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Oral Squamous Carcinoma Cells Secrete RANKL Directly Supporting Osteolytic Bone Loss

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Abstract

OBJECTIVE—Local invasion of bone is a frequent complication of oral squamous cell carcinoma (OSCC). Development of these osteolytic lesions is mediated by osteoclasts. Receptor activation of NF- κ B ligand (RANKL) signaling, counteracted by osteoprotegerin (OPG), regulates osteoclastogenesis. Previous studies in rodent models have demonstrated that inhibition of RANKL decreases tumor growth and lesions within bone. However, the contributory role of OSCC cells to this disease process has yet to be defined.

METHODS—RANKL expression was assessed in a panel of OSCC cell lines by qPCR, flow cytometry, and ELISA. Induction of osteoclastogenesis was assessed by co-culture with macrophages or with OSCC-derived conditioned medium. In an animal model of bone invasion, nude mice were injected intratibially with UMSCC-11B cells expressing a RANKL luciferase promoter to detect tumor-derived RANKL activity. Osteolytic lesions were analyzed by X-ray, micro-CT, and histological methods. RANKL expression was assessed in human OSCC tissues by immunohistochemistry.

RESULTS—We demonstrated that OSCCs express varied levels of all RANKL isoforms, both membrane-bound and soluble RANKL. Both co-culture and treatment with OSCC-conditioned media induced osteoclastogenesis. In mice, we demonstrated human RANKL promoter activity during bone invasion. Over the course of the experiment, animals suffered osteolytic lesions as RANKL-driven luciferase expression increased with time. After 8 weeks, human-derived RANKL was detected in areas of bone resorption by immunohistochemistry. Similar epithelial RANKL expression was detected in human OSCC tissues.

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CONCLUSION—These data demonstrate the ability of OSCCs to produce RANKL, directly altering the tumor microenvironment to increase osteoclastogenesis and mediate local bone invasion.

Introduction

Oral squamous cell carcinomas represent the majority of oral cavity cancers^{1,2}. Despite diagnostic and therapeutic advances, the 5-year disease-specific survival for OSCC patients has not significantly improved over the past several decades and remains near 60% for all stages of disease.³ These tumors frequently invade the mandible and maxilla, contributing to severe morbidities and poorer prognoses.^{4–6} Previous reports have suggested osteoclasts mediate skeletal destruction in these osteolytic lesions, rather than tumor cells. The RANK/RANKL/OPG signaling axis regulates osteoclastogenesis and bone resorption.^{7,8} Receptor activator of NF- κ B ligand (RANKL) is critical for osteoclast differentiation, function, and survival.^{9,10} RANKL exerts its activity through binding its surface receptor RANK on the surface of osteoclast precursors.¹¹ As a soluble decoy receptor, OPG inhibits osteoclastogenesis by competing with RANK for RANKL. Alterations in the balance between OPG and RANKL levels in sites of bone metastases have been observed to stimulate osteoclastogenesis.¹² Experiments with total blockade of RANKL signaling in animal models have demonstrated an absolute dependence of tumor-induced osteolysis on RANKL signaling.^{13,14} In a murine model of mandibular bone invasion, mRNA expression of the cytokines interleukin-6 (IL-6), tumor necrosis factor (TNF)- α , and parathyroid hormone-related protein (PTHrP) associated with osteoclast activation are present in the tumor tissue.¹⁵ OSCC cell lines have been shown to express these osteotrophic factors, including IL-6, TNF- α , PTHrP, and IL-11, which stimulate RANKL production in stromal cells such as osteoblasts, to indirectly mediate osteoclast differentiation and permit tumor invasion.^{16–18} However, the direct contribution of OSCC to bone invasion through RANKL expression has remained undefined. The aim of this study is to investigate the role of tumor-derived RANKL in OSCC-mediated bone destruction. We provide experimental evidence showing that RANKL, derived from OSCCs, stimulates osteoclastogenesis in vitro and bone invasion in vivo with similar expression patterns in human tissues.

Materials and Methods

Reagents

Recombinant human IL-1, IL-6, TNF- α , PTH, RANKL, M-CSF, and antibodies against IL-6, TNF- α were purchased from R&D Systems Inc. (Minneapolis, MN). PTH (hPTH-(1–34)) was purchased from Bachem (Torrance, CA). RANKL ELISA kit and flow cytometry antibodies were purchased from BioVision, Inc. (Mountain View, CA). Anti-Human RANKL (Clone 366) for murine immunohistochemistry was obtained from Amgen, Inc. through a Material Transfer Agreement. Anti-RANKL (N-19) antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used for human immunohistochemistry. An oral squamous cell carcinoma tissue microarray was obtained from US Biomax, Inc. (Rockville, MD). Frozen specimens of OSCC and adjacent normal tissue from patients with clinical record of local bone invasion were obtained from the Hollings Cancer Center Tissue Biorepository using protocols approved by the institutional review board of the Medical University of South Carolina.

Cell culture

The cell lines Saos-2, HaCaT, HeLa, UMSCC-11A, UMSCC-11B, UMSCC-22A, UMSCC-22B, UMSCC-74A, UMSCC-74B, and OSCC3 were cultured in DMEM, PC3 cells in RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY). Human Osteoclast

Precursors were obtained as a source of human peripheral blood mononuclear cells (hPMBC) and cultured in Poietics™ media per manufacturer's instructions (Lonza, North America). All media was supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), 100IU/mL penicillin, 100 g/mL streptomycin, and 50 g/mL L-glutamine in a humidified atmosphere of 5% CO₂ at 37°C.

RNA isolation and PCR

Total RNA was isolated using TRIzol® reagent, from which complementary DNA was transcribed by random hexamer priming with TaqMan™ Reverse Transcription reagents (Applied Biosystems, Inc., Foster City, CA). Gene of interest expression was analyzed using Assay on Demand™ primer probe sets and the ABI 7500 (Applied Biosystems). Primer pairs used for RANKL (accession# AF019048): sense 5'-CAGCACTCACTGCTTTTATAGAATCC-3'; antisense 5'-AGCTGAAGATAGTCTGTAGGTACGC-3'; GAPDH (accession# NM002046): sense 5'-CACCATGGAGAAGGCCGGGG-3'; antisense 5'-GACGGACACATTGGGGTAG-3'. For RANKL isoform expression, pan-specific PCR primers were designed that would recognize all 3 RANKL isoforms: sense 5'-GGAGCGGGAGAGGGAGGAGAGC-3'; antisense 5'-CCCCGATCATGGTACCAAGAGGAC-3'. RANKL isoform PCR products were resolved in 1.5% agarose gels and stained with 0.5µg/mL ethidium bromide with the predicted sizes: hRANKL1 (620bp), hRANKL2 (577bp), and hRANKL3 (401bp). Images and densitometry measurements were obtained with a digital documentation system (Gel Doc XR, Bio-Rad). RT-PCR Reactions were performed in triplicate.

ELISA and Flow Cytometry

Soluble human RANKL was measured in culture medium by an ELISA kit according to the manufacturer's protocols (BioVision, Inc., Mountain View, CA). The concentration of cytokines was normalized to protein concentrations from whole cell lysates (Bradford Assay, BioRad). Surface RANKL expression was determined by flow cytometry using anti-RANKL antibody (BioVision, Inc.) or IgG isotype control (R&D Systems) and allophycocyanin (APC)-conjugated anti-Alexa488 secondary antibody on a FACSCalibur 1 (BD Biosciences, San Jose, CA).

Luciferase reporter assays

Stable UMSSC-11B cell lines were generated following co-transfection with pGL3 containing 1974bp of the human RANKL gene promoter along with pcDNA3.1neo, as described previously¹⁹. G418-resistant clones were screened using whole cell lysates with Dual Luciferase Assay (Promega Corp., Madison, WI) on a LMaxII 384 luminometer (Molecular Devices, Sunnyvale, CA). One clonally expanded stable RANKL proximal reporter cell line, UMSSC-11b-RANKL, was treated with indicated cytokines and hormones for 24hrs, and luciferase activity was measured.

Co-Culture Osteoclastogenesis Assays

SCC cells were plated on 6-well dishes before a 24h serum starvation followed by a 48h treatment with cytokines or hormones before RAW264.7 macrophages were plated. RANKL (50 ng/mL) and OPG (100 ng/mL) were used as positive and negative controls for RAW264.7 osteoclast differentiation. The co-cultures were maintained for 6 days before TRAP staining, as described on the Technical Bulletin #445, BD BioSciences, using reagents from Sigma-Aldrich. Cells were fixed with 10% glutaraldehyde for 15 minutes, washed with PBS and stained for 10 minutes with the staining solution, pH 5.0 (50 mM Sodium Acetate buffer, 30 mM Sodium Tartrate, 0.1 mg/ml Naphtol AS-MX phosphate, 0.1% Triton X-100 and 0.3 mg/ml of Fast Red Violet LB stain). These cells were observed

on an inverted microscope at 40X magnification using bright field, and the numbers of stained cells containing three or more nuclei were counted.

Animal model

All animal-related work was performed in accordance with the National Institute of Health guidelines. The institutional animal care and use committee of the University of Michigan approved all experimental protocols. UM-SCC-11B-RANKL-Luc cells were grown to 70% confluence prior to injection. Six week-old athymic nude mice (NCR-nu/nu strain, NCI, Frederick, MD) weighing approximately 18–25g, were anesthetized via intraperitoneal injection with 100 mg/kg ketamine and 10 mg/kg xylazine. Cells were suspended in 0.2 mL of DMEM and injected intratibially. Every two weeks up to 8 weeks, promoter activity was assessed by bioluminescence following intraperitoneal injection of D-luciferin substrate in PBS at 150 mg/kg body weight, using the Xenogen IVIS imaging system (Xenogen Corp., Alamenda, CA). Faxitron radiographs were also used to image tibias over the 8 week time frame at indicated time points.

Microcomputed tomography

Mice tibiae (n = 4) were scanned by a cone beam microcomputed tomography system (GE Healthcare BioSciences, Chalfont St. Giles, UK). Each scan was reconstructed at a mesh size of 18 μ m, and three-dimensional digitized images were generated for each specimen. Using GEHC MicroView software version viz.+2.0build 0029 (GE Healthcare BioSciences), the images were rotated into a standard orientation and threshold to distinguish between mineralized and non-mineralized tissue. For each specimen, a gray-scale voxel value histogram was generated to determine an optimal threshold value.

Histological Analysis

Formalin-fixed specimens were decalcified in a 10% EDTA solution for 2 weeks at 4°C, with fresh EDTA solution added three times per week. For immunohistochemical staining, 4 μ m paraffinembedded tibiae tissue sections were deparaffinized in xylene and rehydrated in graded ethanol. Following antigen retrieval, slides were incubated with 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. Sections were blocked in 5% normal goat serum for 30 minutes before incubation in primary antibody at 4°C overnight with rabbit anti-human RANKL antibody (Amgen) or preimmune serum to assess nonspecific staining. Frozen human tissue sections were thawed and fixed with ice-cold methanol before antigen retrieval. Sections were incubated in primary antibody overnight with goat anti-RANKL antibody (Santa Cruz N-19) or goat IgG isotype control. The primary antibody was labeled by biotin-streptavidin method using the VectaStain Universal ABC kit (Vector Laboratories Inc., Burlingame, CA). Slides were counterstained with hematoxylin. Immunohistochemical scores were obtained by categorizing the product of stain intensity (0: negative, 1: weakly positive, 2: positive, 3: strongly positive) and total area stained (0: <10%, 1: 10–25%, 2: 25–50%, 3: 50–75%, 4: >75%) on a scale of 0, 1 (1–3), 2 (4–6), 3 (7–9), and 4 (10–12). Slides were scored by a pathologist blinded to clinical history and primary antibody used.

Statistical analysis

Results are expressed as the mean $\pm$ SEM of three independent experiments. Analyses were performed using Prism software (GraphPad Software, San Diego, CA). For comparison of two groups, unpaired student t-test was carried out to determine the statistical differences. For data comparisons of three groups or more, one-way analysis of variance (ANOVA) with Tukey's post-test was used. A probability value of $p < 0.05$ was considered to be statistically significant.

Results

OSCC cell lines present varied levels of expression of RANKL

Since oral squamous cell carcinoma bone invasion is a common finding, associated with severe morbidity due to the extensive osteolytic lesions, we evaluated expression of RANKL, a cytokine crucial for osteoclast differentiation, activity and survival in various OSCC cell lines. Constitutive levels of RANKL mRNA and protein expression varied among the cell lines, but mRNA levels were higher than those expressed by cell lines from osteosarcoma (SaoS2) and prostate cancer (PC3), primary and secondary osteolytic tumors, respectively. Constitutive RANKL mRNA expression in OSCC cells was higher than other epithelial cell lines, both neoplastic (HeLa) and non-neoplastic (HaCaT) (Figure 1A). RANKL protein in whole cell lysates was not detected by immunoblot analysis, due to difficulties with commercially available primary antibodies; however, flow cytometry analysis revealed that 4 of seven OSCC cell lines (namely, UM-SCC-11B, UM-SCC-74A, UM-SCC-22B and OSCC3) expressed detectable levels of membrane-bound RANKL (Figure 1B). Interestingly, we detected RANKL protein in conditioned media from all unstimulated OSCC cell lines by ELISA, demonstrating constitutive levels of soluble RANKL protein expression (Figure 1C). These data indicate OSCC cell lines are capable of expressing the main cytokine involved in osteoclast differentiation, activation, and survival, representing one possible mechanism for OSCC tumor extension into bone.

OSCC cell lines express all RANKL mRNA isoforms and are differentially regulated by osteotrophic hormones and cytokines

As RANKL protein was detected on the conditioned medium from all OSCC cell lines, but only a subset of those expressed detectable levels of membrane-bound RANKL, we used RT-PCR to evaluate if there was differential expression of the three known isoforms of RANKL mRNA. The results show that the OSCC cell lines expressed all RANKL mRNA isoforms; however, higher levels of isoform 1 were expressed by the cell lines in which membrane-bound RANKL protein was detected by flow cytometry. We also observed that OPG mRNA was constitutively expressed at comparable levels by all OSCC cell lines, except for UM-SCC-11A, which presented lower levels (Figure 2A). UM-SCC-11B cells were transfected and generated a stable cell line harboring a 2 kb fragment of RANKL promoter driving the expression of firefly luciferase as a reporter. Using this cell line, we show that this proximal promoter is constitutively active and is not regulated by osteotrophic hormones and cytokines (Figure 2B). We chose three of the seven OSCC cell lines tested to perform further experiments, to include two cell lines that expressed both soluble and membrane-bound RANKL (UMSCC-11B and OSCC3) and one that only expressed soluble RANKL (UM-SCC-74B). The constitutive activation of the RANKL proximal promoter agrees with the lack of regulation of RANKL mRNA in these three OSCC cell lines upon stimulation with inflammatory cytokines and osteotrophic hormones (Figure 2C). These results support the constitutive expression of RANKL mRNA and suggest that regulation of RANKL gene expression by these stimuli requires distant promoter elements.

Osteoclastogenesis in vitro is supported by OSCC cell lines and involves tumor-derived RANKL

We went on to verify that the RANKL expressed by OSCC cell lines is functional and can support osteoclastogenesis in vitro. We used a mouse macrophage cell line (Raw 264.7) for the in vitro osteoclastogenesis experiments. To assess possible cell line or species-specific influences, the results were confirmed using human mononuclear cells as osteoclast precursors. Due to the ease of culture and greater homogeneity of the mouse macrophage cell line, quantitation was performed using these cells as osteoclast precursors (Figure 3A), and only representative images of co-culture experiments with Raw 264.7 and human

peripheral blood monocytic cells (hPBMC) are shown (Figure 3B and 3C, respectively). Cross-species RANKL reactivity was validated in Raw 264.7 cells, as osteoclast formation was induced by rhRANKL and blocked by the simultaneous addition of rhOPG or hRANKL antibody (data not shown). Treatment of UM-SCC-74B with 1,25(OH)₂D₃ and PTH resulted in a significant increase in osteoclastogenesis (Figure 4A). When recombinant human OPG or a blocking antibody against RANKL was added to the co-culture system, osteoclastogenesis was partially inhibited (Figure 4B), indicating that RANKL was involved in the process. To confirm the role of RANKL in OSCC-induced osteoclastogenesis, we performed a co-culture experiment in which hRANKL blocking antibodies were introduced at the beginning of the 6-day co-culture period and led to a significant reduction, although not complete ablation, of osteoclastogenesis in vitro (Figure 4C). Interestingly, incubation of osteoclast precursor cells with conditioned medium from OSCC cells also induced osteoclastogenesis, supporting the findings of soluble RANKL expression by OSCC cells (Figure 4D).

The RANKL proximal promoter is active in OSCC cells in the bone microenvironment, and these cells produce osteolytic lesions in vivo

Since OSCC cells can support osteoclastogenesis in vitro, we wanted to find out if these cells were capable of producing osteolytic lesions when introduced to the bone microenvironment in vivo. For this purpose, we injected the UM-SCC-11B-RANKL-Luc stable cell line (UM-SCC-11B cells stably transfected with a firefly luciferase reporter construct driven by a 2 kb fragment of hRANKL proximal promoter) into the bone marrow of the left tibia of nude mice. The cells proliferated and over the 8-week experimental period produced a large visible erythematous mass that impaired mobility of the animal. Since the hRANKL proximal promoter was constitutively active, the increase of bioluminescence over time (Figure 5A and 5B) likely represents an increase on luciferase-producing cells due to cell proliferation, resulting in stronger bioluminescence signal over time. Faxitron and microcomputer tomography analyses demonstrated that the tibia bones were almost completely resorbed during this period (Figure 5C). Histological analysis shows functional osteoclasts on the remaining tibia bone, (Figure 5D) and the immunohistochemistry (Figure 5E) demonstrates human RANKL expression by the UM-SCC-11B-RANKL-Luc cells. These results indicate that OSCC cells can proliferate in the bone microenvironment and, moreover, that as these cells proliferate and expand, they induce bone resorption through RANKL expression. The data also show that these OSCC cells expand locally and did not produce metastasis over the course of the 8-week experimental period, as indicated by the localized bioluminescence signal.

Human OSCC tissues express varied levels of RANKL

We sought to validate our animal model findings in human tissues from patients with oral squamous cell carcinoma. A commercial tissue microarray consisting of specimens from fifty oral squamous cell carcinomas and ten cancer-adjacent tissues from the oral cavity were stained with an anti-RANKL antibody (Figure 6). Positive staining was identified by a pathologist in predominantly epithelial tissues with some expression in endothelial and smooth muscle tissues (Figure 6B). Staining intensity and area were evaluated to generate an immunohistochemical score for each specimen. Staining of OSCC tissues included the full range of scores from no expression (Figure 6C) to weak (Figure 6D), moderate (Figure 6E), and strong expression (Figure 6F) in epithelial tissues, also seen in non-tumor adjacent tissues (Figure 6I). To characterize RANKL expression in the context of OSCC bone invasion, frozen tissues collected from two patients diagnosed with oral squamous cell carcinoma with documented local invasion to the mandibular bone as well as tissue from an OSCC patient with documented absence of bone invasion were stained for RANKL expression (Figures 6G–H). Although limited by the number of available human OSCC

tissues with clinical record of local bone invasion, these results echo findings from our murine model, demonstrating the expression of RANKL by oral squamous carcinoma cells.

Discussion

Osteoclast-mediated bone resorption is an important step in the process of local bone invasion as well as bone metastases in several malignancies, including oral cancer^{20,21}. Recent studies suggest OSCC cells are capable of regulating osteoclastogenesis as well as mature osteoclast function through numerous mechanisms, including RANKL/OPG signaling²². In vitro studies have demonstrated in multiple myeloma as well as two oral squamous cell carcinoma cell lines, the ability of cancer cells to produce RANKL and induce osteoclastogenesis^{23,24}. As an essential factor for osteoclastogenesis, RANKL expression can be induced in stromal cells, including osteoblasts, by a variety of hormonal and cytokine stimuli including vitamin D₃²⁵, IL-1, IL-6, PTHrP, and IL-17²⁶. However, further studies are necessary to elucidate the particular role of OSCC-derived RANKL in the tumor microenvironment and its function in local bone invasion.

In the present study, we observed in a panel of OSCC cell lines that RANKL mRNA and protein is constitutively expressed. Impressively, the level of expression detected exceeds that of cell lines obtained from both primary and metastatic osteolytic tumors. Closer examination reveals differential levels of membrane-bound versus soluble RANKL. Cell lines with detectable levels of membrane-bound RANKL also demonstrated increased expression of RANKL isoform 1, although the shorter isoforms 2 and 3 were also detected²⁷. RANKL is cleaved to produce a soluble form with biological activity. The shedding of membrane-bound RANKL appears to be mediated by expression of matrix metalloproteinase (MMP) 14 and ADAM10. Suppression of MMP14 in primary osteoblasts increases membrane-bound RANKL and promotes osteoclastogenesis in co-cultures with macrophages. Therefore, RANKL shedding seems to be an important process that down-regulates local osteoclastogenesis. Data presented here indicate that all three isoforms are expressed suggesting the potential for differential capacity of OSCC-derived RANKL to induce osteoclastogenesis. As an additional point to regulate osteoclastogenesis, the proximal promoter activity for the 2kb upstream promoter region of the RANKL promoter was determined. These data demonstrated constitutive transcriptional activity in these cell lines, irrespective of osteotrophic cytokine and hormone stimulation. These results suggest the constitutive expression of RANKL in OSCC cells is not transcriptionally regulated by its proximal promoter, but perhaps by more distant regulatory elements upwards of 120kb as previously described²⁸. Thus, both isoform expression and cleavage along with inducible RANKL may be operative in OSCC in vitro and in vivo.

Previous studies have demonstrated OSCC-conditioned medium can stimulate osteoclast differentiation of human peripheral blood mononuclear cells, an effect blocked by osteoprotegerin²⁹. In a co-culture system, we have confirmed these findings with a panel of cell lines we have characterized as expressing both membrane-bound and soluble forms of RANKL or soluble alone. As previously described in a human OSCC cell line, we demonstrate multiple sources of OSCC-conditioned media can stimulate osteoclast differentiation in vitro¹⁶. Furthermore, antibodies targeted to RANKL inhibited osteoclastogenesis partially, demonstrating a direct paracrine interaction between OSCC-derived RANKL and the osteoclast precursor. These data also suggest that other osteoclast promoting factors could be operative in the tumor-bone microenvironment.

Some evidence suggests stromal tissue is the key regulator of the RANKL:OPG expression ratio, which governs osteoclastogenesis in both physiologic and pathologic conditions^{30,31}. In particular, osteoblasts are well characterized as responding to osteotrophic stimuli by

increasing or decreasing expression of RANKL and OPG. Previous co-culture studies of human OSCC cells with primary osteoblasts suggested OSCC cells decrease osteoblast production of OPG, to support osteoclastogenesis³². In malignancies, RANKL expression by T-regulatory cells has been demonstrated to regulate bone resorption^{33,34}. Other stromal factors that may regulate RANKL function include osteoclast, as well as tumor-derived, secretion of MMPs, such as MMP-7, to mobilize RANKL in the tumor microenvironment and enhance osteolysis^{35,36}. To determine the function of OSCC-derived RANKL, we performed an in vivo assay of osteoclastogenesis using a human RANKL-luciferase reporter construct in athymic nude mice. Luciferase-producing cells proliferated within the tibiae, generating osteolytic lesions, visible by small animal imaging. In these areas of bone resorption, osteoclasts were histologically identified and an antibody exclusively recognizing the human homolog detected human RANKL. Our results demonstrate OSCC cells are a direct cellular source of proosteoclastogenic RANKL in an animal model of local bone invasion.

To validate cancerous epithelium as a cellular source of RANKL in oral squamous cell carcinoma, we characterized its expression in a panel of oral squamous cell carcinoma specimens available through a commercial tissue microarray. The broad distribution of RANKL staining in these tissues reflects the molecular heterogeneity of OSCC observed within established cell lines. Similar staining in non-tumor adjacent tissues suggests RANKL may participate in the “field effect” first described in OSCC where histologically “normal tissue” in close proximity to the tumor bear genetic alterations, playing a role in the high number of local recurrences and second primary tumors seen in OSCC patients³⁷. Stromal expression of RANKL was also observed in a minority of samples, as suggested by previous studies, but was limited to smooth muscle and endothelial cells (Figure 6B). These results contribute to efforts to define the molecular mechanisms that can distinguish the subset of human oral squamous cell carcinomas with the predilection to invade bone. Despite limitations in the number of available specimens from patients with bone invasion, we have confirmed the expression of RANKL by cancerous oral epithelium with high levels in a patient with local bone invasion as well as moderate expression in a patient with no bone invasion. Determining the prognostic potential of RANKL expression is well beyond the scope of this study, but understanding the multiple mechanisms involved in osteolytic disease will enhance development of future diagnostic and therapeutics.

Altogether, these data underscore the functional importance of RANKL signaling in the pathophysiology of OSCC bone invasion. Furthermore, these data demonstrate the direct role of tumor-derived RANKL in the invasive activity of oral squamous cell carcinoma.

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References

1. Funk GF, Karnell LH, Robinson RA, Zhen WK, Trask DK, Hoffman HT. Presentation, treatment, and outcome of oral cavity cancer: A national cancer data base report. *Head Neck*. Feb; 2002 24(2): 165–180. [PubMed: 11891947]
2. Silverman S Jr. Demographics and occurrence of oral and pharyngeal cancers. The outcomes, the trends, the challenge. *J Am Dent Assoc*. Nov; 2001 132(Suppl):7S–11S. [PubMed: 11803655]
3. Horner, MJRL.; Krapcho, M.; Neyman, N.; Aminou, R.; Howlader, N.; Altekruse, SF.; Feuer, EJ.; Huang, L.; Mariotto, A.; Miller, BA.; Lewis, DR.; Eisner, MP.; Stinchcomb, DG.; Edwards, BK. *Seer cancer statistics review, 1975-2006*. 2009. http://seer.cancer.gov/csr/1975_2006/

4. Nomura T, Shibahara T, Cui NH, Noma H. Patterns of mandibular invasion by gingival squamous cell carcinoma. *J Oral Maxillofac Surg.* Oct; 2005 63(10):1489–1493. [PubMed: 16182917]
5. Patel RS, Dirven R, Clark JR, Swinson BD, Gao K, O'Brien CJ. The prognostic impact of extent of bone invasion and extent of bone resection in oral carcinoma. *Laryngoscope.* May; 2008 118(5):780–785. [PubMed: 18300706]
6. Shaw RJ, Brown JS, Woolgar JA, Lowe D, Rogers SN, Vaughan ED. The influence of the pattern of mandibular invasion on recurrence and survival in oral squamous cell carcinoma. *Head Neck.* Oct; 2004 26(10):861–869. [PubMed: 15390204]
7. Kearns AE, Khosla S, Kostenuik PJ. Receptor activator of nuclear factor kappa ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev.* Apr; 2008 29(2):155–192. [PubMed: 18057140]
8. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. *Nature.* May 15; 2003 423(6937):337–342. [PubMed: 12748652]
9. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, et al. Opgl is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* Jan 28; 1999 397(6717):315–323. [PubMed: 9950424]
10. Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, et al. Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. *Am J Pathol.* Aug; 2000 157(2):435–448. [PubMed: 10934148]
11. Matsuzaki K, Udagawa N, Takahashi N, Yamaguchi K, Yasuda H, Shima N, et al. Osteoclast differentiation factor (odf) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem Biophys Res Commun.* May 8; 1998 246(1):199–204. [PubMed: 9600092]
12. Roodman GD. Mechanisms of bone metastasis. *N Engl J Med.* Apr 15; 2004 350(16):1655–1664. [PubMed: 15084698]
13. Morony S, Capparelli C, Sarosi I, Lacey DL, Dunstan CR, Kostenuik PJ. Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. *Cancer Res.* Jun 1; 2001 61(11):4432–4436. [PubMed: 11389072]
14. Jones DH, Nakashima T, Sanchez OH, Kozieradzki I, Komarova SV, Sarosi I, et al. Regulation of cancer cell migration and bone metastasis by rankl. *Nature.* Mar 30; 2006 440(7084):692–696. [PubMed: 16572175]
15. Nomura T, Shibahara T, Katakura A, Matsubara S, Takano N. Establishment of a murine model of bone invasion by oral squamous cell carcinoma. *Oral Oncol.* Mar; 2007 43(3):257–262. [PubMed: 16920384]
16. Deyama Y, Tei K, Yoshimura Y, Izumiyama Y, Takeyama S, Hatta M, et al. Oral squamous cell carcinomas stimulate osteoclast differentiation. *Oncol Rep.* Sep; 2008 20(3):663–668. [PubMed: 18695921]
17. Kayamori K, Sakamoto K, Nakashima T, Takayanagi H, Morita K, Omura K, et al. Roles of interleukin-6 and parathyroid hormone-related peptide in osteoclast formation associated with oral cancers: Significance of interleukin-6 synthesized by stromal cells in response to cancer cells. *Am J Pathol.* Feb; 2010 176(2):968–980. [PubMed: 20035059]
18. Shibahara T, Nomura T, Cui NH, Noma H. A study of osteoclast-related cytokines in mandibular invasion by squamous cell carcinoma. *Int J Oral Maxillofac Surg.* Oct; 2005 34(7):789–793. [PubMed: 15982855]
19. Henson B, Li F, Coatney DD, Carey TE, Mitra RS, Kirkwood KL, et al. An orthotopic floor-of-mouth model for locoregional growth and spread of human squamous cell carcinoma. *J Oral Pathol Med.* Jul; 2007 36(6):363–370. [PubMed: 17559499]
20. Aoki J, Yamamoto I, Hino M, Shigeno C, Kitamura N, Sone T, et al. Osteoclast-mediated osteolysis in bone metastasis from renal cell carcinoma. *Cancer.* Jul 1; 1988 62(1):98–104. [PubMed: 3383123]
21. Semba I, Matsuuchi H, Miura Y. Histomorphometric analysis of osteoclastic resorption in bone directly invaded by gingival squamous cell carcinoma. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology.* Sep; 1996 25(8):429–435.

22. Tada T, Shin M, Fukushima H, Okabe K, Ozeki S, Okamoto M, et al. Oral squamous cell carcinoma cells modulate osteoclast function by rankl-dependent and -independent mechanisms. *Cancer Lett.* Feb 8; 2009 274(1):126–131. [PubMed: 18930344]
23. Farrugia AN, Atkins GJ, To LB, Pan B, Horvath N, Kostakis P, et al. Receptor activator of nuclear factor-kappa ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. *Cancer Res.* Sep 1; 2003 63(17):5438–5445. [PubMed: 14500379]
24. Nagai M, Kyakumoto S, Sato N. Cancer cells responsible for humoral hypercalcemia express mrna encoding a secreted form of odf/trance that induces osteoclast formation. *Biochem Biophys Res Commun.* Mar 16; 2000 269(2):532–536. [PubMed: 10708588]
25. Kitazawa R, Kitazawa S. Vitamin d(3) augments osteoclastogenesis via vitamin d-responsive element of mouse rankl gene promoter. *Biochem Biophys Res Commun.* Jan 18; 2002 290(2):650–655. [PubMed: 11785948]
26. Hashizume M, Hayakawa N, Mihara M. Il-6 trans-signalling directly induces rankl on fibroblast-like synovial cells and is involved in rankl induction by tnfr-alpha and il-17. *Rheumatology.* Nov; 2008 47(11):1635–1640. [PubMed: 18786965]
27. Ikeda T, Kasai M, Utsuyama M, Hirokawa K. Determination of three isoforms of the receptor activator of nuclear factor-kappa ligand and their differential expression in bone and thymus. *Endocrinology.* Apr; 2001 142(4):1419–1426. [PubMed: 11250921]
28. Fu Q, Manolagas SC, O'Brien CA. Parathyroid hormone controls receptor activator of nf-kappa ligand gene expression via a distant transcriptional enhancer. *Molecular and cellular biology.* Sep; 2006 26(17):6453–6468. [PubMed: 16914731]
29. Pandravadu SN, Yuvaraj S, Liu X, Sundaram K, Shanmugarajan S, Ries WL, et al. Role of cxc chemokine ligand 13 in oral squamous cell carcinoma associated osteolysis in athymic mice. *Int J Cancer.* May 15; 2010 126(10):2319–2329. [PubMed: 19816883]
30. Giuliani N, Bataille R, Mancini C, Lazzaretti M, Barille S. Myeloma cells induce imbalance in the osteoprotegerin/osteoprotegerin ligand system in the human bone marrow environment. *Blood.* Dec 15; 2001 98(13):3527–3533. [PubMed: 11739153]
31. Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, et al. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/rankl but not macrophage colony-stimulating factor: Receptor activator of nf-kappa b ligand. *Bone.* Nov; 1999 25(5):517–523. [PubMed: 10574571]
32. Tada T, Jimi E, Okamoto M, Ozeki S, Okabe K. Oral squamous cell carcinoma cells induce osteoclast differentiation by suppression of osteoprotegerin expression in osteoblasts. *Int J Cancer.* Aug 20; 2005 116(2):253–262. [PubMed: 15800904]
33. Tan W, Zhang W, Strasner A, Grivennikov S, Cheng JQ, Hoffman RM, et al. Tumour-infiltrating regulatory t cells stimulate mammary cancer metastasis through rankl-rank signalling. *Nature.* Feb 24; 2011 470(7335):548–553. [PubMed: 21326202]
34. Giuliani N, Colla S, Sala R, Moroni M, Lazzaretti M, La Monica S, et al. Human myeloma cells stimulate the receptor activator of nuclear factor-kappa b ligand (rankl) in t lymphocytes: A potential role in multiple myeloma bone disease. *Blood.* Dec 15; 2002 100(13):4615–4621. [PubMed: 12393684]
35. Lynch CC, Hikosaka A, Acuff HB, Martin MD, Kawai N, Singh RK, et al. Mmp-7 promotes prostate cancer-induced osteolysis via the solubilization of rankl. *Cancer Cell.* May; 2005 7(5):485–496. [PubMed: 15894268]
36. Watanabe H, Iwase M, Ohashi M, Nagumo M. Role of interleukin-8 secreted from human oral squamous cell carcinoma cell lines. *Oral Oncol.* Oct; 2002 38(7):670–679. [PubMed: 12167419]
37. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer.* Sep; 1953 6(5):963–968. [PubMed: 13094644]

Figure 1. Heterogeneous levels of constitutive RANKL gene expression in OSCC cell lines
 (A) RT-qPCR was used to assess the level of RANKL mRNA in seven different OSCC cell lines, an osteosarcoma cell line (SaoS2), a prostate cell line (PC3), and both neoplastic (HeLa) and non-neoplastic (HaCaT) epithelial cell lines. Each cell line was grown to 80% confluency, the culture medium was changed and total RNA was harvested after 24h. After reverse transcription into cDNA, the levels of RANKL mRNA were determined by real-time PCR using TaqMan gene expression assays and normalized to the levels of housekeeping gene GAPDH using the relative quantitation method. (B) Flow cytometric analysis indicated that a subset of the OSCC cell lines expressed membrane-bound form of RANKL. The black line indicates background fluorescence in samples where the primary anti-hRANKL antibody was omitted. 10,000 cells were gated under the same experimental conditions and the graphs shown are representative of three independent experiments. (C) The conditioned culture medium from the indicated cell lines was collected 72h after changing the medium, and the presence of RANKL protein was determined by ELISA. The results were normalized to the concentration of total protein in the samples of conditioned medium. In both graphs, bars indicate averages and vertical lines standard error of the mean of a minimum of three independent experiments.

Figure 2. Three isoforms of RANKL mRNA are expressed by OSCC cell lines, and RANKL proximal promoter is constitutively active and not regulated by inflammatory cytokines and osteotropic hormones

(A) Representative image of RT-PCR results for the expression of the isoforms of RANKL and also for OPG expression. Each cell line was grown to near-confluency, washed and fresh medium was added. 24h later, the cells were washed and total RNA was harvested and reverse-transcribed. Expression of each isoform of RANKL, of OPG and of the housekeeping gene GAPDH was evaluated with specific primers under optimized cycling conditions. The reaction products were resolved by agarose gel electrophoresis, and the image is representative of two independent experiments. (B) UM-SCC-11B cells stably-transfected with a luciferase reporter construct in which luciferase expression was under the control of a 2kb fragment of RANKL human promoter were grown to near-confluency in 6-well plates. Culture medium was changed and the cells stimulated with IL-1 (1 ng/mL), IL-6 (25 ng/mL), TNF- α (10 ng/mL), 1,25(OH) $_2$ D $_3$ (10^{-8} M), PTH (10^{-7} M) or ethanol vehicle for 24 hours. Cell lysates were harvested and assayed for luciferase expression. The data are presented as fold change of luciferase activity over the empty vector transfected control (average values for the background Relative Luciferase Units – RLU – was set to 1). (C) Regulation of RANKL mRNA expression by inflammatory cytokines and osteotropic hormones was evaluated by RT-qPCR in the indicated OSCC cell lines. Cells were grown to 80% confluency, the culture medium was changed and the IL-1 (1 ng/mL), IL-6 (25 ng/mL), 1,25(OH) $_2$ D $_3$ (10^{-8} M), TNF- α (10 ng/mL), PTH (10^{-7} M) or ethanol vehicle were added. Total RNA was harvested after 24h, reverse transcribed into cDNA and the levels of RANKL mRNA were determined by real-time PCR using TaqMan gene expression assays and normalized to the levels of housekeeping gene GAPDH by the relative quantitation method. In (B) and (C), bars indicate averages and vertical lines standard error of the mean of a minimum of three independent experiments.

Figure 3. OSCC cell lines directly induce osteoclastogenesis in vitro through expression of RANKL

(A) Raw 264.7 cells were cultured for 6 days in the presence of RANKL (100 ng/mL), RANKL + OPG (200 ng/mL) or PBS/BSA vehicle and then stained for expression of Tartrate-resistant acid phosphatase (TRAP) as a marker of osteoclast differentiation. The images demonstrate that Raw cells respond to RANKL treatment with increased number of large (> 3 nuclei) TRAP+ cells and that OPG inhibited this event. (B) The indicated OSCC cell lines were co-cultured with Raw 264.7 cells for 6 days in the presence of ethanol vehicle (control), OPG (100 ng/mL), 1,25 (OH)₂D₃ (10⁻⁸M). 'Conditioned media' indicate that only 1 mL (50% of total volume of medium) from unstimulated OSCC cells was added to the Raw 264.7 cells for 6 days. (C) Human peripheral blood mononuclear cells (hPMBC) were treated with PBS/BSA vehicle, RANKL (100 ng/mL), or co-cultured with UMSSC-11B cells and then stained for TRAP+ cells. Images are representative of three independent experiments.

Figure 4. OSCC cell line-derived RANKL is functional and induces osteoclastogenesis in vitro (A) OSCC cell lines were treated with either 10^{-8} M of $1,25(\text{OH})_2\text{D}_3$ or 10^{-7} M PTH for 72 hours and then plated onto Raw264.7 for 6-day co-cultures, after which the cells were submitted to TRAP staining and the large (>3 nuclei) TRAP+ cells were counted (* $p < 0.05$, * $p < 0.01$ in comparison to untreated cells). (B) Addition of 100 ng/mL of OPG at the start of the co-culture period decreased the number of large (> 3 nuclei), TRAP+ cells formed (* $p < 0.05$ in comparison to no OPG treated cells). (C) Relative decrease on the number of large, TRAP+ cells counted in 6-day co-cultures of Raw264.7 and the indicated OSCC cell lines in the presence of 1 ug/mL of blocking antibodies against hRANKL. (D) Raw 264.7 cells were cultured for 6 days in the presence of 1 mL of conditioned medium from the indicated OSCC cell lines treated as indicated in (A) and then the number of large (> 3 nuclei), TRAP+ cells was counted (* $p < 0.05$).

Figure 5. OSCC cells induce osteolytic lesions in vivo

(A) Increase on bioluminescence over the 8-week experimental period. Each data point (2, 4, 6 and 8 weeks) represents the average value of three animals. (B) Bioluminescence scan images of a control, DMEM-injected mouse (top) and an experimental animal, injected with UM-SCC-11B-RANKL-Luc cells in the left tibia. The increase on the intensity of bioluminescence indicates proliferation of the cells over time. (C) Radiographic (Faxitron) of the same animal over the 8-week experimental period showing the growth of soft tissue mass. Representative 3D reconstructions from CT scans of one control and one experimental animal at 8 weeks show severe destruction of tibia bone. (D) H&E stained section of UM-SCC-11B-RANKL-Luc invaded tibia presents a large number of tumor cells. Only 'islands' of bone were present and the arrows indicate multinucleated osteoclasts at tumor-bone interface. (E) Immunohistochemical staining with monoclonal antibodies against human RANKL in sections of a tibia invaded by UM-SCC-11B-RANKL-Luc cells demonstrates high levels of RANKL expression by these cells in the bone microenvironment in vivo (40X).

Figure 6. Human OSCC tissues display heterogeneous expression of RANKL

(A) Normal tongue tissue stained with a goat IgG isotype control antibody. (B) Oral squamous cell carcinoma tissue stained with anti-RANKL antibody demonstrating stromal expression of RANKL. (C) OSCC tissue stained with anti-RANKL antibody demonstrating no RANKL expression. (D) OSCC tissue stained with anti-RANKL antibody demonstrating mild epithelial RANKL expression (E) OSCC tissue stained with anti-RANKL antibody demonstrating moderate epithelial RANKL expression (F) OSCC tissue stained with anti-RANKL antibody demonstrating severe epithelial RANKL expression (G) OSCC tissue from a patient with documented mandibular invasion stained with anti-RANKL antibody. (H) OSCC tissue from a patient with documented absence of local bone invasion stained with anti-RANKL antibody. (I) Distribution of immunohistochemical staining scores for non-tumor adjacent tissue (n = 11) and OSCC (n = 53). All images were taken with a 20X objective lens.