The inhibition of RANKL/RANK signaling by osteoprotegerin suppresses bone invasion by oral squamous cell carcinoma cells

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Oral squamous cell carcinomas (OSCCs) are malignant tumors that frequently invade the maxilla and mandibular bone. However, the molecular mechanisms underlying bone invasion by OSCC are unclear. Recent studies showed that receptor activator of nuclear factor κB (RANK) was expressed not only in osteoclast precursors but also in tumor cells. Therefore, we examined whether RANK ligand (RANKL)/RANK signaling regulates bone invasion by OSCC cells in vivo and in vitro. We first injected human OSCC B88 cells into the masseter region of nude mice. Mice were treated for 3 weeks with osteoprotegerin (OPG), the decoy receptor for RANKL. Treatment with OPG decreased bone invasion by B88 cells, reduced the number of osteoclasts and increased B88 cell apoptosis. However, OPG did not affect apoptosis and proliferation in B88 cells in vitro, suggesting that the effects of OPG on apoptosis in B88 cells are restricted in a bone environment. RANK was expressed in the B88 cells and in OSCC cells from patients. RANKL induced NF-κB activation and extracellular signal-regulated kinase phosphorylation in B88 cells and enhanced B88 cell migration in a modified chemotaxis chamber equipped with a gelatin-coated filter. OPG inhibited RANKL-induced NF-κB activation, extracellular signal-regulated kinase phosphorylation and cell migration. Our data clearly indicate that RANKL/RANK inhibition suppresses bone invasion by inhibiting osteoclastogenesis and cancer cell migration and by inducing apoptosis of cancer cells via indirect anticancer action in vivo.

Introduction

Squamous cell carcinoma of the oral cavity is a malignant tumor that is clinically characterized by diverse morphological features and pathologically characterized by marked local invasiveness. A major clinical problem is tumor invasion into the adjoining maxilla and mandible (1,2). Advanced oral squamous cell carcinoma (OSCC) has a high mortality rate, and treatment is complicated by the disruption of speech and swallowing after surgery. Patients with mandibular invasion should be treated by radiation and chemotherapy to reduce tumor size and subsequently treated surgically. Mandibulectomy greatly affects the patient’s quality of life and is a critical determinant of the postoperative functional outcome (3,4). The extent of bone invasion can only be assessed by diagnostic imaging. Furthermore, the cellular and molecular mechanisms regulating bone invasion by OSCC are poorly understood.

Although controversial, bone destruction that occurs with OSCC invasion is thought to be mediated by osteoclasts rather than by the carcinoma itself. Three proteins crucial for osteoclast development and activation are the receptor activator of NF-κB ligand (RANKL), its receptor (RANK) and its decoy receptor osteoprotegerin (OPG) (5–11). RANKL is a member of the tumor necrosis factor family and stimulates osteoclastogenesis (5–7). RANK is a member of the tumor necrosis factor receptor family and is the cognate receptor of RANKL. RANK is expressed at high levels in osteoclast precursors. Interactions between RANKL and RANK induce differentiation of preosteoclasts into osteoclasts, ultimately resulting in the destruction of bone (10,11). Thus, osteoclastogenesis is modulated through a balance between OPG and RANK.

Recent studies have established that bone resorption by osteoclasts is an important step in the process of bone invasion and metastasis in several types of malignancies (12). Several in vitro and animal experiments using human OSCC cells have shown that tumor cells produce several cytokines, including interleukin-6, interleukin-11, tumor necrosis factor-α and parathyroid hormone-related protein. These cytokines regulate the expression of RANKL and OPG (12). The RANKL/RANK signaling pathway also regulates bone invasion or metastasis in several types of human cancer, including breast cancer (13,14), prostate cancer (15,16), myeloma (17,18) and oral SCC (19). It has been reported that RANKL-expressing cancer cells induce osteoclastogenesis in the absence of other accessory cells (19,20). However, not all cancers express RANKL, and cell-to-cell contact between cancer cells and host cells does not always lead to RANKL expression (21,22). One of our previous studies used BHY cells that highly invade into mandibular bone when inoculated into the masseter of nude mice. Although these cells express RANKL on their surface, they fail to induce osteoclastogenesis in co-cultures of mouse bone marrow cells and BHY cells (23). However, osteoclastogenesis is markedly induced in the absence of osteotropic factors when BHY cells are added to a co-culture of mouse primary osteoblasts and bone marrow cells.

Materials and methods

All protocols for the present experiments were reviewed and approved by the Fukuoka Dental College Research Ethics Committee (Approval number 61) and by the Council on Animal Care at Kyushu Dental College (Approval number 07-010).
Reagents
Recombinant human RANKL was obtained from PeproTech (Rocky Hill, NJ). Recombinant human OPG was kindly provided by Daichi-Sankyo (Tokyo, Japan). Anti-phosphorylated extracellular signal-regulated kinase (ERK) (sc-7383) and anti-rabbit immunoglobulin G (sc-2004) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK antibodies (#9102) and anti-mouse immunoglobulin G antibodies were obtained from Cell Signaling (Beverly, MA) and Amersham Bioscience (Piscataway, NJ), respectively.

Cell lines
The original B88 cell tumor was moderately differentiated SCC of the tongue and cells are capable of invading mandibular bone when injected into the masseter muscle of nude mice (28). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO2 at 37°C.

In vivo tumor invasion to mandibular bone
B88 cells were injected into the left masseter region of 7- to 10-week-old male nude mice (Balb/cA/Jl-nu). One week after injection, mice were injected between the left masseter region and the surface of the left mandibular bone with vehicle (100 μl of PBS/mouse, n = 10) or OPG (5 μg/100 μl of PBS/mouse, n = 10) three times a week for a total of 3 weeks. The insulin injection syringe with 27G needle was inserted between the tumor and the bone surface. When the needle reached the periosteum, the syringe was pulled a little to prevent periosteum from damage and then we injected OPG solution slowly. Tumors were allowed to progress until day 28, and mice were sacrificed at this time. Tumor sizes were assessed using calipers at the point, and tumor volume was calculated using the following formula: width2 × length × 0.52 (29). Mandibular bone and periiphery tissue were collected for histological analysis. Briefly, tissues were fixed in 3.7% formaldehyde, decalcified with 10% EDTA (pH 8.0) for 2 weeks and then embedded in paraffin. Formaldehyde-fixed paraffin-embedded sections were cut at 5 μm and stained with hematoxylin and eosin, tartrate-resistant acid phosphatase (TRAP) and an anti-human RANKL monoclonal antibody (clone; 12A668, 1:250; Merck, Darmstadt, Germany) using the immunoperoxidase technique (Dako LSAB2 System, HRP; Dako, Carpenteria, CA). The non-immune immunoglobulin G antibody was used as negative control for the immunohistochemistry. All incubations were performed at room temperature. All samples were observed under a microscope (Biorevo, BZ-9000; Keyence, Tokyo, Japan). Five fields of tumor in each specimen were randomly selected and counted to determine the number of TRAP-positive multinucleated cells. Data are expressed as the number of TRAP-positive multilobulated cells/bone surface/mm2 section.

Tissue samples and histology
Following resection of the mandibles of 15 patients with OSCC, tumor and mandibular blocks were obtained from the Pathology Department of Fukushima Dental College. The blocks were fixed with 10% paraformaldehyde, decalcified in formic acid and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin and processed for immunohistochemical staining with anti-human RANKL monoclonal antibody (clone: MAR6831; R&D Systems, Minneapolis, MN) using the immunoperoxidase technique (Dako LSAB2 System). The non-immune immunoglobulin G antibody was used as negative control for the immunohistochemistry.

Polymerase chain reaction amplification of reverse-transcribed messenger RNA
For reverse transcription–polymerase chain reaction, total RNA from OSCC cells was prepared using Trizol (Invitrogen, Carlsbad, CA) and amplified using Superscript II and Taq polymerase (Invitrogen). The primers used in our study were as follows: human RANK forward-CATCCATGTACAGTGAAGTTT and reverse-CAAGGAAAGTTTACCGTGG (amplicon size 441 bp); human RANKL forward-TGGATCACAGCACATCAGAGCAG and reverse-ACTGCGGTCACTTATGTCCAAGG (amplicon size 588 bp); human OPNG forward-AGGGACACAAATGACAGTTT and reverse-AGCGGACACCTCAACATC (amplicon size 409 bp); and human GAPDH forward-TGACAGACATGTTCTGAAGG and reverse-ACCACTGACGGTTACAGT (amplicon size 737 bp).

Western blotting
For immunoblotting, whole-cell lysates were resolved in sodium dodecyl sulfate–polyacrylamide gels, transferred to polyvinylidene difluoride membranes and incubated at 4°C overnight with antibody dilutions ranging from 1:500 to 1:1,000 in a 5% dry milk solution containing 0.01% azide. The blots were then washed in TTBS (10 mM Tris-HCl, 50 mM NaCl and 0.25% Tween 20) and incubated with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were visualized using ECL (Amersham).

Electrophoretic mobility shift assay
Preparation of nuclear extracts and sequences NF-xB and NF-kB binding oligonucleotides were described previously (30). The oligonucleotides were labeled with the biotin 3’ end DNA Labeling Kit (Thermo, Rockford, IL) for use in electrophoretic mobility shift assay. The DNA-binding reaction was performed at room temperature in a volume of 20 μl that contained binding buffer (10 nM Tris-HCl, pH 7.5; 50 mM KCl; 1 mM dithiothreitol; 0.05% Nonidet P-40; 2.5% glycerol), 1 μg of poly(dI-dC)·2 nM of nuclear extract proteins. Following a 20-min incubation, the samples were subjected to electrophoresis on native 5% acrylamide/0.5× TBE gels. The reactions were transferred to a nylon membrane. The biotin-labeled DNA was detected with the LightShift chemiluminescent electrophoretic mobility shift assay kit (Thermo).

Apoptosis of B88 cells
Apoptosis in B88 cells was determined with TUNEL-stained sections. TUNEL staining was performed with the ApopTag Plus In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD) according to the manufacturer’s protocol. Five fields of tumor in each specimen were randomly selected and counted to determine the number of apoptotic cells. Data are expressed as the number of TUNEL-positive cells/mm2 tumor area.

In vitro apoptosis assay
B88 cells were cultured in the presence or absence of either RANKL (100 ng/ml) or OPG (100 ng/ml) for 24 h. Cells were stained with Annexin V-FITC and propidium iodide (R&D Systems) and analyzed by flow cytometry (FACS Calibur, Becton Dickinson, UK). Bay11-7082 (100 ng/ml) was added to the culture medium to induce apoptosis and to serve as a positive control.

Proliferation assay
B88 cells (2 × 103 cells) were seeded into 96-well plates in the presence or absence of OPG (100 ng/ml) and with or without RANKL (100 ng/ml) for indicated time periods. Proliferation of tumor cells was measured using the Cell Counting Kit 8 (Dojin, Kunitomo, Japan), according to the manufacturer’s protocol.

Migration assay
The modified Boyden chamber with a gelatin-coated membrane was used for this experiment. B88 (3 × 105 cells) were placed in 200 μl of Dulbecco’s modified Eagle’s medium + 1% fetal bovine serum in the upper well. RANKL with or without OPG at 100 ng/ml were added to 700 μl of Dulbecco’s modified Eagle’s medium + 1% fetal bovine serum in the bottom well. After a 7-h incubation, cells attached to the upper surface of the membrane (8.0-μm pore size) were removed. Cells that migrated onto the lower surface were fixed with 3.7% formaldehyde and stained with Mayer’s hematoxylin. Results are expressed as the mean number of cells per five high-power fields of view that passed through the gelatin-coated membrane.

Data analysis
Comparisons were made by factorial ANOVA. Data were expressed as mean ± SD; P values < 0.05 were considered significant.

Results

Tumor sizes and body weights of animals treated with OPG
B88 cells were injected into the masseter region of nude mice, and mice were treated with or without various concentrations of OPG for 3 weeks to examine whether inhibition of RANKL signaling by OPG altered cell invasion to mandibular bone. To choose an optimal dosage of OPG, we preliminarily injected various concentrations of OPG (0, 1, 2, 5, and 10 μg/mouse) and the effect was judged by reduced tumor size. OPG reduced tumor size in a dose-dependent manner. OPG (5 μg/100 μl of PBS/mouse, n = 10) treatment resulted in decreased tumor size compared to the control (Figure 1A and B). At 5 μg, OPG reduced tumor size at the similar level of 10 μg of OPG treatment (data not shown). Therefore, we used 5 μg/mouse of OPG in the following experiments. Mice injected with tumor cells weighed significantly less than control mice at 21 days, suggesting that the progression of tumor and bone invasion interfered with food intake and increased bone mass in mice. In contrast, the weights of mice treated with OPG were almost comparable to those of the control mice (Figure 1C).
OPG reduced bone invasion by inhibiting osteoclastogenesis

Microscopic examination showed that the shape of the mandible in mice bearing tumors had an irregular margin with both bone formation and bone resorption compared to the control mice (Figure 2A and C). Invasion was observed microscopically in all mice, and an increased number of TRAP-positive osteoclasts at the tumor/bone interface (Figure 2C, D and G). In contrast, treatment with OPG eliminated both tumor invasion and tumor-induced osteoclasts (Figure 2E, F and G). No RANKL-positive cells were observed in normal mandible (Figure 3A). Tumor-bearing mice demonstrated that strongly RANKL-positive osteoblastic cells (Figure 3B and C) and inflammatory lymphocytes (Figure 3B and D) were located at the interface between bone and B88 cells, presumably reflecting the increases in osteoclast numbers observed (Figure 2D and 3), while B88 cells did not express RANKL (Figure 3B). In contrast, weakly RANKL-positive cells were observed in OPG-treated mice (Figure 3E). The non-immune immunoglobulin G antibody was used as negative control for the immunohistochemistry (Figure 3F).

Expression of RANK on OSCC cells in patients and human OSCC cell lines

We next examined the expression of RANK on bone-resorbing OSCC cells from 15 patients by immunostaining with anti-RANK antibody. Figure 4 shows representative hematoxylin and eosin staining in a section of mandibular bone invaded by OSCCs in a patient. RANK is expressed on OSCC cells (Figure 4A and B). The non-immune immunoglobulin G antibody was used as negative control for the immunohistochemistry (Figure 4C). We further examined the messenger RNA expression levels of RANK, RANKL and OPG in B88 cells using reverse transcription–polymerase chain reaction analysis. B88 cells express RANK, but not RANKL and OPG messenger RNA (Figure 4D). BHY cells that were derived from a highly differentiated human SCC of the lower alveolus and express RANK, RANKL, and OPG (31) were used as a positive control.

RANKL-mediated activation of signaling pathways in B88 cells

We examined whether RANKL activates NF-κB and ERK through RANK on the cell surface of B88 cells as well as osteoclast progenitors. RANKL-induced NF-κB activation and RANKL-induced ERK phosphorylation was observed up to 30 min. OPG prevented RANKL-induced NF-κB activation and ERK phosphorylation (Figure 4E and F). Thus, RANK is expressed in B88 cells and can activate specific downstream signaling pathways induced by RANKL.
Effects of OPG on the progression of B88 cells

Upon microscopic examination, there was a decrease in tumor burden (Figure 1C) and cell density (data not shown) in OPG-treated mice, suggesting that OPG induced significantly more cell death compared with the control treatment. We next examined whether a decrease in B88 cell bone invasion following OPG treatment was associated with increased B88 cell apoptosis. The TUNEL intranucleosomal DNA fragment end-labeling technique was used to quantify the number of apoptotic B88 cells. TUNEL-positive cells were clearly identified by their brown stain. OPG treatment significantly increased the number of apoptotic B88 cells (Figure 5A and B). In contrast, histomorphometric analysis showed no changes in mitosis in B88 cells (data not shown).

Effects of OPG on apoptosis and proliferation in B88 cells in vitro

To determine the effects of OPG on B88 cell growth and apoptosis in vitro, we assessed apoptotic cells using Annexin V staining and measured proliferation. OPG treatment did not alter B88 cell apoptosis or proliferation in the presence (Figure 5C and D) or absence of RANKL. These data are consistent with a mechanism of action whereby inhibiting tumor-induced bone turnover deprives tumor cells of bone-derived growth factors and cytokines that would otherwise be released and further stimulate tumor growth (12).

The RANKL/RANK system regulated migration of B88 cells in vitro

We next examined the functional roles of RANKL on bone invasion by OSCC. The effect of RANKL on B88 cell migration was examined using a modified chemotaxis chamber with a gelatin-coated filter. RANKL was placed in the lower chamber, and the chemotaxis chamber was incubated for 7 h. The cells that migrated to the lower surface of the membrane were counted. RANKL markedly enhanced the migration of the B88 cells (Figure 6). Cell migration was strongly inhibited by the addition of OPG into the upper chamber 1 h before RANKL treatment (Figure 6). These data suggest that RANKL may be a chemotactic or chemokinetic factor for OCSS cells.

Discussion

Breast cancer and multiple myeloma are common metastatic cancers. Approximately 65–75% of breast cancers and 95–100% of multiple myelomas metastasize to bone (32). The mechanism of OSCC invasion to bone is unclear, however. This is because patients with oral cancer that metastasizes to lymph nodes in the neck or to the lung die early, and
histological analysis of the bone was not performed (1–4). OSCC cells directly invade into the mandibular bone, whereas other cancers metastasize through many steps to invade the bone. It has been suggested that RANKL signaling plays important roles in bone metastasis and in mandibular bone invasion by OSCC cells (1–4). Importantly, OPG suppresses OSCC cell-induced osteoclastogenesis (23), and it is possible that OPG reduces mandibular bone invasion by OSCC cells. Thus, in this study, we examined the effects of OPG in a mouse model of mandibular bone invasion by OSCC cells.

B88 cells inoculated into the masseter region of nude mice invaded into mandibular bone. Bone invasion was strongly reduced by local treatment with OPG that was administered every other day starting 1 week after the B88 cells were inoculated. It is suspected that OPG suppressed mandibular bone invasion by three mechanisms. First, OPG suppressed osteoclastogenesis and bone resorption activity (Figure 7). We have previously shown that OSCC cells induce osteoclast differentiation by suppression of OPG expression in osteoblasts and that OSCC cells enhance multinucleation, survival and bone resorption of osteoclasts (23,24). Recently, Tan et al. reported that stromal/osteoblastic cells and inflammatory T cells recruited to host/tumor junction, but not tumor cells, expressed RANKL (34). In our model, RANKL expression was observed in osteoblastic cells and inflammatory lymphocytes that faced the bone surface, but not B88 cells, suggesting that tumor cells changed the balance of RANKL and OPG in the host environment through the release of cytokines and growth factors from tumor cells (33). Consistent with these results, osteoclasts were present in mice inoculated with B88 cells, but few osteoclasts were observed in mice treated with OPG.

Second, OPG may regulate the tumor microenvironment of OSCC. We show that OPG increased cell death in B88 cells and decreased tumor burden, whereas B88 cell mitosis was unchanged (data not shown). These results suggest that OPG promotes death of B88 cells,
insulin-like growth factor and transforming growth factor β (35), these growth factors are released into the bone microenvironment in active forms as a consequence of osteoclastic bone resorption (36). OPG likely limits the supply of such bone-derived growth factors that facilitate proliferation and survival of OSCC cells that colonize bone through the inhibition of osteoclastic bone resorption. This growth factor-deficient condition may cause an increase in B88 cell death (Figure 7). Indeed, OPG-induced apoptotic B88 cells in bone environment led to less production of cytokines, which induce RANKL. Thus, the numbers of RANKL-positive cells were reduced in OPG-treated mice. It is possible that OPG, at least in part, promotes B88 cell death in bone through inhibition of osteoclastic bone resorption.

Third, OPG suppressed RANKL-induced OSCC cell migration (Figure 7). Several tumors expressed RANK, and it has been suggested that RANKL/RANK signaling not only contributes to osteoclastogenesis but also regulates cancer cell functions (25–27). Primary tumor cells from OSCC patients expressed RANK. OSCC cell lines, including BHY and B88, expressed RANK, and NF-κB and ERK were activated by RANKL. These results indicate that RANKL regulates OSCC functions. Tumors reached the bone in mice inoculated with B88 cells; however, tumors were farther from the bone in mice treated with OPG. We also demonstrated that RANKL promoted OSCC cell migration, an effect that was abrogated by OPG. We propose that one of the effects of RANKL signaling in OSCC cells is to facilitate cancer cell migration.

Recently, various drugs have been used as osteoporotic therapeutics, such as bisphosphonate, which is problematic because bisphosphonate induces necrosis of jaw bone as its side effect (37–39). As bisphosphonate accumulates in the bone for a long term, bone remodeling halts due to strong inhibition of osteoclast function. OPG has been tested in clinical trials of bone metastasis and osteoporosis. A phase I study testing recombinant OPG in patients with multiple myeloma or breast carcinoma-related bone metastases is underway (40). Thus far, OPG has few side effects when administered as a single subcutaneous injection to patients (41), and OPG was used subcutaneously in bone metastases of multiple myeloma cells and breast cancer cells (25). In the case of mandibular bone invasion, it is possible to treat locally with OPG because OSCC cells invade from cortical bone, and the tumor and defect area are at the surface of the body. Therefore, OPG may oppose OSCC cell bone invasion and have few side effects. However, it is important to carefully consider the administration dosage and schedule to avoid the changing balance between bone formation and resorption by local injection of OPG before the clinical application.

B88 cells invaded through tumor-reacting new bone that formed around mandibular bone. This differs from how human OSCC cells invade. A new mouse model will be necessary for further analysis. In conclusion, our study reveals that RANKL promotes migration of RANK-expressing OSCC cells, induces osteoclasts and results in facilitating mandibular bone invasion of OSCC. It appears that OPG inhibits RANKL signaling and suppresses mandibular bone invasion by OSCC cells. Consequently, RANKL/RANK inhibition is expected to be a therapeutic target to prevent bone invasion by OSCC cells.

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References


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