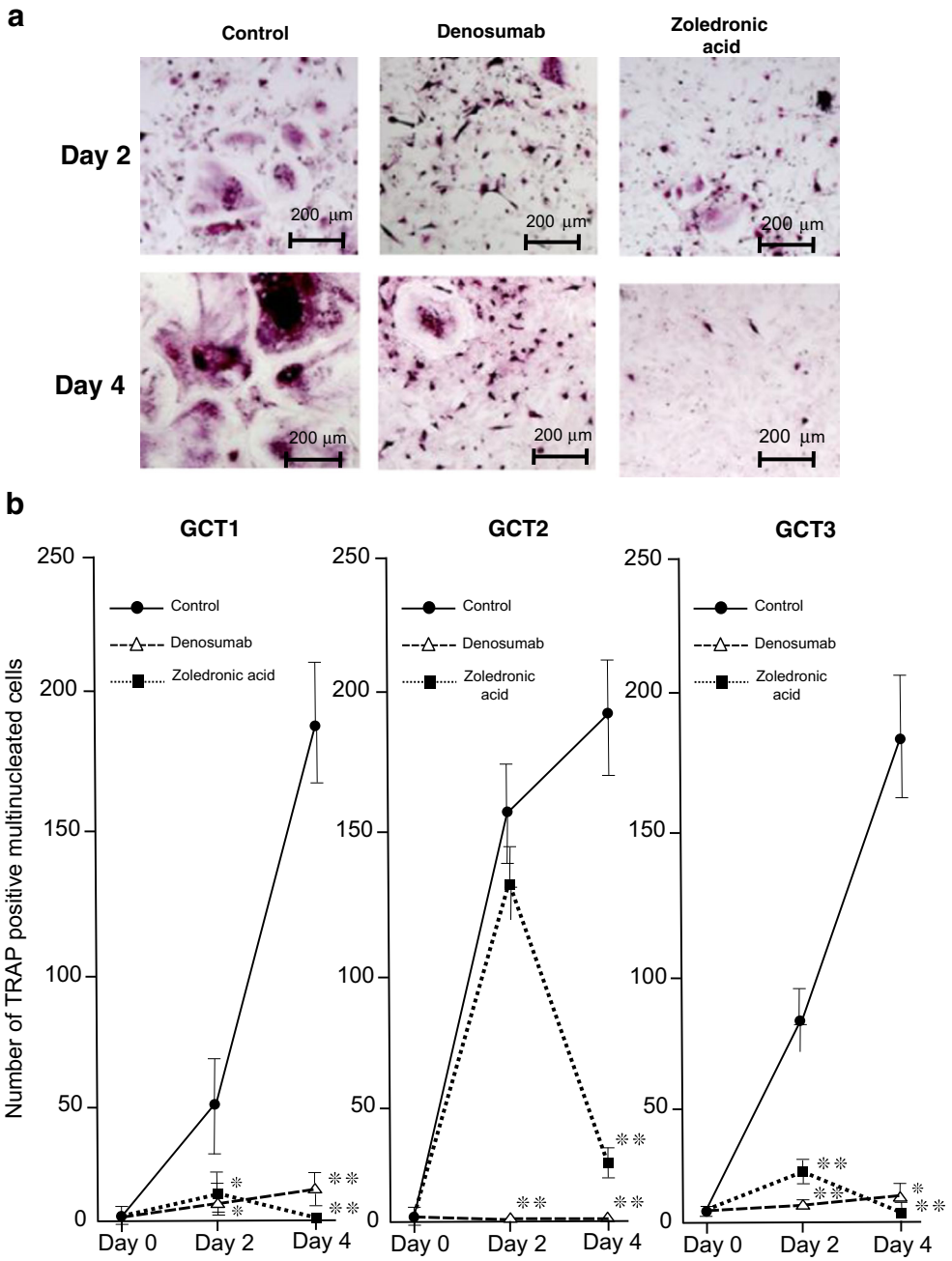

Fig. 3 Effects of denosumab and zoledronic acid on osteoclast differentiation from mononuclear cells prepared from GCTB. Mononuclear cells prepared from GCTB were cultured for 2 or 4 days with or without (closed circles) denosumab (30 μg/mL) (open triangles) or zoledronic acid (2 μg/mL) (closed squares). Then, the cells were stained for TRAP (**a**) and TRAP-positive cells were counted as osteoclasts (**b**). Data are shown as means ± SD. * $p < 0.05$, ** $p < 0.01$ compared with control

RANKL has been previously established, the exact target events in GCTB comprising multiple cell populations—for example, whether it influences osteoclast differentiation, activation and/or survival—remain unknown. We investigated the mechanism of action of denosumab in GCTB because the differentiation and survival of osteoclasts occur in GCTB tissue cultures.

Clinical data previously showed that administration of denosumab resulted in pathological changes, namely, loss of multinuclear giant cells [31]. This clearly indicates the loss of osteoclasts, but whether denosumab affects existing osteoclasts in GCTB or new osteoclast differentiation remains unclear. We confirmed that, in the culture of mononuclear cells from GCTB tissues, administration of denosumab resulted in a lack of multinuclear osteoclast differentiation. Considering that multinucleated giant cells emerge from the mononuclear cell population in the control group, denosumab appeared to inhibit differentiation of osteoclast precursor cells into osteoclasts. Branstetter et al. showed the appearance of many RANK-positive mononuclear cells as well as the loss of osteoclasts in denosumab-administered GCTB tissues in vivo [42]. Meanwhile, Atkins et al. reported that RANK was expressed on osteoclast precursor cells, but not on other mononuclear tumor cells [51]. Taken together, the findings

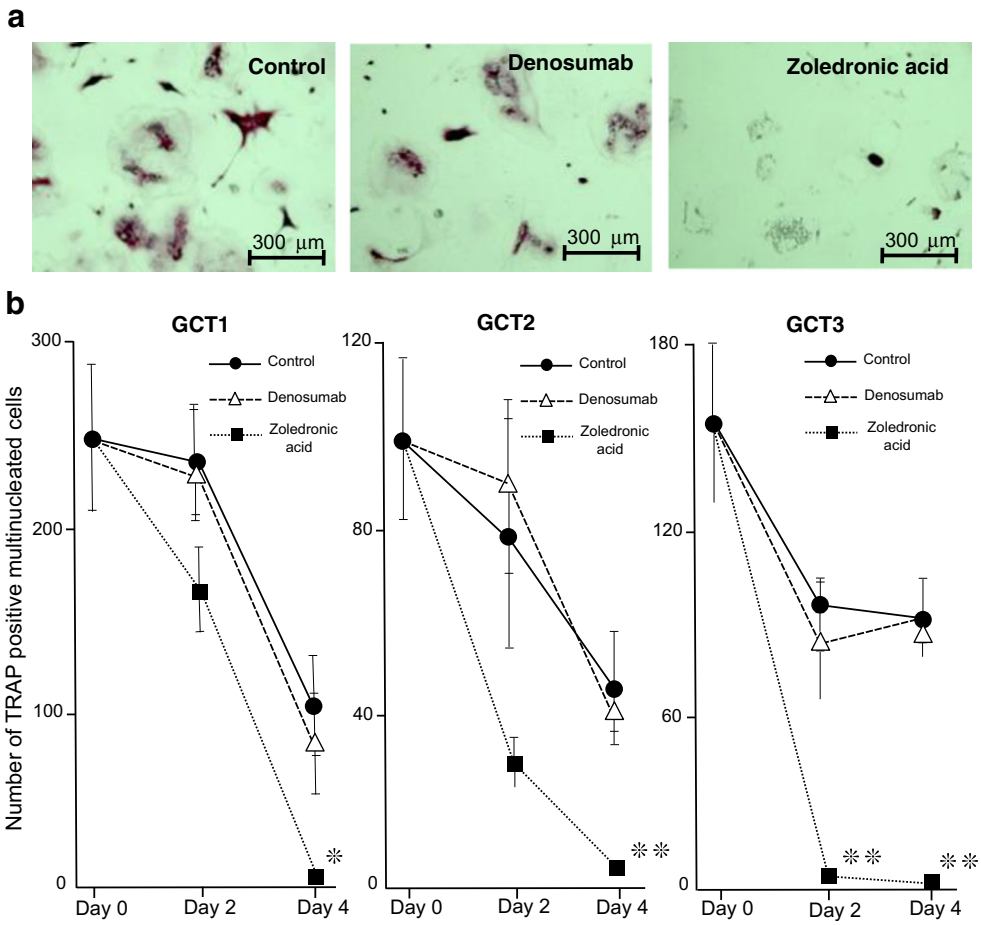

Fig. 4 Effects of denosumab and zoledronic acid on the survival of osteoclasts prepared from GCTB. Osteoclasts prepared from GCTB were cultured for 2 or 4 days with or without (closed circles) denosumab (30 μg/mL) (open triangles) or zoledronic acid (2 μg/mL) (closed squares). Then, the cells were stained for TRAP (**a**) and TRAP-positive cells were counted as osteoclasts (**b**). Data are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$ compared with control

of the study by Branstetter et al. support our results demonstrating the inhibitory effect of denosumab on osteoclast differentiation.

The inhibitory effects of zoledronic acid on osteoclast differentiation in GCTB cell cultures were similar to those of denosumab. Liverani et al. used a cell culture model of monocyte–osteoclast differentiation and found aggregates of vacuolated osteoclast in the presence of zoledronic acid, whereas osteoclasts were not generated in the presence of denosumab [52], suggesting that zoledronic acid acts on differentiated osteoclasts. In our experiments, osteoclast differentiation was inhibited by denosumab but a transient increase in the number of osteoclasts was observed in the presence of zoledronic acid. Thus, our results obtained from the experiments using GCTB cell cultures are consistent with the findings of Liverani et al.

We removed mononuclear cells from the primary cell population and used the remaining fraction to evaluate the survival of osteoclasts in GCTB. It was previously reported that bisphosphonates induce apoptosis of osteoclasts in GCTB [53], and we also found a marked reduction of osteoclasts induced by zoledronic acid, whereas osteoclasts disappeared in a time-dependent manner in the control group. By contrast, there was no difference between the denosumab-treated and the control groups, indicating that this agent does not suppress survival of osteoclasts themselves. None of the previous studies examined the inhibitory effects of denosumab on differentiation and survival separately and, to the best of our knowledge, no study has demonstrated the cytocidal effect of denosumab on osteoclasts. Thus, the finding of this study indicates that the suppression of osteoclast differentiation is the underlying mechanism of action of the denosumab-induced disappearance of osteoclasts in GCTB tissue.

RANKL, the effect of which is blocked by denosumab, has an essential role in osteoclast survival, and is expressed in neoplastic cells [33]. The osteoclast survival assay in the present study did not contain neoplastic cells, only osteoclasts. Thus, no difference should have been observed in the cells in the osteoclast survival assay even if denosumab (which blocks RANKL) was added, as there would be no RANKL-expressing cells to affect. Yet, osteoclasts have been reported in some other studies to express RANKL [45, 46]. However, if this were the case, we would expect to have seen a change after the addition of denosumab to the osteoclast survival assay in the present study, which contained only osteoclasts.

The bone resorption assay showed fewer resorption cavities in the group treated with denosumab than in the control group. As shown in the results regarding the effect of

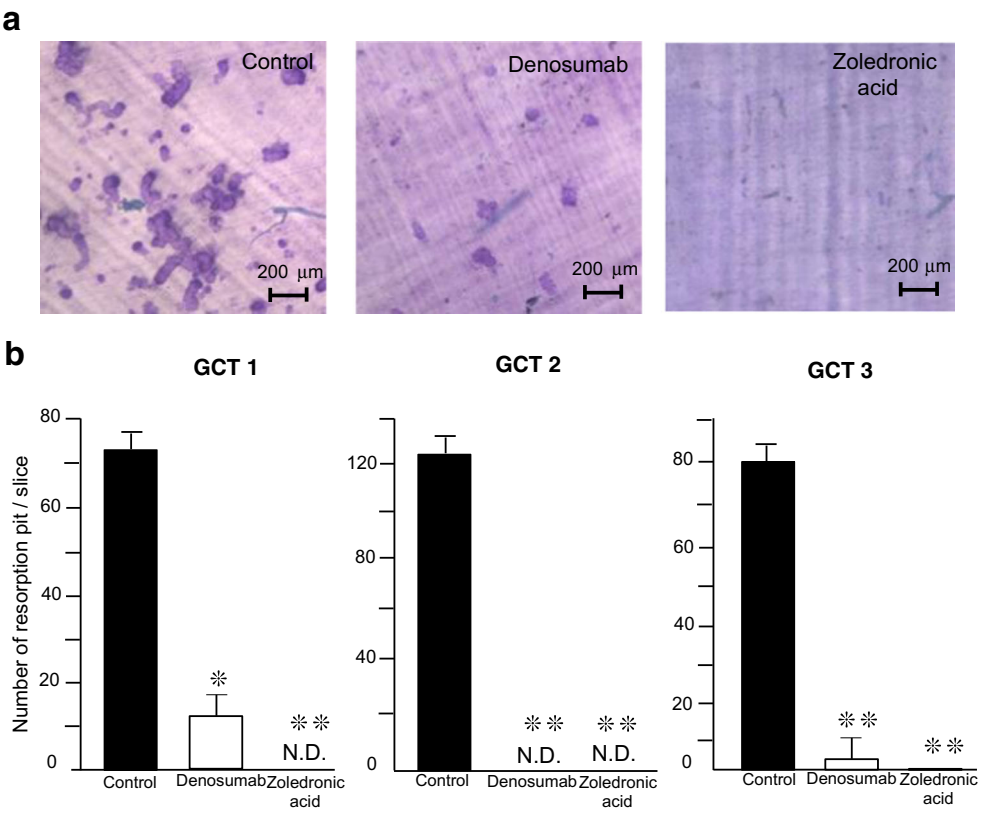

Fig. 5 Effects of denosumab and zoledronic acid on the pit-forming activity of primary cells prepared from GCTB. **a** Primary GCTB cells were cultured on dentin slices for 4 days with or without (closed circles) denosumab (30 μg/mL) (open triangles) or zoledronic acid (2 μg/mL) (closed squares). Then, the cells were removed from the dentin slices and resorption pits formed on the slices were visualized by staining with toluidine blue. **b** The number of pits on the slice was counted. Data are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$ compared with control

denosumab on osteoclast differentiation and survival, the numbers of existing osteoclasts on a dentin slice were similar between the two groups, whereas the differentiation of precursor cells into osteoclasts ceased after the addition of denosumab and, consequently, there were fewer osteoclasts in the denosumab-treated group than in the control group. The number of resorption pits reflects the number and function of osteoclasts, and indeed denosumab decreased both the number of osteoclasts and the number of resorption cavities. However, zoledronic acid suppressed both differentiation and survival, and the almost complete loss of resorption cavities in the presence of zoledronic acid was consistent with the findings of culture experiments: the lack of newly generated osteoclasts and disappearance of existing osteoclasts in culture. Balke et al. also performed the bone resorption assay to examine the effect of zoledronic acid in GCTB, and reported results that were similar to ours [26]. Thus, the present study is the first to show the corresponding findings for denosumab.

We analyzed the mononuclear cell population containing osteoclast precursor cells and the osteoclast population to examine differentiation and survival of osteoclasts, respectively. The effect of the two agents on the remaining cell population of GCTB, namely, the neoplastic cell population, was also examined in this study. Zoledronic acid was clearly shown to have a suppressive effect on tumor cells in GCTB, whereas few studies support a similar effect of denosumab [29]. Zoledronic acid induces apoptosis of tumor cells in a dose-dependent manner in GCTB [43, 54], and we also found dose-dependent suppression of tumor cells by this agent, but not by denosumab, in the present study. These results suggest that denosumab acts via a single mechanism that causes inhibition of osteoclast differentiation in GCTB, whereas zoledronic acid has multiple effects in GCTB, such as inhibition of osteoclast differentiation, inhibition of osteoblast survival and suppression of tumor cells.

In contrast to our findings, previous in vivo data demonstrated a marked anti-tumor effect of denosumab, such as reduction of tumor size as shown by image analysis and pathological changes [42, 55]. Also, an anti-RANKL antibody reduced the metastasis rate of osteosarcoma [56], suggesting the presence of a mechanism through which RANKL indirectly affects tumor cells themselves, in addition to the mechanisms through which RANKL is involved in the differentiation and survival of osteoclasts in tumor tissue. James et al. and Murata et al. demonstrated that tumor cells in GCTB shared many characteristics with osteoblasts, and showed a tendency to differentiate into osteoblasts [57, 58]. Also, denosumab induced bone neoplasticity within GCTB tissue [42]. Osteogenesis within the tumor tissue suggests differentiation of tumor cells into osteoblasts, but whether this is due to inhibition of the action of RANKL remains to be determined. Although details of the mechanism underlying the induction of differentiation remain unclear, it is likely that induction of tumor cell differentiation impairs their proliferation ability,

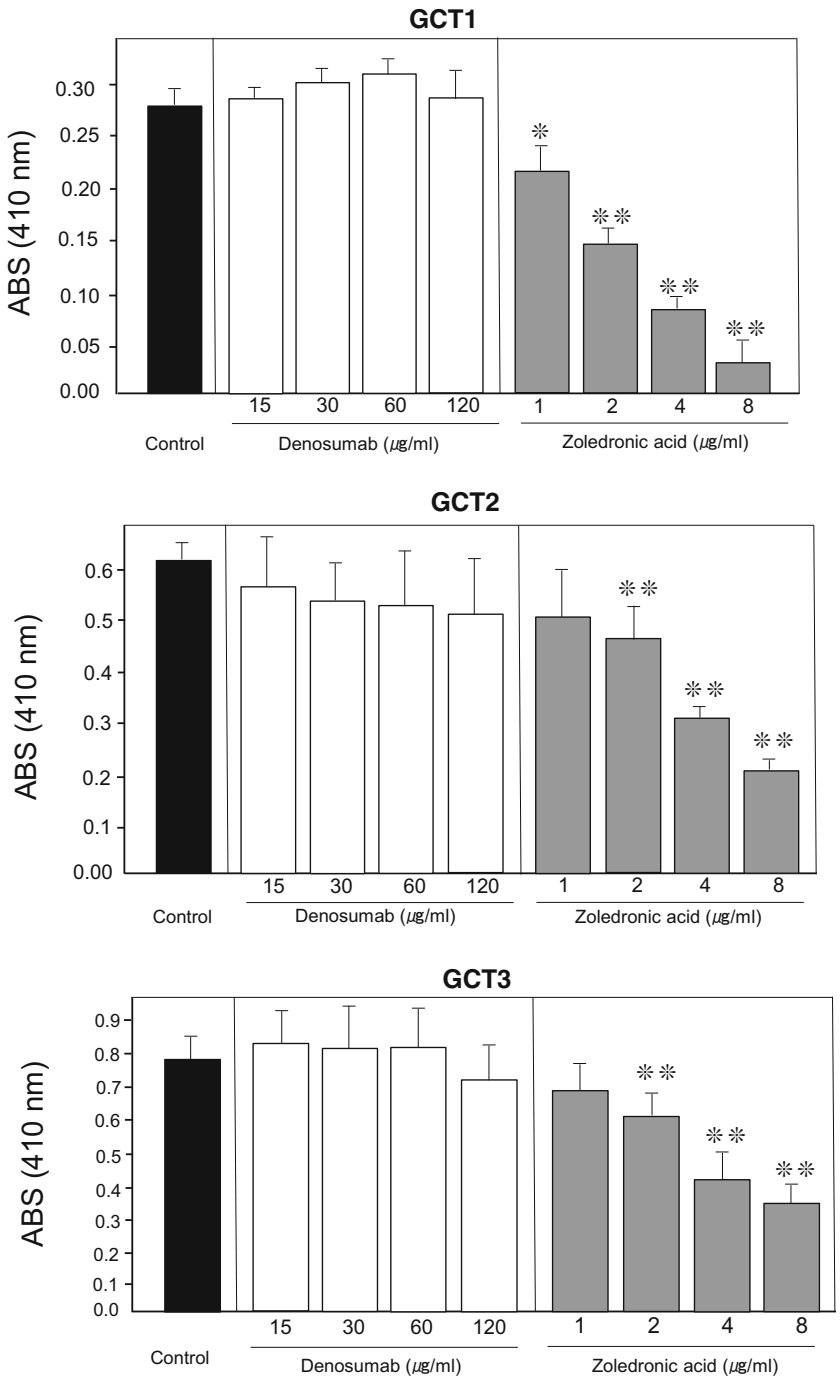

Fig. 6 Effects of denosumab and zoledronic acid on the proliferation of neoplastic cells. Cells prepared from GCTB were cultured for 6 days in the presence of the indicated concentrations of denosumab (white bar) or zoledronic acid (gray bar). Controls are shown by a black bar. Then, cell proliferation was evaluated using the MTS assay. Data are shown as means ± SD. * $p < 0.05$, ** $p < 0.01$ compared with control

thereby reducing the tumor size and suppressing metastasis. Whether the anti-tumor effect of denosumab, which is superior to that of zoledronic acid, is the result of the induction of tumor cell differentiation via RANK–RANKL interaction is an intriguing question to be addressed in future studies.

Various cell populations exist in GCTB: neoplastic cells and multinuclear giant cells that possess the functions of osteoclasts, and their precursors. These populations affect one another to influence differentiation and survival of osteoclasts, which collectively explain active bone resorption in GCTB. In this study, we confirmed that denosumab suppresses osteoclast differentiation and bone resorption in GCTB, but it does not have a significant effect on osteoclast survival. Given the differences between denosumab and zoledronic acid, both of which are classified as antiresorptive agents, the findings of this study may lead to new drug discovery via investigation of the complex characteristics of GCTB and phenomena triggered by RANKL inhibition.

Acknowledgements This work was supported by the Japan Society for the Promotion of Science (Grant Numbers 26293398 and 16 K20655) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Drafting the article or revising: Isao Shibuya, Masamichi Takami, Akira Dezawa, Shigeru Nakamura, Ryutaro Kamijo.

GUARANTOR: Isao Shibuya.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This study has been carried out with the approval of the ethics committee of Teikyo University School of Medicine (approval No: 12–001) and Showa University School of Dentistry (approval No: 2006–12). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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