SPANDIDOS PUBLICATIONS

Oral squamous cell carcinomas stimulate osteoclast differentiation

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Abstract. Matrix metalloproteinases (MMPs) play important roles in the invasion and metastasis to soft tissues of carcinomas including, oral squamous cell carcinomas (SCCs). Although, osteoclastic bone resorption is an important step in bone involvement in a variety of malignancies, the mechanism of bone involvement of oral SCC remains unclear. Once cancer cells arrest in bone, the bone is a storehouse of a variety of cytokines and growth factors and thus provides an extremely fertile environment for cell growth. The bone-invasive oral cancer cell line, BHY, transcriptionally expressed detectable levels of TGF-ß, IL-1ß, IL-8, parathyroid hormone-related protein (PTHrP) and vascular endothelial growth factor (VEGF) mRNAs and failed to express GM-CSF, IL-6, and TNF-α. Furthermore, the BHY-conditioned medium greatly upregulated IL-6 and RANKL/ODF mRNA expression in osteoblasts, suggesting a potential indirect stimulation of osteoclastogenesis via the osteogenic lineage. Seven out of eleven patients with carcinomas of the lower alveolus and gingiva showing infiltrative bone involvement expressed PTHrP mRNA. These data suggest that the occurrence of PTHrP may be an indication of developing oral malignant carcinomas.

Introduction

Oral carcinomas, especially oral squamous cell carcinomas (SCCs), frequently invade maxillary or mandibular bone (1-5). This bone involvement is a commonly occurring clinical problem in the treatment of patients with oral carcinomas. Physiological bone remodelling is maintained by osteoclasts and osteoblasts (6) and bone tissue is rich in cytokines and local factors that, along with systemic hormones, regulate the activity of bone cells (7). Bone resorption occurs due to the activity of osteoclasts, multinucleated cells derived from the haematopoietic colony forming a unit-granulocyte macrophage (CFU-GM)/monocyte-macrophage family (8). The mechanism whereby osteolytic lesions occur in bone invasion is not fully elucidated. It has been reported that bone resorption by osteoclast is an important procedure in the process of bone invasion in malignancies with oral carcinomas. Cancer cells invade surrounding tissue in order to enlarge the tumor mass by secreting proteolytic enzymes such as asparatic, cysteine and serine proteases and matrix matalloproteinases (MMPs). Osteoclastic bone resorption provides the space and nutrients necessary for the development of cancer cells in bone. This study investigated the mechanism by which BHY oral carcinoma cell line stimulates osteoclastogenesis *in vitro*. PTHrP has been identified as a mediator of the humoral hypercalcemia of malignancies. It is produced by human breast cancer and prostate cancer cell lines and induces osteoclast formation *in vivo* and *in vitro* (9). PTHrP has been purified from breast cancer cells by Burtis *et al* (10) and cloned by Suva *et al* (11). PTHrP binds to the PTH receptor and can cause hypercalcemia, osteoclast-mediated bone destruction and increased renal reabsorption of calcium and the excretion of phosphate. Breast cancer metastases (>90%) to bone express PTHrP (12) and 70% of visceral breast cancer metastases also express PTHrP. These data suggest that the bone microenvironment enhances PTHrP production by breast cancer cells. In oral cancer, it was reported that although PTHrP was present in all of the 24 primary SCC of mandibles studied, there was no correlation between PTHrP expression and the pattern of tumor invasion (13). The present study investigated the mechanism by which the highly bone invasive oral SCC cell line, BHY, stimulates osteoclastogenesis. Moreover, the expression of PTHrP mRNA in patients with carcinomas of the lower alveolus and gingiva were investigated.

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Materials and methods

Reagents. Cell culture media were obtained from Invitrogen Corp. (Carlsbad, CA); fetal bovine serum (FBS) was from Roche Molecular Systems, Inc. (Braunchburg, NJ); 1α,25(OH)$_2$ vitamin D$_3$ [1α,25(OH)$_2$D$_3$] and Dexamethasone (Dex) were from Sigma Chemical Co. (St. Louis, MO); human recombinant parathyroid hormone-related protein (PTHrP) was from Bachem Bioscience Inc. (King of Prussia, PA) and murine vascular endothelial growth factor (VEGF) was from Peprotech, Inc. (Rocky Hill, NJ).

Oral cancer cell culture. The human oral SCC cell line, BHY (14), which is highly invasive in the mandibular bone of nude mice was provided by Dr Mitsunobu Sato (Tokushima University, Japan). Cells were cultured in Dulbecco's modified minimum Essential medium (DMEM) and supplemented with 10% FBS.

Conditioned media. Oral cancer cells were plated into culture dishes and grown to confluence. The medium was then replaced with a fresh medium containing 0.5% FBS and cultured for a further 48 h. The media were then collected and stored in aliquots at -80˚C until use. The controls used the media prepared from gingival fibroblasts.

Osteoblast culture. Osteoblastic MC3T3-E1 (E1) cells derived from the calvaria of C57/BL6 new born mice were plated at a density of 5x10^4 cells/ml into 100-mm plastic dishes in α-modified minimum essential medium (α-MEM), containing 10% FBS with 60 mg/ml kanamycin.

Preparation of bone marrow cells and co-culture with osteoblasts. Bone marrow cells were isolated from 3- to 5-week old C57/Bl6 mice, as described elsewhere (15). The mice were then sacrificed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut and the marrow cavity was flushed out into a petri dish by slowly injecting the medium at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a pipette to obtain single cell suspension. The bone marrow cells (2x10^5 cells/well of a 24-well plate) were then co-cultured with E1 cells for 6 days in α-MEM containing 10% FBS with 10^{-8} M 1α,25(OH)$_2$D$_3$ (Sigma) and 10^{-7} M Dex (Sigma). Osteoclast-like cells (OCL) were identified by their characteristic multinucleation and tartrate-resistant acid phosphatase (TRAP) staining.

Patients. Thirty-seven patients with carcinomas of the lower alveolus and gingiva were treated surgically at the Hokkaido University Dental Hospital from 1995 through to 2002. The two biopsy specimens required to examine the expression of PTHrP and the surgical specimens to estimate the histopathological bone involvement of the tumors were available in 11 of the 37 patients (Table I). The T and N stages were classified by the UICC classification of 1997. The dissected mandibles were divided into several blocks, basically by buccolingual or labiolingual transection and examined pathologically with H&E staining. The pattern of bone involvement and its extent were evaluated and compared to the amplification of PTHrP. The classification of the histopathological patterns and extent of bone involvement were according to Totsuka *et al* (16).

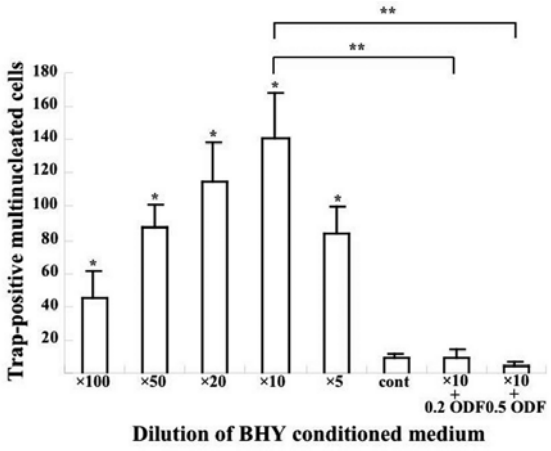

Figure 1. Mouse osteoblastic MC3T3-E1 and bone marrow cells co-cultured for 6 days with 1,25(OH)$_2$D$_3$ (10^{-7} M), Dex (10^{-8} M), CM from BHY cells at indicated concentrations or an ODF/RANKL antibody and the number of tartrate-resistant acid phosphatase-positive osteoclast-like cells with >3 nuclei were counted. A neutralizing antibody against ODF/RANKL (ODF Ab) was added at 0.2 or 0.5 μg/ml, respectively. Data represent means ± SE (n=5-7) and are shown as a percentage of the number of osteoclast-like cells formed in the presence of 1,25(OH)$_2$D$_3$ (control, far left). *P<0.01 vs. control and **P<0.001 vs. 1:10 diluted CM treatment.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using Isogen (Nippon gene, Toyama, Japan) from E1 cells, BHY and tumor tissue, as instructed by the manufacturer. First-strand cDNA was synthesized from the total RNA using the ReverTra Ace moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan) and random primers (Toyobo) and was subjected to PCR amplification with AmpliTaq Gold DNA Polymerase (Roche Molecular Systems, Inc.) using specific PCR primers: human PTHrP, 5'-ATG CAG CGG AGA CTG GTT CAG-3' and 5'-CCT CGT CGT CTG ACC CAA A-3'; mouse ODF/RANKL, 5'-TAT GAT GGA AGG CTC ATG GT-3' and 5'-TGT CCT GAA CTT TGA AAG CC-3'; mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. For human IL-1ß, IL-6, IL-8, GM-CSF, TNF-α and TGF-ß, we used MPCR kits for the Human Inflammatory Cytokine Genes Set (Maxim Biotech, Inc., San Francisco, CA) and for mouse IL-1ß, IL-6, GM-CSF, TNF-α and TGF-ß, MPCR kits for Mouse Inflammatory Cytokine Genes Set (Maxim Biotech, Inc.). The PCR products were separated by electrophoresis on a 1% agarose gel.

Results

Osteoclast generation by a medium of conditioned oral cancer cells. To test whether BHY cells affected osteoclast differentiation, co-cultures of osteoblasts and bone marrow cells in which the percent of FBS was progressively reduced

The relationship between the expression of PTHrP and histopathological bone involvement.

Patient no.	T and N stage	Size of the tumor (mm)	PTHrP mRNA expression	Type of bone involvement	Extent of bone involvement
1	T1N0	12x8	(-)	N.A.	N.A.
2	T1N0	15x10	(-)	N.A.	N.A.
3	T2N0	25x18	(-)	N.A.	N.A.
4	T4N0	37x26	(-)	Expansive	S
5	T2N0	37x15	(+)	Infiltrative	M
6	T2N0	25x10	(+)	Infiltrative	M
7	T4N0	30x15	(+)	Infiltrative	D
8	T4N0	40x25	(+)	Infiltrative	M
9	T4N1	32x18	(+)	Infiltrative	M
10	T4N2c	50x30	(+)	Infiltrative	D
11	T4N2c	60x36	(+)	Infiltrative	D

N.A., not available; M, exceeded the alveolar but did not extend beyond the mandibular canal; S, limited to alveolar bone and D, included or extended beyond the mandibular canal.

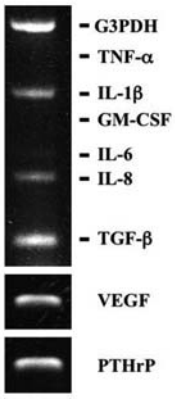

Figure 2. Expression of GM-CSF, IL-1, IL-6, IL-8, PTHrP, TGF-ß, TNF-α, VEGF and G3PDH mRNA expression in BHY cells. BHY cells were grown in conditions identical to those used for obtaining the conditioned medium. Cells were lysed and the RNA was extracted and reverse-transcribed. The equivalent of 0.05 μg RNA was subjected to PCR.

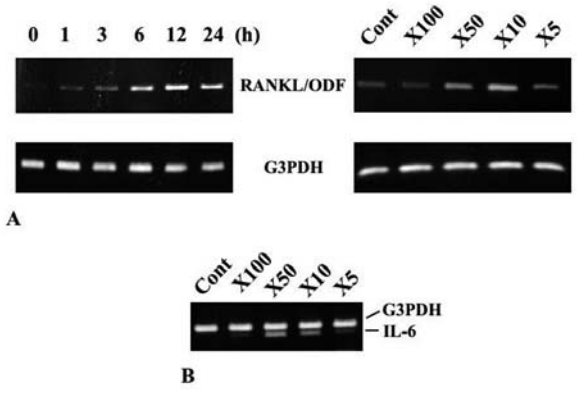

Figure 3. Effects of CM derived from BHY cells in the mRNA expression of ODF/RANKL and IL-6 in MC3T3-E1 cells. (A) E1 cells treated with CM derived from BHY cells (1:10 dilution) for the indicated times (left). E1 cells exposed to various concentrations of CM from BHY for 6 h (right). The mRNA levels of RANKL/ODF and G3PDH were determined by RT-PCR. (B) E1 cells were exposed to various concentrations of CM from BHY for 6 h and the level of IL-6 mRNA was determined by RT-PCR.

from 10 to 3% were established. This was done in order to minimise the effects of the serum, which can modify the responses to the factors released by the cancer cells. It was observed that, although reduced, osteoclastogenesis can be induced by 1α,25$(OH)_2D_3$ in low serum conditions and that 3% FBS still allowed sufficient cell growth (data not shown). Therefore, this concentration was selected for subsequent experiments where conditioned media from BHY cell cultures were tested. To obtain conditioned media, BHY confluent cells were starved in 0.5% FBS-containing medium for 48 h and the conditioned media were collected and used diluted with fresh medium to a final FBS concentration of 3%. Parallel co-cultures were then established with or without BHY conditioned media and with or without 1α,25$(OH)_2D_3$. Using this experimental design, it was observed that cultures treated with BHY-conditioned media were able to undergo osteoclastogenesis (Fig. 1). In these cultures, TRAP-positive multinucleated cells were observed, measured and compared with the control. The effects of the addition can be observed when the conditiond media and 1α,25$(OH)_2D_3$ are used in combination. The CM from BHY markedly accelerated osteoclast formation in a dose-dependent manner (Fig. 1). Furthermore, the addition of the anti-mouse ODF/RANKL antibody significantly inhibited osteoclast formation, which was stimulated by CM derived from BHY CM (Fig. 1).

Gene expression levels in oral cancer cells. Among the numerous cell-derived factors, GM-CSF, IL-1α, IL-1ß, IL-6, IL-8, OPG/OCIF, PTHrP, RANKL/ODF, TGF-ß, TNF-α and VEGF are likely to play pivotal roles in regulating osteoclastogenesis and have been referred to as potent modulators of tumor-induced osteolytic lesions (17-21). Therefore, RT-PCR was used to assess whether BHY cells expressed these molecules. This series of experiments used primary gingival fiblablast as reference cells. Fig. 2 demonstrates that BHY cells failed to express detactable levels of GM-CSF, IL-6 and TNF-α mRNAs. The expression

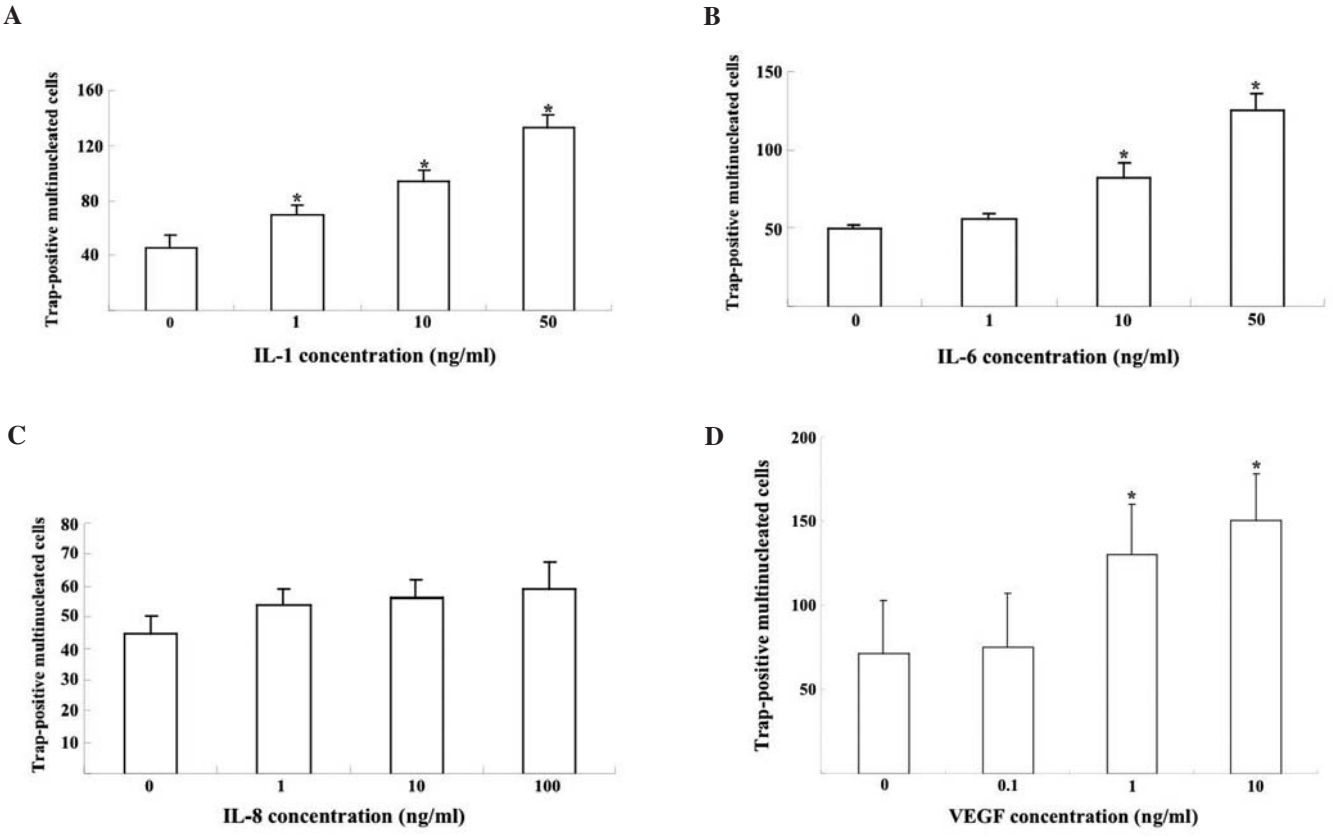

Figure 4. Osteoclast generation by cytokines. E1 and bone marrow cells were co-cultured in a medium containing 3% FBS and mouse recombinant IL-1ß (A), IL-6 (B), IL-8 (C), VEGF (D) and human recombinant PTHrP (E) for 8 days. TRAP-positive multinucleated cells were counted. Data are the means ± SE of three independent experiments. *P<0.01 vs. the control.

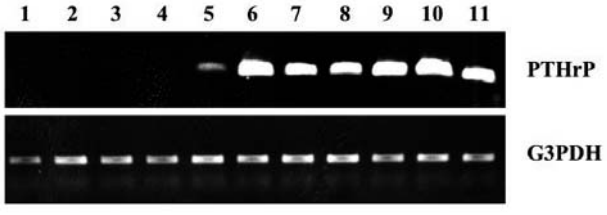

Figure 5. PTHrP mRNA expression in carcinomas of the lower alveolus and gingiva. Fresh primary tumors were collected following biopsy or surgical resection. Total RNA was extracted from tumor samples, cDNA synthesized and then subjected to PCR. Lane numbers are the patient number, as described in Table I.

of TGF-ß was instead clearly apparent, though at the same level as in primary gingival fibroblasts. Expressions of PTHrP and VEGF mRNA were clearly stronger than gingival fibroblasts. These results suggest that PTHrP and VEGF may directly support osteoclast generation.

Gene expression levels in osteoblast and bone marrow cells by a conditioned medium of oral cancer cells. To determine whether BHY-conditioned media stimulated the expression of the paracrine factors in the osteogenic cells, the osteoblastic E1 cells were treated with BHY-conditioned medium and diluted in α-MEM containing 3% FBS. RT-PCR showed IL-6 and RANKL/ODF were enhanced in a dose-dependent manner (Fig. 3A and B), indicating that osteoblasts could be targeted by BHY-conditioned medium.

Osteoclast formation by cytokines. To address the question of whether the factors released from BHY cells and upregulated by BHY CM contributed to the regulation of osteoclast generation, E1 and bone marrow cells were co-cultured and treated for 6 days with mouse recombinant IL-1ß, IL-6, IL-8, VEGF and human recombinant PTHrP. All of these except IL-8 induced osteoclast formation (Fig. 4A-E).

Highly bone invasive oral cancer expressed PTHrP. Normal gingiva obtained from patients without a history of head or

cer did not express IL-1ß, IL-6, IL-8, VEGF or mRNA (data not shown). The carcinoma samples augmented fragments of the IL-1ß, IL-6, IL-8, and VEGF transcript. PTHrP was amplified in 7 of the 11 cases (Fig. 5). Of the four patients with no amplification of PTHrP, three had no bone involvement one patient showed expansive bone involvement limited to the alveolar cortical bone. Among the seven patients with expression of PTHrP, infiltrative bone involvement was observed in all cases and in 3 of the 7 patients, the extent of bone involvement included the mandibular canal (Table I). In the carcinoma of the lower alveolus and gingiva, the expression of PTHrP correlated with the type and extent of bone involvement.

Discussion

This study demonstrated that oral carcinoma cells induce osteoclast generation. Tumor-induced osteoclast generation is likely to be due to indirect, osteoblast-mediated stimulation through IL-6 and ODF/RANKL. Oral cancer, especially SCC, frequently invades the maxillary mandibular bone and this bone invasion is a common clinical problem in patients with oral cancer. However, the mechanism of bone invasion by the tumor remains to be established. Studies have shown that bone resorption by osteoclasts is an important step in the process of bone invasion and metastasis in several malignancies, including oral cancer (22). Although, RANKL/ODF and various cytokines, such as PTHrP, IL-1ß, IL-6, M-CSF, GM-CSF, TGF-ß and TNF-α have been shown to generate and activate osteoclasts and osteoclastic bone resorption, it is has not been clearly shown whether these factors that can be produced by cancer cells are directly involved in bone invasion and metastasis of malignancies. PTHrP has been reported to play an important role in the bone metastasis of human lung and breast carcinoma cells (23). Furthermore, IL-6 secreted by oral cancer cells also plays a significant role in bone invasion (24). Usually, osteoclast generation occurs only if RANKL/ODF in osteogenic cells binds to a receptor present on osteoclast precursors called RANK (24). Osteoblasts also secrete paracrine factors (25,26), which regulate osteoclast development and function.

It has been reported that breast cancer cells stimulate osteoclastogenesis when they are co-cultured with bone marrow cells (ref.?). Our results clearly demonstrated that cell-to-cell contact was not critical in achieving this and that paracrine stimulation induced the generation of osteoclasts. However, the data appear to rule out any direct involvement of GM-CSF or TNF-α as paracrine regulators released from oral cancer cells. TGF-ß was expressed by BHY cells, however, only a weak expression was shown in gingival fibroblasts. This suggests that it is unlikely that, at least in the experiments here, these cytokines have a direct role in inducing the potential of osteoclast generation of this cell line. However, it was shown that IL-1ß, IL-8, PTHrP and VEGF mRNA were expressed in BHY and that BHY-conditioned media enhanced the transcription of ODF/RANKL as well as of IL-6 in osteoblastic E1 cells. Therefore, there are two possible mechanisms that may be employed by BHY to stimulate osteoclast formation. Either the cytokines released from BHY directly induced osteoclastogenesis or BHY stimulates osteoclast generation indirectly via the enhancement of cytokine expression by osteoblasts. In confirmation, the addition of recombinant mouse IL-1ß, IL-6, IL-8, PTHrP, RANKL and VEGF to the co-culture with bone marrow cells and osteoblasts induced osteoclast formation, whereas IL-8 had no effect. The expression pattern of these genes in carcinoma tissues of the oral cavity by RT-PCR was therefore examined. Normal gingiva obtained from patients without a history of head or neck cancer did not express these genes. Each of the carcinoma samples augmented fragments of the IL-1ß, IL-6, IL-8 and VEGF transcript and PTHrP was amplified in 7 of the 11 cases. Results of the present study indicate that the extent of infiltrative bone involvement correlates with the expression of PTHrP for carcinomas of the lower alveolus and gingiva. Although further study is needed to clarify the relationship between the amplification of PTHrP and type or extent of bone involvement, the amplification of PTHrP may be one factor predicting bone involvement of a carcinoma of the lower alveolus and gingiva.

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