TUMORIGENESIS AND NEOPLASTIC PROGRESSION

RANKL Synthesized by Both Stromal Cells and Cancer Cells Plays a Crucial Role in Osteoclastic Bone Resorption Induced by Oral Cancer

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The molecular mechanisms underlying bone destruction by invading oral cancer are not well understood. Using IHC, we demonstrated that receptor activator of nuclear factor-κB ligand (RANKL)—positive fibroblasts and cancer cells were located at sites of bone invasion in human oral cancers. HSC3 and HO-1-N-1, human oral cancer cell lines, expressed RANKL and stimulated Rankl expression in the UAMS-32 murine osteoblastic cell line. We discriminated the roles of RANKL synthesized by stromal cells and cancer cells in cancer-associated bone resorption by using species-specific RANKL antibodies against murine RANKL and human RANKL, respectively. Osteoclastogenesis induced by the conditioned medium of HSC3 and HO-1-N-1 cells in a co-culture of murine bone marrow cells and UAMS-32 cells was inhibited by the addition of antibodies against either mouse or human RANKL. HSC3-induced bone destruction was greatly inhibited by the administration of anti-mouse RANKL antibody in a xenograft model. HO-1-N-1—induced bone destruction was inhibited by the administration of either anti-mouse or anti-human RANKL antibody. Bone destruction induced by the transplantation of human RANKL-overexpressing cells (HSC3-R2) was greatly inhibited by the injection of anti-human RANKL antibody. The present study revealed that RANKL produced by both stromal and cancer cells is involved in oral cancer—induced osteoclastic bone resorption. These results provide important information for understanding the cellular and molecular basis of cancer-associated bone destruction and the mechanism of action underlying RANKL antibody (denosumab) therapy. (Am J Pathol 2013, 182: 1890–1899; http://dx.doi.org/10.1016/j.ajpath.2013.01.038)

Cancer-associated bone destruction is caused by direct invasion of cancer cells or metastasis of cancer cells into the bone. The former is frequently observed in oral squamous cell carcinoma (OSCC). Although the destruction of bone caused by invading OSCC is associated with a poor prognosis,1,2 the molecular mechanisms underlying this process are not well understood. Osteoclasts are the cells responsible for bone resorption, and osteoclastogenesis is regulated by a complex signaling system that involves the receptor activator of nuclear factor-κB (RANK), the RANK ligand (RANKL), and osteoprotegerin (OPG).3 RANKL is expressed in osteoblasts and bone marrow stromal cells. It critically regulates the differentiation and function of osteoclasts by binding to its receptor RANK,3,5 which is expressed in osteoclast lineage cells. Furthermore, osteoblasts and...
stromal cells synthesize OPG, which is a decoy receptor for RANKL. Thus, the balance between RANKL and OPG expression levels is crucial for regulating osteoclast differentiation and function.

We have previously reported that fibroblastic stromal cells located at the interface between the cancer and bone play a critical role in osteoclastic bone resorption by producing RANKL, which had been stimulated by IL-6 and parathyroid hormone-related peptide, in OSCC.\(^6\) Expression of RANKL in osteoblast and stromal cells has been reported in breast cancer,\(^8\)–\(^10\) prostate cancer,\(^11\)\(^,\)\(^12\) and multiple myeloma.\(^13\) Cancer cells have been reported to express RANKL in several types of human malignant tumors, including breast cancer,\(^9\)\(^,\)\(^14\) prostate cancer,\(^15\) renal cancer,\(^16\) multiple myeloma,\(^17\) and oral cancer,\(^18\)\(^–\)\(^20\) suggesting that RANKL expressed by cancer cells is capable of inducing osteoclastogenesis; however, the precise role of RANKL produced by the tumor in cancer-associated bone destruction has not been well documented.

A fully human monoclonal antibody to human RANKL, denosumab, has been successfully developed and clinically applied to prevent osteoclastic bone resorption in patients with osteoporosis\(^21\) and metastatic bone lesions,\(^22\)\(^–\)\(^24\) but the precise target of this drug in cancer-induced bone destruction has not been well elucidated. It is important to clarify the roles of RANKL produced by both stromal cells and cancer cells during cancer-associated bone destruction. These results will provide more definitive information for establishing RANKL antibody as a mechanism-based drug. In the present study, we investigated the roles of RANKL produced by stromal cells and cancer cells in cancer-associated bone destruction. To discriminate the roles of RANKL derived from stromal cells and cancer cells, we used species-specific RANKL antibodies in a mouse xenograft model of transplantation of human oral cancers. We show that RANKL produced by both stromal cells and cancer cells is involved in osteoclastic bone resorption induced by oral cancer and RANKL antibody inhibits cancer-associated bone resorption by neutralizing RANKL activity released by both stromal cells and cancer cells. These results provide important information for elucidating the mechanism of action underlying RANKL antibody therapy.

Materials and Methods

Antibodies

Rabbit anti-human RANKL polyclonal antibody (sc-9073; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for immunohistochemical (IHC) analysis. Rat anti-mouse RANKL neutralizing monoclonal antibody (clone OYC1) with no cross-reactivity with human RANKL\(^25\) and mouse anti-human RANKL neutralizing monoclonal antibody with no cross-reactivity with mouse RANKL\(^26\) were generated as described previously.

IHC Staining

The fixed-surgical specimens were decalcified in 10% EDTA at 4°C and embedded in paraffin. For IHC staining, the sections were prepared from 13 cases of gingival squamous cell carcinomas, and they were pretreated by microwaving in 0.01 mol/L citric acid for 1 hour at 80°C, followed by quenching of endogenous peroxidase activity by incubation in 3% hydrogen peroxide solution for 30 minutes. The specimens were treated with 100 mmol/L glycine solution (pH 3.0) for 20 minutes before the blocking step. The sections were incubated overnight at 4°C with 1:50 rabbit anti-human RANKL antibody. After washing with PBS, the sections were incubated with peroxidase-conjugated secondary antibody (Envision+ Dual Link System Peroxidase Kit; Dako, Glostrup, Denmark) for 1 hour. Diaminobenzidine was used as a chromogen. For negative controls, nonimmunized rabbit IgG or PBS was used as a substitute for the primary antibody. We also performed antibody absorption test by adding excess human recombinant RANKL (ratio of the antibody and the RANKL was 1 mol:20 mL).

Cell Culture

The human OSCC cell lines, HSC3, and HO-1-N-1 were obtained from the Japanese Collection of Research Resources Cell Bank (Osaka, Japan). Human RANKL-overexpressing HSC3 cells (HSC3-R2) were generated by transfection with qCXN2-Flag-RANKL vector as described previously.\(^20\) The osteoblast/stromal cell line UAMS-32, which is a capable of inducing osteoclastogenesis, was kindly provided by O’Brien et al.\(^27\) ST-2 cells, mouse bone marrow-derived stromal cells, were purchased from RIKEN BioResource Center (Tukuba, Japan). RAW264 cells were purchased from RIKEN Bioresource Center (Tsukuba, Japan). The HSC3 cells, UAMS-32 cells, and RAW264 cells were maintained in α-modified minimum essential medium (α-MEM; Nacalai Tesque, Inc., Kyoto, Japan). The ST-2 cells were maintained in RPMI 1640 medium. The HSC3-R2 cells were cultured in complete Dulbecco’s modified Eagle’s medium containing 0.2 mg/mL of G418. All media except for the conditioned medium (CM)

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Table 1 Primer Sequences Used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tr>
<td>RANKL</td>
<td>5′-AGGACGCCAGATGGATCCTAA-3′</td>
<td>3′-ATCCACCACATCGTTTCTCTG-5′</td>
</tr>
<tr>
<td>OPG</td>
<td>5′-AGGAATGCCAACACAGCA-3′</td>
<td>3′-FCCTTTGGTCGCCAGGCAA-5′</td>
</tr>
<tr>
<td>Rankl</td>
<td>5′-ATGATGGAAAGCATGTCTT-3′</td>
<td>3′-CCAGAGGGCAGAGATGTTT-5′</td>
</tr>
<tr>
<td>Ogp</td>
<td>5′-CTGCTGGGAGAGAGATCAG-3′</td>
<td>5′-CTGCTGTGGAAGAGATCAG-5′</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5′-GTAACCCCGTGAGAAGCTTATT-3′</td>
<td>3′-CCATCCAATCGGTAGCAG-5′</td>
</tr>
</tbody>
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contained 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 50 U/mL of penicillin G, and 50 μg/mL of streptomycin.

Preparation of CMs from Cancer Cell Lines

All of the cancer cells were grown to confluence in 100-mm dishes. After washing with PBS 3 times, the cells were cultured for an additional 48 hours in 4 mL of serum-free α-MEM. The collected culture supernatants were centrifuged at 250 × g for 5 minutes, filtered using a 0.22-μm filter unit, and kept at −80°C until use. These supernatants were used as the CM.

Measurements of RANKL and OPG in the CM of Cancer Cell Lines

The concentrations of RANKL and OPG in the CM or serum were determined using an ampli-soluble (sRANKL) enzyme-linked immunosorbent assay (ELISA) kit and an osteoprotegerin ELISA kit (Biomedica, Wien, Austria), respectively.

RT-PCR Analysis

For RT-PCR analysis, total RNA extracted from the cultured cells was reverse-transcribed into cDNA using a First-Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Indianapolis, IN). The mRNA expression levels were quantified by real-time RT-PCR using a Light-Cycler System (Roche Diagnostics, Basel, Switzerland) and a Platinum SYBR Green quantitative PCR SuperMix UDG kit (Invitrogen, Carlsbad, CA) with the specific primers listed in Table 1. The relative expression level of each mRNA was normalized to the 18S rRNA expression level.

Osteoclast Formation

To investigate the direct effects of the CM on osteoclast progenitors, we used RAW264 cells.28 After culture of these cells in 96-well plates for 24 hours, the medium was changed to 0.2 mL of α-MEM containing 0%, 5%, 10%, or 20% of CM derived from HSC3, HSC3-R2, or HO-1-N-1 cells in the presence of 20 μmol/L MEK1 inhibitor (PD98059; Cell Signaling Technology, Inc., Danvers, MA). We also co-cultured 3 × 105 RAW264 cells per well with HSC3, HO-1-N-1, and 1 × 105 HSC3-R2 cells per well to assess the direct effects of cancer cells on osteoclast formation in the absence of osteoblast and stromal cells.

To explore the effects of cancer cells on osteoclast formation through stromal cells, bone marrow stromal cells (BMCs) obtained from the tibiae of 4- to 6-week-old male ddY mice were co-cultured with UAMS-32 cells in 0.5 mL of α-MEM containing various concentrations of the CM derived from HSC3, HSC3-R2, or HO-1-N-1 cells in 24-well plates. In each experiment, osteoclast formation was identified by tartrate-resistant acid phosphatase (TRAP) staining as described previously.7

![Figure 1](image1.jpg)  
**Figure 1** Histologic characteristics of the interface between oral cancers and the resorbing bone. A: Low magnification of a H&E-stained section. Numerous cancer nests are observed around the resorbing bone. B: IHC for human RANKL. Note that immunoreaction (brown) is observed in both stromal cells and cancer cells. Immunoreaction was diminished by incubation of an excess amount of recombinant RANKL with the primary antibody (A). Scale bars: 500 μm (A); 50 μm (B–D). Ca, cancer nest; Bo, bone.

![Figure 2](image2.jpg)  
**Figure 2** Stimulations of RANKL expression in stromal cells and osteoclastogenesis by cancer cells. Effects of CM derived from HSC3 and HO-1-N-1 cells on Rankl (A) and Opg (B) expression in the mouse stromal cell line UAMS-32. A and B: UAMS-32 cells were cultured for 24 hours with various concentrations of CMs derived from HSC3 or HO-1-N-1 cells, and then mRNA expression of Rankl (A) and Opg (B) was determined by RT-PCR. *P < 0.05, **P < 0.01, significantly different from cultures without CM supplement. C and D: Effects of the CM derived from HSC3 and HO-1-N-1 cells on osteoclastogenesis in co-culture of bone marrow cells and UAMS-32 cells. C: TRAP staining. D: Number of TRAP-positive cells. **P < 0.01, significantly different from cultures without CM supplement. Data are representative of at least three independent experiments (A–D).
Xenograft Experiments of Human Oral Cancer Cells into Athymic Mice

We injected $5 \times 10^5$ OSCC cells per mouse onto the periosteal region of the parietal bone in athymic mice (BALB/c Slc-nu/nu) as previously described. Immediately after transplantation of the cancer cells, 100 µg/100 µL per mouse-rat anti-mouse RANKL neutralizing monoclonal antibody or 100 µg/100 µL per mouse-mouse anti-human RANKL neutralizing monoclonal antibody was injected i.p. or s.c. at tumor-injected sites. Mice xenografted with HSC3 cells or HSC3-R2 cells were sacrificed on day 14, whereas the mice xenografted with HO-1-N-1 cells were sacrificed on day 21. Serum levels of rat IgG were measured by ELISA using a rat IgG ELISA (quantitation set; Bethyl Laboratory, Inc., Montgomery, TX). We measured TRAP5b by a mouse TRAP assay kit (Immunodiagnostic Systems, Boldon, UK). After fixation of the samples in 4% paraformaldehyde, soft X-ray photographs and microtomography images were taken. Data obtained from the former analysis were used to calculate the bone destruction area using a software package (ImageJ version 1.43; NIH, Bethesda, MD). The tissues were embedded in paraffin after decalcification with 20% EDTA at 4°C.

To assess the effects of RANKL antibody injection on bone structure in femur, we analyzed trabecular bone in femur by a micro–computed tomography (micro-CT) system (SMX-100CT; Shimadzu, Kyoto, Japan). The specimens were treated with sodium hypochlorite and hydrogen peroxide for removing soft tissue. The images consisted of 1200 sections with a voxel size of 8 µm in all three axes. All experimental procedures were reviewed and approved by the Animal Care and Use Committees and the Ethical Review Committees at Tokyo Medical and Dental University.

Statistical Analysis

All results are expressed as means ± SD for all data. Significance of differences was determined by Student’s t-test or analysis of variance with a Dunnett’s test. $P < 0.05$ was considered significant.

Results

Histologic Characteristics and Expression of RANKL at the Bone Invasion Region in Surgical Specimens of Human Oral Cancers

Varying amounts of fibrous connective tissues were observed between the tumor cells and bone in surgical specimens obtained from gingival squamous cell carcinoma patients (Figure 1). We occasionally detected cancer nests located close to the resorbing bone surface (Figure 1). The IHC staining revealed that many fibroblastic cells located between cancer nests and the resorbing bones were positive for RANKL (Figure 1B). Cancer cells were also positive for RANKL (Figure 1B). Among 13 cases of OSCC examined, RANKL-positive stromal cells were observed in 10 cases, and RANKL-positive cancer cells were observed in 11 cases. Immunoreaction was diminished by incubation of an excess amount of recombinant RANKL with the primary antibody (Figure 1C) and incubation with nonimmunized rabbit IgG instead of the primary antibody (Figure 1D).

![Figure 3](image)

**Figure 3** The expression of RANKL and OPG in human cancer cell lines. The expression of RANKL (A) and OPG (D) mRNA in oral cancer cells. Protein levels of RANKL in cell lysates (B) and culture supernatants (C) were determined by ELISA. E and F: Protein levels of OPG protein levels in cell lysates and culture supernatants, respectively. *$P < 0.05$, **$P < 0.01$. Data are representative of at least three independent experiments (A–F). G: Induction of osteoclastogenesis by co-culture of RAW264 cells with HSC3 cells (RAW+HSC3) or HO-1-N-1 cells (RAW+HO-1-N-1). Osteoclastogenesis was identified by TRAP staining.
Induction of Osteoclastogenesis by Oral Cancer Cells through RANKL Expression in Stromal Cells

We confirmed whether the oral cancer cell lines, HSC3 and HO-1-N-1, induced RANKL expression in osteoblast and stromal cells using a mouse osteoblast and stromal cell line, UAMS-32. The CMs derived from these oral cancer cell lines stimulated the expression of Rankl (Tnfsf11) mRNA. HSC3 cells greatly induced Rankl expression after the addition of 10% and 20% of the CM, and HO-1-N-1 cells induced Rankl expression after the addition of only 20% of the CM (Figure 2A). The expression of Opg mRNA was not significantly altered by treatment with CMs derived from HSC3 cells and HO-1-N-1 cells (Figure 2B). Similar effects were observed in ST-2 cells by adding CMs isolated from HSC3 cells and HO-1-N-1 cells (Supplemental Figure S1). We next investigated the expression of RANKL (TNFSF11) in human OSCC cell lines. Although the expression level of OPG mRNA in HO-1-N-1 cells was significantly higher than that in HO-1-N-1 cells (Figure 2D), the OPG protein levels in cell lysates were almost similar among the cells and HO-1-N-1 cells (Supplemental Figure S1). We next investigated the osteoclast-inducing activity of these CMs using a co-culture of BMCs and UAMS-32 cells. Both CMs derived from HSC3 cells and HO-1-N-1 cells generated TRAP-positive multinucleated cells (MNCs) (Figure 2C). The CM derived from HSC3 cells and HO-1-N-1 cells dose dependently increased the number of TRAP-positive MNCs (Figure 2D). These results indicate that HO-1-N-1 cells are capable of inducing osteoclastogenesis by stimulating the expression of Rankl in osteoblast and stromal cells as HSC3 cells did in ST-2 cells in our previous report.7

RANKL Production by Oral Cancer Cells and Its Role in Osteoclastogenesis

We next investigated the expression of RANKL (TNFSF11) and OPG (TNFSF11B) in human OSCC cell lines. Although the expression level of RANKL mRNA in HSC3 cells was higher than that in HO-1-N-1 cells (Figure 3A), RANKL protein levels in cell lysates were almost similar among the cell lines tested by ELISA (Figure 3B). The RANKL protein level in the culture supernatant of HO-1-N-1 cells was significantly higher than that of HSC3 cells (Figure 3C). The mechanism underlying the uncoupling expression levels of RANKL mRNA and its protein in these cancer cells is further investigated. The expression level of OPG mRNA in HO-1-N-1 cells was significantly higher than that in HSC3 cells (Figure 3D). The OPG protein levels in cell lysates and culture supernatants of HSC3 cells were significantly lower compared with those of HO-1-N-1 cells (Figure 3, E and F).

We investigated whether HSC3 and HO-1-N-1 cells can stimulate osteoclastogenesis in the absence of osteoblast and stromal cells using an osteoclast precursor cell line, RAW264. A small number of TRAP-positive MNCs were observed in co-culture of RAW264 cells with HSC3 cells but not with HO-1-N-1 cells (Figure 3G). This finding might be due to the higher level of OPG production in HO-1-N-1 cells than that of HSC3 cells (Figure 3, D–F). Further, these results suggest that RANKL produced by HSC3 cells and HO-1-N-1 cells is involved in osteoclastogenesis; however, the amounts of RANKL and OPG produced by these cell lines was critical to effectively induce osteoclastogenesis in RAW264 cells.

Discrimination of the Roles of RANKL Produced by Stromal Cells and Cancer Cells in in Vitro Osteoclastogenesis

To discriminate the roles of RANKL produced by stromal cells and cancer cells, we investigated the effects of neutralizing antibodies specific to mouse RANKL and human RANKL, respectively, on the formation of TRAP-positive MNCs using co-cultures of mouse BMCs and UAMS-32 cells. Supplementation with 10% CM derived from HSC3 cells greatly increased the number of TRAP-positive MNCs, and this activity was inhibited by the addition of antibodies against mouse RANKL and human RANKL (Figure 4A). Figure 4, B and C, summarizes the dose-dependent effects of antibodies against human RANKL and mouse RANKL on the formation of TRAP-positive MNCs in the co-culture system. These antibodies dose dependently inhibited the number of TRAP-positive MNCs; however, the inhibitory effect of the anti-mouse RANKL antibody was greater than that of the anti-human RANKL antibody.

**Figure 4** The effects of RANKL antibodies on osteoclastogenesis in co-culture of bone marrow cells and UAMS-32 cells. A: Osteoclastogenesis was induced by the addition of 20% CM derived from HSC3 cells to the co-culture of BMCs and UAMS-32 cells in the presence or absence of various concentrations of anti-human and mouse RANKL antibodies and assessed by TRAP staining. B and C: Quantitative analysis of the effects of RANKL antibodies on the formation of TRAP-positive MNCs induced by supplementation with the CM derived from HSC3 cells (B) or HO-1-N-1 cells (C). *P < 0.05, **P < 0.01, significantly different from cultures treated with each CM in the absence of any antibodies. Data are representative of at least three independent experiments (B and C).
anti-human RANKL antibody in both co-cultures supplemented with CMs derived from HSC3 cells and HO-1-N-1 cells (Figure 4, B and C). The effect of simultaneous addition of both antibodies was the same as that achieved by the addition of anti-mouse RANKL antibody alone (Figure 4, B and C).

**In Vivo Application of RANKL Antibodies to Prevent Bone Destruction Induced by Cancer Cells in a Xenograft Model**

We compared the effects of two methods of antibody administration, i.p. injection and s.c. local injection, using rat anti-mouse RANKL neutralizing antibody because the local injection would be useful for the application of the antibody to oral cancer patients. After 2 weeks of i.p. or s.c. local injections of rat anti-mouse RANKL neutralizing antibody, substantial levels of rat IgG were detected in the sera of both groups (Figure 5A). The administration of anti-mouse RANKL antibody by either i.p. or s.c. injection into HSC3-transplanted mice markedly inhibited the bone destruction (Figure 5, B and C), serum levels of TRAP5b (Figure 5D), and number of osteoclasts on the bone surface at the bone resorbing area (Figure 5, E and F). These results indicated that both systemic and local injections of anti-mouse RANKL antibody inhibited the bone destruction induced by HSC3 cells. Therefore, we used the s.c. local injection of the antibody for the following experiments. The s.c. local injection of the mouse RANKL antibody increased trabecular bone mass in femur by micro-CT analysis (Supplemental Figure S2).

**Discrimination of the Roles of RANKL Produced by Stromal Cells and Cancer Cells in Cancer-Associated Bone Destruction in a Xenograft Model**

The bone destruction induced by transplantation of HSC3 cells was almost completely blocked by the injection of anti-mouse RANKL antibody, whereas the injection of anti-human RANKL antibody had no such effect (Figure 6, A and B). These results suggest that RANKL synthesized by stromal cells plays an indispensable role in the bone destruction induced by HSC3 cells. In contrast, injection of anti-human RANKL antibody significantly inhibited the bone destruction induced by transplantation of HO-1-N-1 cells, although this effect was smaller than that of anti-mouse RANKL antibody (Figure 6, C and D). Simultaneous injection of both anti-human and anti-mouse RANKL antibodies almost completely inhibited the bone destruction induced by HO-1-N-1 cells (Figure 6, C and D). These results suggest that RANKL synthesized by both stromal cells and cancer cells plays a crucial role in the bone destruction induced by HO-1-N-1 cells. Taken together, these findings indicate that the participation of RANKL synthesized by stromal cells may be common mechanism underlying bone destruction by oral cancers; however, the role of RANKL produced by cancer

![Figure 5](image-url)
cells may vary among oral cancers, depending on the level of RANKL production.

Osteoclastogenesis and Bone Resorption Induced by RANKL-Overexpressing HSC3 Cells

We next conducted in vitro and in vivo experiments using human RANKL-overexpressing HSC3 cells (HSC3-R2) to clarify the role of RANKL produced by cancer cells. The expression level of RANKL in HSC3-R2 cells was substantially higher than that in the maternal HSC3 cells (Figure 7A). The HSC3-R2 cells also secreted a large amount of RANKL protein into the culture supernatant compared with that produced by maternal HSC3 cells (Figure 7B). The HSC3-R2 cells greatly increased the number of TRAP-positive MNCs compared with that induced by the maternal HSC3 cells when they were co-cultured with RAW264 cells (Figure 7, C and D). The CM derived from HSC3-R2 cells increased the number of TRAP-positive MNCs in RAW264 cells compared with that induced by CM derived from HSC3 cells (Figure 7E). These results indicate that RANKL synthesized by cancer cells is capable of inducing osteoclastogenesis in vitro when a substantial amount of the RANKL protein is produced. The CM derived from HSC3-R2 cells significantly increased the expression of Rankl mRNA in UAMS-32 cells (Figure 7F) and inhibited the expression of Opg mRNA (Figure 7G) at a concentration of 10%. The CM from HSC3-R2 generated a larger number of TRAP-positive MNCs in co-culture of BMCs and UAMS-32 cells than that derived from HSC3 cells (Figure 7H).

Xenografting of HSC3-R2 cells induced bone destruction 2 weeks after transplantation (Figure 7, I and J). The injection of anti-human RANKL antibody greatly inhibited the bone destruction induced by HSC3-R2 cells, whereas the injection of anti-mouse RANKL antibody did not (Figure 7, I and J), suggesting that the expression of RANKL in stromal cells might be different, depending on cancer cells transplanted. Simultaneous injection of anti-human RANKL and anti-mouse RANKL antibodies almost completely inhibited the bone destruction induced by HSC3-R2 cells (Figure 7, I and J). These results imply that RANKL synthesized by cancer cells plays a major role in the osteoclastic bone resorption induced by RANKL-overexpressing HSC3-R2 cells.

Finally, we evaluated the serum levels of human RANKL in HSC3-R2 transplanted mice with or without RANKL antibody treatments. As shown in Figure 7K, we detected the substantial level of serum RANKL in the mice without RANKL antibody treatment and with anti-mouse RANKL antibody treatment. In contrast, serum RANKL levels in the mice with anti-human RANKL treatment and simultaneous treatment with anti-mouse RANKL and anti-human RANKL antibodies were extremely low.

Discussion

We previously reported that RANKL synthesized by stromal cells plays an important role in osteoclastic bone resorption during oral cancer—associated bone destruction,7 as reported in cases of multiple myeloma,30 breast cancer,8–10 and prostate cancer.11,12 Production of RANKL by tumor cells has also been reported in multiple myeloma,17 breast cancer,9,14 renal cancer,16 prostate cancer,15 and oral cancers.18–20 Although these results suggest that RANKL produced by tumor cells is also involved in cancer-associated bone destruction, it has not been demonstrated...
that the RANKL produced by tumor cells actually participates in cancer-associated bone resorption. In the present study, we first recognized the expression of RANKL in human OSCC cases and cell lines. The CMs derived from HSC3 cells but not HO-1-N-1 cells failed to induce osteoclastogenesis in RAW264 cells (Figure 7E and unpublished data), but co-culture of RAW264 cells with HSC3 cells generated TRAP-positive MNCs, whereas co-culture with HO-1-N-1 cells did not. These results might be due to excess production of OGP in HO-1-N-1 cells (Figure 3F). Alternatively, the amounts of sRANKL produced by these OSCC cells were insufficient to directly induce osteoclastogenesis in RAW264 cells. Indeed, CM derived from HSC3-R2 cells, which overexpressed human RANKL, induced TRAP-positive MNCs in RAW246 cells (Figure 7E). These results indicate that RANKL synthesized by cancer cells is capable of inducing osteoclastogenesis directly in the absence of osteoblasts and stromal cells in vitro when substantial RANKL protein was produced.

We measured the concentrations of RANKL in supernatants of OSCC cell lines by using a sRANKL human ELISA kit, which consisted of human OPG as an immobilized capture component and biotinylated human RANKL antibody. Because OSCC cell lines also produced substantial amounts of OPG, almost binding sites of RANKL might be occupied by OPG in this assay system. Therefore, the concentration of sRANKL in CM derived from HSC3-R2 cells, which could induce TRAP-positive MNCs in RAW246 cells, was 5 pmol/L (equivalent to 100 pg/mL). Because 10 to 100 ng/mL of sRANKL was usually required to induce osteoclastogenesis in RAW246 cells, the amounts of sRANKL in CM derived from HSC3-R2 cells might be underestimated.

Several in vivo experiments have been conducted to explore the role of RANKL in cancer-associated bone resorption by using anti–bone resorption agents. Morrissey et al.13 applied an antibody specific to human RANKL that did not cross-react with murine RANKL to distinguish the role of RANKL synthesized by human cancer cells from that synthesized by mouse host cells in the xenograft bone metastasis model by using C4-2 human prostate cancer cells. They demonstrated that the osteolytic response was not inhibited after the administration of the human RANKL-specific monoclonal antibody, and suggested that murine...
RANKL, which had been produced by host stromal cells, was a major mediator of bone resorption induced by C4-2 human prostate cancer cells, but their study did not confirm the role of RANKL synthesized by mouse cells. Several groups applied OPG to prevent RANKL-induced bone resorption in xenograft experiments by transplantation of human tumor cells into athymic mice. Although the administration of OPG inhibited cancer-induced bone resorption, these experiments were unable to distinguish the roles of RANKLs derived from cancer cells (human) or stroma cells (mouse) in cancer-associated bone resorption because OPG blocked RANKLs synthesized by both human and mouse cells. To understand the precise mechanism underlying the roles of RANKL in cancer-associated bone resorption, it is important to discriminate the roles of RANKLs synthesized by stromal cells from RANKL synthesized by cancer cells.

To discriminate the roles of RANKL synthesized by stromal cells and cancer cells, we applied species-specific RANKL antibodies against the murine RANKL and human RANKL, respectively. In our in vitro and in vivo experiments, the cancer cells were always derived from humans, and the osteoblastic and stromal cells were derived from mice. When we applied these antibodies to our experiments, the mouse RANKL antibody specifically abolished RANKL activity synthesized by the stromal cells and the human RANKL antibody specifically inhibited RANKL activity produced by the cancer cells. Our results clearly revealed that RANKLs produced by both stromal cells and cancer cells participate osteoclastic bone resorption during OSCC-associated bone destruction. These antibodies will be useful tools for investigating the precise roles of RANKL in cancer-associated bone resorption induced by various tumors in xenograft models.

The function of RANKL produced by cancer cells during cancer-associated bone resorption might be influenced by two factors: the expression level of RANKL in each case of cancer and the localization of RANKL-producing cancer cells. We have previously reported that the mRNA expression of RANKL greatly varied among different cases of human OSCC. Others also reported variation in RANKL expression in oral, breast, and renal cancers. These results suggest that the involvement of cancer-derived RANKL in osteoclastic bone resorption may be dependent on its expression level in each case of cancer. Regarding the distribution of RANKL-producing cancer cells, it is likely that such cells adjacent to the bone surface effectively stimulate osteoclastic bone resorption. We have demonstrated that RANKL-producing cancer cells are occasionally distributed in the vicinity of the resorbing bone surface at the site of bone invasion. In such cases, RANKL produced by cancer cells may participate in osteoclastic bone resorption.

We revealed that RANKL produced by cancer cells flowed into the bloodstream in an HSC3-R2 xenograft model (Figure 7K). The serum levels of RANKL were well correlated with bone resorption activity in this model. These results suggest that serum levels of RANKL reflect bone resorption activity in patients with OSCC and prompted us to measure the serum levels of RANKL in human patients with OSCC.

Because bone invasion in OSCC is a critical factor for poor prognosis, RANKL antibody therapy may be useful to prevent osteoclastic bone resorption in cases of OSCC-associated bone destruction. Our experiments demonstrated that the local injection of RANKL antibody exerted similar inhibitory effects on bone resorption as systemic injection, suggesting the clinical application of local RANKL antibody injection to prevent or inhibit OSCC-associated bone destruction in human cases.

Although the results of this study provide important information on the mechanism of action of RANKL antibody (denosumab) therapy to prevent cancer-associated bone resorption, we previously reported that RANKL expression in OSCC promoted epithelial mesenchymal transition and tumor progression. RANK/RANKL signaling has been reported to regulate cancer cell migration and bone metastasis and tumor initiation, progression, and metastasis in human mammary epithelial cells inducing stemness and epithelial-mesenchymal transition. Further studies are necessary to investigate the role of RANK/RANKL signaling in tumor progression of OSCC other than bone resorption using the species-specific RANKL antibody. The findings in these studies will provide important information for understanding the precise mechanism underlying cancer therapy using RANKL antibody.

**Supplemental Data**

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.01.038.

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