Bone Destruction by Invading Oral Squamous Carcinoma Cells Mediated by the Transforming Growth Factor-β Signalling Pathway

TAKESHI GODA[1], TSUYOSHI SHIMO[1], YASUTO YOSHIHAMA[1,2], NUR MOHAMMAD MONSUR HASSAN[1], SOICHIRO IBARAGI[1], NAITO KURIO[1], TATSUO OKUI[1], TATSUKI HONAMI[1], KOJI KISHIMOTO[1] and AKIRA SASAKI[1]

[1]*Department of Oral and Maxillofacial Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan;*
[2]*Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University, Tokyo, Japan*

Abstract. *Background: Gingival squamous cell carcinoma (SCC) cells frequently invade mandibular bone, and this destruction is associated with a worse prognosis. However, the relationship between bone destruction and associated factors is unclear. In this study, the role and diagnostic utility of transforming growth factor-β (TGF-β) type I receptor (TβRI) in bone destruction of the mandible was investigated. Patients and Methods: The expression of TβRI was explored by using an immunohistochemical method on paraffin-embedded tissues from 21 cases of mandibular SCC. An inhibitor of the kinase activity of the TβRI (TβRI-I) was used to assess the role of TβRI in bone destruction by a human oral SCC cell line (HSC-2) that highly expresses TβRI. Results: TβRI-positive signals were closely associated with destructive invasion of the mandible by oral SCC cells. Consistent with these results, TβRI-I greatly reduced HSC-2 cell-induced bone destruction and osteoclast formation in vivo and in vitro. TβRI-I treatment reduced the expression of TNF-α, RANKL and connective tissue growth factor (CTGF/CCN2), all of which were up-regulated by TGF-β in HSC-2 cells. Conclusion: These data demonstrated an important role for TGF-β signalling in bone invasion by oral SCC cells, and suggest that the bone destruction is mediated by RANKL, TNF-α and CCN2.*

Gingival squamous cell carcinoma (SCC) induced mandible bone destruction is associated with worse prognosis, and should be treated surgically by resection. The ensuing physical damage results in disruption of speech and swallowing (1, 2).

Transforming growth factor-β (TGF-β) is a pleiotrophic cytokine that plays a crucial role in regulating the pathogenesis of a wide variety of disorders including cancer, fibrosis and inflammation (3-5). In early tumourigenesis, TGF-β acts as a growth inhibitory cytokine (6). However, as shown by several studies, in the late stage, primary tumour cells can reprogram their response to TGF-β by dys-regulation or mutational activation of various components of the TGF-β signalling pathway and through cross-interaction with other oncogenic pathways. Consequently, TGF-β signalling promotes lung and bone metastasis (7). In the case of osteolytic bone metastasis, it has been proposed that TGF-β released from the decaying bone matrix stimulates neighboring tumour cells, establishing a vicious cycle that exacerbates the growth of the metastatic lesion (8).

TGF-β transduces its signal through two highly conserved single transmembrane serine/threonine kinase receptors, termed type I (TβRI) and type II (TβRII). TβRII activates TβRI upon formation of a ligand–receptor complex by hyperphosphorylating serine/threonine residues in the GS region of TβRI. Activated TβRI in turn phosphorylates Smad2 and Smad3, which interact with Smad4. Their complex is translocated to the nucleus, where it regulates the transcription of target genes. This signalling cascade initiates broad cellular and noncellular processes including proliferation and differentiation, migration and motility, and deposition of extracellular matrix and induction of cytokines contributing to tumourigenesis, metastasis and angiogenesis (9, 10). Due to its central role in TGF-β signalling, TβRI is emerging as a novel target for the blockade of the tumour-promoting activity and metastasis of the TGF-β pathway (11). However, the precise mechanism by which TGF-β exerts its complex activity in oral SCC-induced bone destruction is still not fully understood. In this study, first, TβRI production and

Correspondence to: Dr. Tsuyoshi Shimo, Department of Oral and Maxillofacial Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8525, Japan. Tel: +81 0862356702, Fax: +81 0862356704, e-mail: shimotsu@md.okayama-u.ac.jp

Key Words: Mandibular bone destruction, TGF-β, osteoclast.

distribution in the lesions of oral SCC in the mandibular region were investigated to clarify whether up-regulation of TβRI is associated with increased bone destruction and clinicopathological features of patients with these tumours. Next, the effect of a systemic administration of an ATP-competitive TβRI serine/threonine kinase inhibitor (TβRI-I) on oral SCC-induced bone destruction was examined

Patients and Methods

Patient data. The study population included 20 patients, 12 men and 9 women, with oral SCC of the mandibular region. Their mean age was 74.6 years, ranging from 47 to 84 years. All patients were examined and treated at the Okayama University Hospital (Okayama, Japan) between 1992 and 2003, and the diagnosis was confirmed clinicopathologically. No patient had received chemotherapy and/or radiation therapy before surgery was performed. All tumour samples were obtained after the consent of the patients.

Reagents. Human TGF-β was obtained from R&D Systems (Minneapolis, MN, USA). TβRI-I, chemically [3-(pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole, was obtained from CALBIOCHEM (La Jolla, CA, USA).

Cell culture. Human oral cancer cell line cells (Human Science Research Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen Corporation, Grand Island, NY, USA) containing 10% foetal calf serum (FCS; JRH Bioscience, Lenexa, KS, USA), and 1% penicillin/streptomycin solution (Invitrogen Corporation) in an atmosphere of 5% CO_2/air at 37˚C. Bone marrow cells were isolated from 5-week-old male C57BL/6 mice obtained from Charles River (Yokoyama, Japan). The cells were cultured in the α modification of minimum essential medium (αMEM; Sigma, St. Louis, MO, USA) supplemented with 10% FCS (Hyclone Laboratories, Logan, UT, USA), and penicillin/streptomycin solution in an atmosphere of 5% CO_2/air at 37˚C.

Classification of bone destruction. From the surgically resected samples, decalcified, haematoxylin-eosin (HE)-stained specimens were prepared. Sections from the deepest part of the invasion and the boundary between the tumour and the bone were evaluated primarily by light microscopic observation. The histological pattern was classified into one of two patterns on the basis of modified classifications of tumour spread in the mandible (12). The erosive pattern exhibited a loss of continuity in the cortex and a smooth, well-defined U-shaped or scalloped margin at the medullary bone with no isolated bony spicules. The invasive pattern had an irregular, ill-defined margin with either bony spicules or isolated fragments.

Immunohistochemistry. The sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. After having been autoclaved in 0.2% citrate buffer for 15 minutes, the sections were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. The slides were then washed in Tris-buffered saline (TBS) and incubated in 2.5% skimmed milk for 20 minutes to reduce nonspecific staining. A primary anti-TGF-β receptor (TβRI) antibody (R&D Systems) was used for the immunohistochemical analysis. The specimens were incubated with a 1:200 dilution of the antibody overnight at 4˚C, followed by 3 washes with TBS. The slides were then treated with a streptoavidin-biotin complex (Envision System Labelled polymer, horseradish peroxidase (HRP); Dako, Carpinteria, CA, USA) for 60 minutes at a dilution of 1:100. The immunoreaction was visualised by using a 3,3'-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako), and counterstaining was performed with haematoxylin. Finally, the sections were immersed in an ethanol and xylene bath and then mounted for examination. For the quantification of TβRI in tissue sections, a section was considered positive when there were more than 50% immunoreactive tumour cells in the section.

Bone xenograft model. A mouse model of bone xenografting was prepared by inoculating mice with tumour cell suspensions of HSC-2 cells (10^5 cells/100 μl of PBS) into the paraperiosteal tissue of the tibial metaphysis. On day 28, radiographs were obtained, and the hind limbs were processed, as described in the following subsections. The Animal Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences approved all experimental procedures.

Radiographs and measurement of osteolytic lesion areas. Osteolytic bone metastases were assessed on radiographs as previously described (13, 14). The mice were anaesthetised with an intraperitoneal injection of pentobarbital (0.05 mg/g body weight), placed laterally in the prone position against the film (22×27 cm; Fuji industrial film FR: Fuji Photo Film Co Ltd, Tokyo, Japan), and exposed to soft X-rays at 35 kV for 15 seconds by use of a Sofron apparatus (Sofron, Tokyo, Japan). The radiolucent bone lesions in the hind limbs were observed microscopically (IX81, Olympus Corporation, Tokyo, Japan), and the areas were quantified with Lumina Vision/OL (MITANI CORPORATION, Tokyo, Japan).

Bone histology and immunochemistry. The hind limb long bones of nude mice that had been injected with cancer cells were excised, fixed in 10% neutral-buffered formalin, decalcified, and then embedded in paraffin. Serial sections (5 μm) were cut cross-sectionally, and the sections were stained with Mayer's HE solution. Images of the growth plate and proximal tibia were photographed by using a camera attached to an Olympus IX81 microscope. For the measurement of the osteoclast number, the sections were stained for tartrate-resistant acid phosphatase (TRAP). The total tumour area and osteoclast number/mm of tumour/bone interface were measured in the midsections of the tibia without knowledge of the experimental groups. Histomorphometric analysis was performed by using Lumina Vision/OL (Mitani Corporation, Tokyo, Japan) analysing software.

Immunoblot analysis. Oral cancer cell monolayer cultures were rinsed with ice-cold PBS and lysed in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1% NP-40, 10 mM NaF, 100 mM leupeptin, 2 mg/ml aprotinin and 1 mM phenylmethyl sulfonyl fluoride). Cell lysates containing 10 μg of total protein in the lysis buffer were electrophoresed in 12% SDS-PAGE gels, and the proteins were then transferred to nylon membranes (Immobilon-P; Millipore Co., Bedford, MA, USA). The membranes were blocked with 2% nonfat dry milk in TBS overnight at 4˚C and then incubated with a 1:200 dilution of the desired antibody. HRP-conjugated goat anti-rabbit or goat anti-mouse IgG was used as the secondary antibody at a 1:1000 dilution (Amersham

Biosciences, Buckinghamshire, UK). Bands were visualised by the ECL chemiluminescence detection method (RPN2109; Amersham Biosciences).

Cell proliferation assay. The HSC-2 cells were plated in a 96-well plate at 2,000 cells per well in the presence of TGF-β. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5,-diphenyltetrazolium bromide (MTT) assay was carried out to obtain relative cell numbers after 5 days of incubation, as described previously (15).

Migration assay. Migration of HSC-2 cells was studied in a Boyden chamber. HSC-2 cells in logarithmic growth phase were detached by trypsin-EDTA, and 1×10^5 cells were seeded onto polycarbonate membranes (pore size 8.0 mm; Becton Dickinson, Franklin Lakes, NJ, USA) and treated with 1 or 5 ng/ml TGF-β in the presence or absence of 100 or 1000 nM TβRI. DMEM/F12 containing 10% FCS was added to the lower chamber, and the system was incubated at 37˚C for 6 h in 5% CO_2. After incubation and fixation, nonmigrating cells were removed with a cotton swab, and the remaining cells were stained with 2% Crystal Violet (Sigma). Filters were examined microscopically for cells on the lower side of the membrane. The number of cells in 4 microscopic fields per well were counted, and the average of 4 wells was determined.

Real-time PCR (RT-PCR). Total RNA was isolated from HSC-2 cells by using TRIZOL reagent (Life Technologies Inc, Gaithersburg, MD, USA) according to the manufacturer's recommendations, and the transcripts were analysed by RT-PCR. The primers used were the following: 5'-GGCACTCACTGCATTTATAG3' (forward) and 5'-GCTTTCTCTGCTCTGATGTG3' (reverse) for *RANKL*; 5'-CCAG GCAGTCAGATCATC3' (forward) and 5'-AGGTACAGGCCCTC TGAT3' (reverse) for *TNF-α*; 5'-GGTAAGGTCCGATTCCT ACCAGG3' (forward) and 5'-CTAGAAAGGTGCAAACATG TAAC3' (reverse) for CCN2; 5'-CACAGCTCACAAGAACAGAC3' (forward) and 5'-AAGGTGAGGTTAGCATGTCC3' (reverse) for *OPG*; 5'-GGCAAGGAGGTGACCGAGTT3' (forward) and 5'-CACTGCCGCCACTTCATCTC3' (reverse) for PGE_2; 5'-ATGCAG CGGAGACTGGTT3' (forward) and 5'-TGTCATGGAGGAGCT GATG3' (reverse) for *PTHrP*; 5'-TGAGAGTAGTGAGGAA CAAGC3' (forward) and 5'-ATTTGTGGTTGGGTCAGG3' (reverse) for IL-6; 5'-ACGGCTGCTGCATTAACATAA3' (forward) and 5'-TGGTTTTGGGTATCTCAGGC3' (reverse) for *IL-1α*; 5' CCCTGATGAGATCGAGTACATCTT3' (forward) and 5'-AGGG GCAAGGCCCACAGGGATTT3' (reverse) for *VEGF*; and 5'-TGAACGGGAAGCTCACTGG3' (forward) and 5'-TCCACCA CCCTGTTGCTGTA3' (reverse) for *GAPDH*. RT-PCR was performed with a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in Light Cycler capillaries by using a commercially available master mix containing Taq DNA polymerase and SYBR-Green I deoxyribonucleoside triphosphates (Light Cycler DNA master SYBR-Green I; Roche Molecular Biochemicals). After the addition of primers (final concentration: 10 μM), $MgCl_2$ (3 mM), and template DNA to the master mix, 65 cycles of denaturation (95˚C for 15 s) and extension (60˚C for 45 s) were performed. After the completion of PCR amplification, a melting curve analysis was performed.

Osteoclastogenesis assay. Mouse bone marrow cells (1×10^6 /well) were cultured in 48-well plates containing αMEM with 30 ng/ml soluble recombinant mouse RANKL (PeproTech EC, Ltd, London, UK) in the presence of 50% HSC-2 or HSC-4 cell-conditioned medium (HSC2-CM, HSC4-CM) or of 50% medium conditioned by cells pretreated for 24 h with 5 ng/ml TGF-β (HSC2-TGFβ-CM, HSC4-TGFβ-CM) or 500 nM TβRI-I (HSC2-TβRI-I, HSC4-TβRI-I) or both (HSC2-TβRI-I+TGFβ-CM, HSC4-TβRI-I+TGFβ-CM) for 6 days until typical multinucleate cells were observed. The cells were then fixed and stained for TRAP activity by using a commercially available kit (Sigma), and TRAP-positive cells containing 3 nuclei were counted as osteoclasts under microscopic examination. The Animal Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences approved all experimental procedures.

Statistical analysis. The data were analysed by using the Fisher's protected least significant difference (Fisher's PLSD) for the analysis of TRAP activity-positive cells and Fisher's exact test for the immunohistochemical analysis of the 21 cases. The results were expressed as mean±S.D and $p<0.05$ was considered statistically significant.

Results

Histological analysis. Figure 1 illustrates a representative histological pattern of erosive (Figure 1A) and invasive (Figure 1B) bone destruction observed in a patient with oral SCC in the mandibular region. The erosive type showed a well-defined border between the tumour and the bone (Figure 1A). In contrast, the invasive type exhibited tumour cells that had filled the bone marrow space and destroyed both the trabecular and cortical bone of the mandible (Figure 1B). Of note, an abundance of TβRI had been produced by the tumour cells that had invaded the bone matrix (Figure 1C).

Relationship between histological classification and TβRI expression. Eighteen out of the 21 cases of mandible SCC showed strong intensity of TβRI immunoreactivity (85.7%). The samples with the invasive pattern of bone destruction significantly expressed TβRI ($p<0.05$, Figure 1D).

Blockade of TGF-β signaling by TβRI-I in HSC-2 cells. To determine whether oral SCC cell lines expressed TβRI *in vitro*, Western blotting was used to examine HSC-2, HSC-4, HSC-3, HO1u1 and HO1N1 cells for the expression of TβRI. As shown in Figure 2A, HSC-2 cells highly expressed TβRI, whereas HSC-4, HSC-3, HO1u1 and HO1N1 cells did so only weakly. Next, the effect of TGF-β on Smad phosphorylation in HSC-2 cells was examined. HSC-2 cells were treated with 1 or 5 ng/ml TGF-β and 100 or 1000 nM TβRI-I, and then examined by Western blot analysis for endogenous phospho-Smad2/3. Phosphorylation of Smad2/3 was up-regulated by the treatment with TGF-β, and the increased Smad2/3 phosphorylation was completely blocked by 100 or 1000 nM TβRI-I (Figure 2B). The total Smad2/3 level remained constant at all doses (Figure 2B). The effect of TGF-β on HSC-2 cell proliferation was then examined. The HSC-2 cells

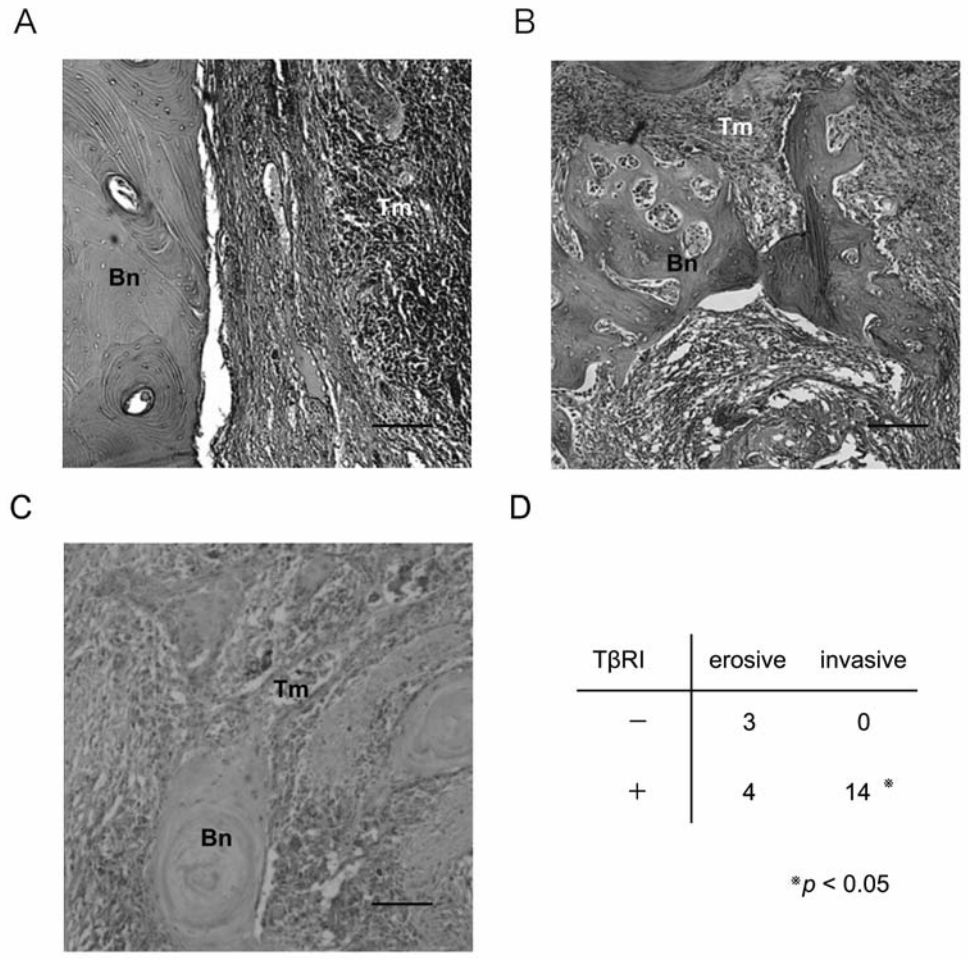

Figure 1. *Histomorphometric and immunohistochemical analysis of oral squamous cell carcinoma of the mandibular region. A: and B: HE-stained sections of resected mandibles showing the erosive type (A) and invasive type (B) of metastasis. C: Immunohistochemical staining of TβRI in a section of a resected mandible showing the invasive pattern. Bn: Bone, Tm: tumour tissue. D: Immunohistochemical analysis of 21 cases of oral SCC of the mandible by use of anti-TβRI antibody. Scale bar: 200 μm.*

did not respond to the treatment with 0.5-10 ng/ml TGF-β with or without 1000 nM TβRI-I (Figure 2C). Cell motility and invasion are critical metastatic events that occur in epithelial cells during cancer progression. Therefore, the effect of TGF-β on the migration of HSC-2 cells was examined in a Boyden chamber assay. Treatment with TGF-β increased the migration of the cells dose dependently, and this stimulated cell migration was significantly blocked by the treatment with 100 or 1000 nM TβRI-I for 6 h (Figure 2D).

Effect of TβRI-I on HSC-2 cell-induced osteolysis. Since a recent study by Ehata *et al*. (16) suggested that an inhibitor of TGF-β type I receptor kinase inhibits bone metastasis of a human breast cancer cell line, the next *in vivo* experiment was designed to determine the effect of TβRI-I on the development of HSC-2 cell-induced osteolysis. Nude mice were administered *i.p*. a 10 mg/kg dose of TβRI-I every day from the day after the bone injection of tumour cells for 28 days and comparatively analysed with similarly inoculated animals treated with control vehicle. Figure 3A illustrates representative radiographs taken 28 days after tumour inoculation. Obvious osteolytic lesions were present in mice that had received the control vehicle, whereas very few osteolytic lesions were present in the mice treated with TβRI-I. Representative histological horizontal sections through the tibial metaphysis are presented in Figure 3B. The tumour cells had filled the bone marrow space and destroyed both trabecular and cortical bone in mice that had received the control vehicle. Osteoclasts at the destroyed bone/tumour cell interface (Figure 3B, arrowheads) and microvessels (Figure 3B, arrows) were observed in the osteolytic lesion of the control vehicle-treated mice. In contrast, most of the TβRI-I-treated mice retained intact cortical bone, and many bones had no evidence of tumour involvement. The total area of radiographic osteolytic

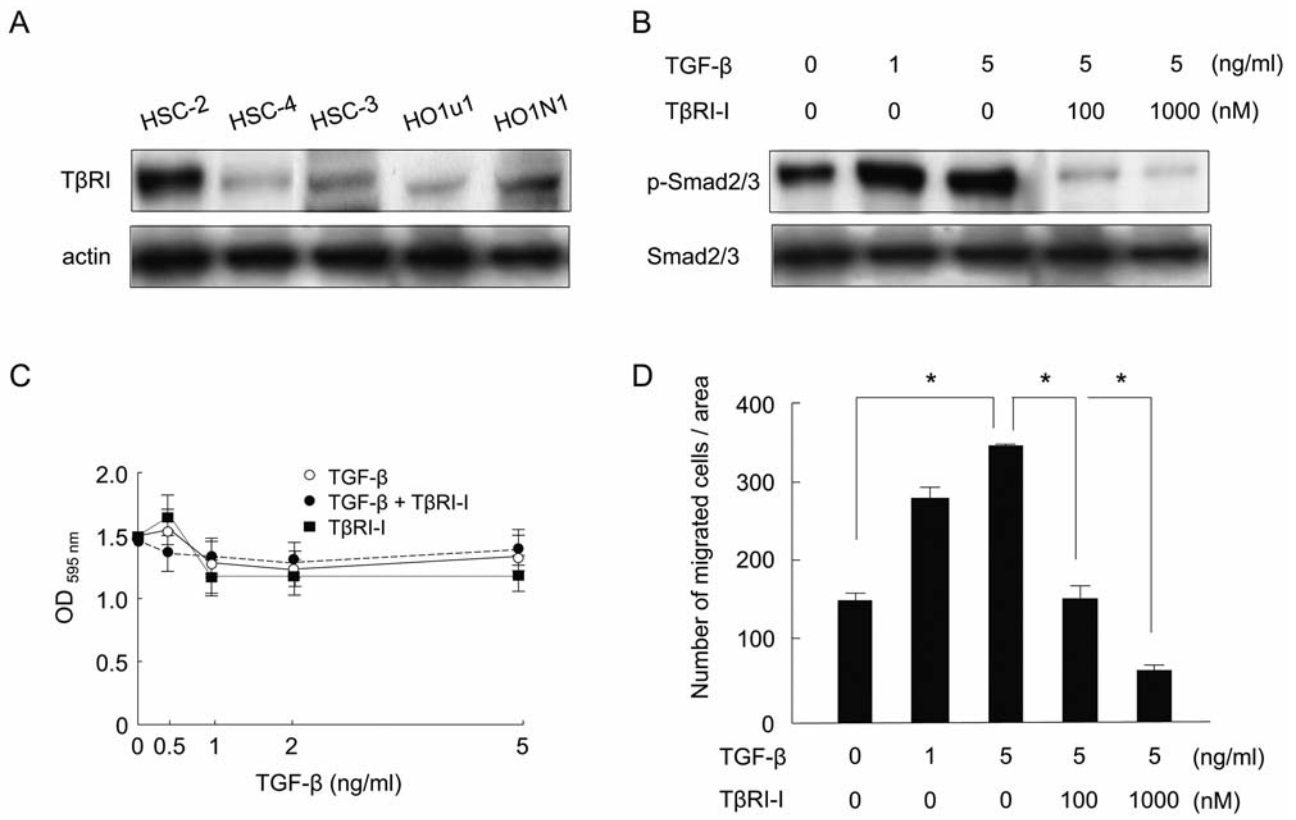

Figure 2. *Inhibition of TGF-β signalling by TβRI-I. A: Oral SCC cell lines HSC-2, HSC-4, HSC-3, HO1u1, and HO1N1 were examined for the expression of TβRI. B: HSC-2 cells were treated or not with TGF-β at 1 or 5 ng/ml or with TGF-β (5 ng/ml) plus 100 or 1000 nM TβRI-I for 60 min. Western blot analysis was performed with the cell lysates to determine the levels of TβRI, activated/phosphorylated Smad2/3 (p-Smad2/3) and Smad2/3. C: MTT assay was conducted to obtain relative cell number as reflected by the absorbance at 595 nm in the presence of the indicated amounts of TGF-β with or without 1000 nM TβRI-I. ○: TGF-β, ●: TGF-β+TβRI-I, ■: TβRI-I. D: In a Boyden chamber migration assay, HSC-2 cells in serum-free medium were placed inside the insert of an invasion chamber and treated with 1 or 5 ng/ml TGF-β in the presence or absence of 100 or 1000 nM TβRI-I for 6 hours. The lower chamber contained the complete medium with serum as a chemoattractant. Cells were removed from the upper surface of the chamber membrane, and the membrane was then stained for the migrated cells, which were then counted under a microscope. Similar results were obtained in at least 3 independent experiments. Statistically significant differences ($p<0.05$) between bracketed groups are marked by an asterisk.*

lesions from all tibia was quantified by using a computerised image-analysis system (Figure 3C). The lesion area was significantly smaller in mice treated with TβRI-I than in those given the control vehicle ($p<0.05$). Histomorphometric analysis of the tibia confirmed the radiographic quantification of the osteolytic lesion area. The tumour tissue area was significantly smaller in mice treated with TβRI-I than in those that had received the control ($p<0.01$, Figure 3D).

CM from HSC-2 cultures pretreated with TGF-β stimulated osteoclast formation. Since TβRI-I reduced the amount of HSC-2 cell-induced bone destruction and the number of osteoclasts in the tumour/bone interface, the effect of HSC2-CM on osteoclast formation was studied. Total bone marrow cells were treated with 50% HSC2-CM or HSC4-CM in the presence of 30 ng/ml RANKL for 6 days. Other bone marrow cells were similarly treated with medium conditioned by HSC-2 cells that had been pretreated with 5 ng/ml TGF-β for 24 h (HSC2-TGFβ-CM). This CM stimulated osteoclast formation more than HSC2-CM or HSC4-TGFβ-CM ($p<0.05$). HSC-2 cells that had been pretreated with 500 nM TβRI-I for 30 min and treated with 5 ng/ml TGF-β for 24 h were also used to prepare conditioned medium (HSC2-TβRI-I-TGFβ-CM), which significantly reduced osteoclast formation induced by HSC2-TGFβ-CM ($p<0.05$, Figure 4). However, osteoclast formation in response to HSC4- TGFβ-CM was no greater than that obtained by the treatment with HSC4-CM (Figure 4).

TGF-β induced RANKL, TNF-α and CCN2 gene expression in HSC-2 cells. To examine the effects of TGF-β signalling on various osteoclastogenic factors in HSC-2 cells, the cells were treated with 5 ng/ml TGF-β for 24 h, total RNA was

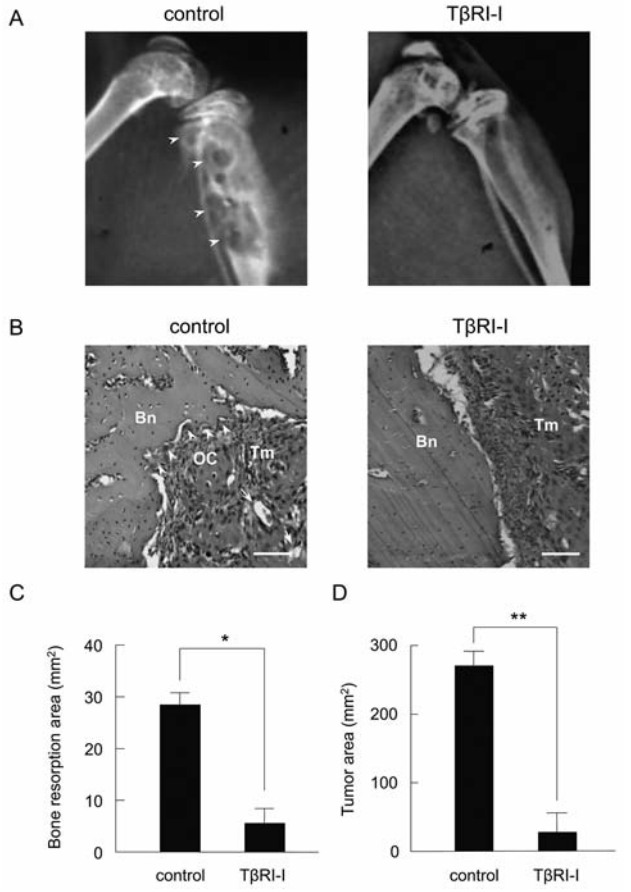

Figure 3. *Radiographic and histomorphometric analysis of bone from mice bearing HSC-2 cells and treated with either control vehicle or 10 mg/kg TβRI-I (n=10/group). Representative radiographs (A) and histology (B) of tibia from mice 28 days after tumour inoculation. The arrowheads indicate osteolytic lesions (A) and osteoclast (B), whereas arrows indicate microvessels (B). C: Osteolytic lesion area (mm²) on radiographs of hind limbs from tumour-bearing mice. D: Histmorphometric analysis of tumour area (mm²) in osteolytic lesions of tumour-inoculated mice. Statistically significant differences (p<0.05) between bracketed groups are marked by an asterisk. Oc: Osteoclast; Bn: bone; Tm: tumour tissue; scale bar: 200 μm.*

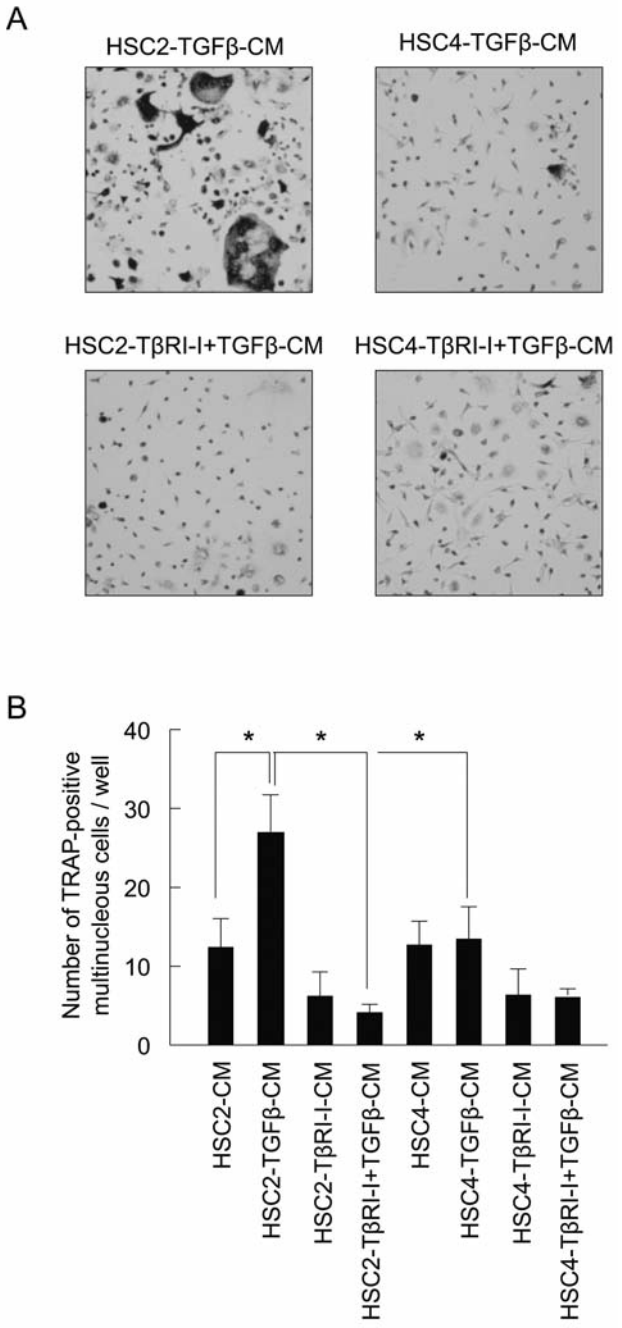

Figure 4. *CM from HSC2 cells pretreated with TGF-β stimulated osteoclast formation. Mouse total bone marrow cells were cultured with 30 ng/ml RANKL in the presence of HSC2-CM or HSC4-CM. Others were similarly cultured, but with CM from HSC2 or HSC4 cells pretreated for 24 hours with 5 ng/ml TGF-β (HSC2-TGFβ-CM, HSC4-TGFβ-CM), or 500 nM TβRI-I (HSC2-TβRI-I-CM, HSC4- HSC2-TβRI-I), or with both (HSC2-TβRI-I+TGFβ-CM, HSC4-TβRI-I+TGFβ-CM). After having been cultured for 6 days, the cells were fixed and stained for TRAP. Representative picture of TRAP staining (A) and number of TRAP-positive multinucleate (>3 nuclei) cells (B) are shown. Similar results were obtained in at least 3 independent experiments. Statistical significant differences (p<0.05) between bracketed groups are marked by an asterisk.*

extracted and RT-PCR was performed. TGF-β induced the expression of *RANKL*, *TNF-α* and *CCN2* mRNAs, and TβRI-I significantly reduced that of all of them ($p<0.05$, Figure 5 A-C). However, osteoclast regulatory genes, such as *OPG, PGE2, PTHrP, IL-6, IL-1α* and *VEGF,* were not responsive to the treatment with TGF-β (Figure 5 D).

Discussion

The activation of TGF-β signalling in mammary tumours has been shown to accelerate the progression of metastasis to bone, lungs and other organs *in vivo* (17, 18). However, the

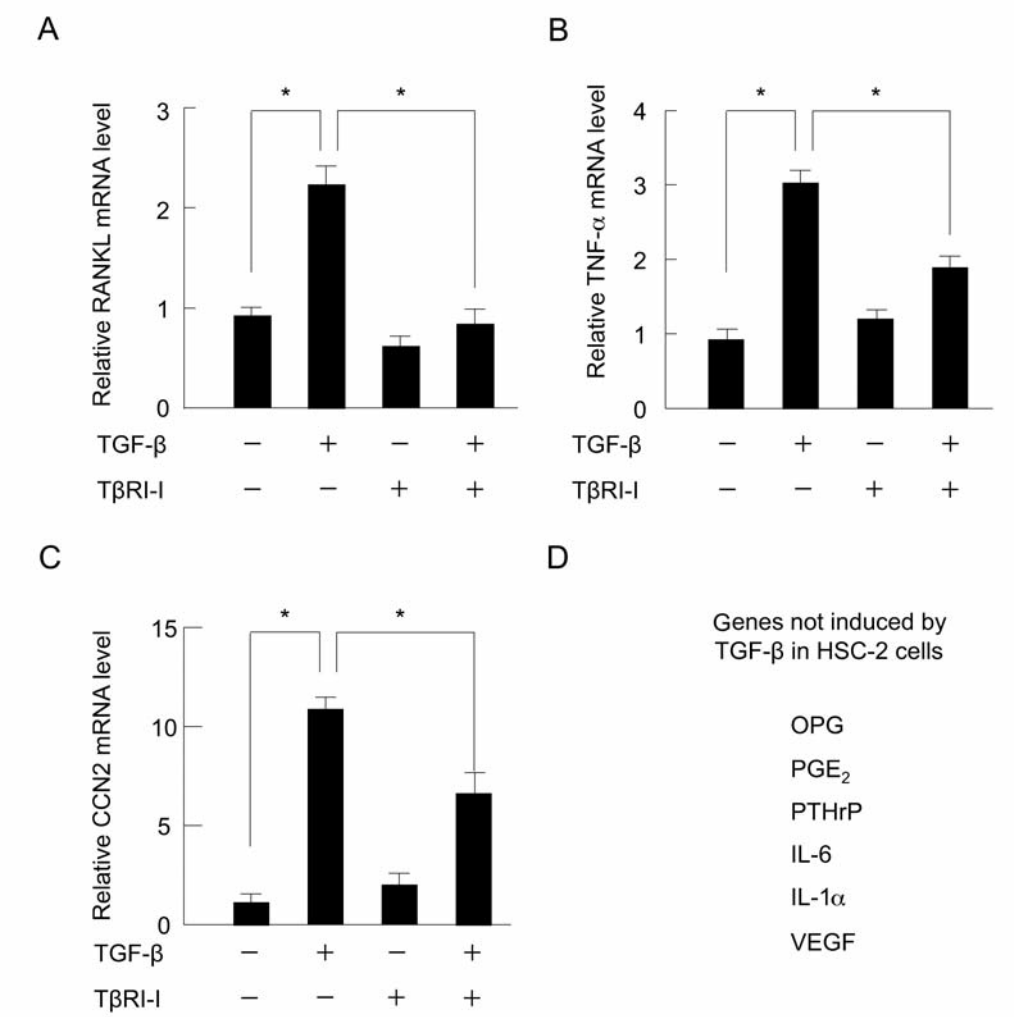

Figure 5. *Effect of TGF-β signalling on the gene expression of various osteoclastogenic factors in HSC-2 cells. HSC-2 cells were left untreated or were treated with 5 ng/ml TGF-β for 24 h in the presence or absence of 1000 nM TβRI-I. Total RNA was extracted, and the RANKL (A), TNF-α (B), CCN2 (C), and OPG, PGE_2, PTHrP, IL-6, IL-1α, and VEGF (D) expression levels were examined by quantitative RT-PCR. Fold-induction by TGF-β stimulation is indicated. Each value is normalised to the expression of GAPDH. Similar results were obtained in at least 3 independent experiments. Statistically significant differences ($p<0.05$) between bracketed groups are marked by an asterisk.*

role of TβRI in oral SCC cells is not well understood. The purpose of the present study was to clarify the role of TβRI in oral SCC cell-induced bone destruction by performing clinical histological examination and using a bone invasion mouse model.

Previous clinicopathological studies have suggested that the invasive type of oral SCC exhibits more aggressive bone destruction than the erosive type (2). The present results demonstrated that the invasive type of SCC showed highly expressed TβRI in all cases examined, whereas the erosive-type SCC expressed it in about half of the cases. These finding suggest that the increase in locally active TGF-β signalling altered tumour-cell behavior to promote growth and bone destruction at the invasion site.

To evaluate the role of TGF-β signalling in oral SCC further, the human oral SCC carcinoma cell line HSC-2 was selected for the *in vitro* and *in vivo* experiments, as it highly expressed TβRI. The results indicate that HSC-2 cells possess an operational TGF-β signalling pathway and that TβRI-I can block TGF-β signalling in cells effectively, as indicated by the lower amount of TGF-β-induced phosphorylated Smad2/3. In contrast, TGF-β did not inhibit the growth of HSC-2 cells, as shown in Figure 2C. As such, its signalling in cells is likely to promote malignant progression.

Many steps are involved in metastasis from the primary site to the skeleton. Acquisition of tumour cell motility plays a fundamental role in the onset and progression of metastatic

cancer, which is induced by TGF-β in experimental models (19). This study hypothesised that osteolytic destruction of bone matrix will release TGF-β to activate a feedback signalling pathway in tumour cells. It was shown that blockade of TGF-β signalling by TβRI-I inhibited TGF-β-induced migration of HSC-2 cells effectively, providing a mechanism by which TβRI-I suppressed TGF-β-induced invasion of bone by tumour cells.

Current experimental studies support the notion that there is a vicious cycle at the bone metastatic site, where metastatic cells stimulate osteoclast-mediated bone resorption, while bone-derived growth factors released from the resorbed bone promote tumour growth (20). The most abundant repository for TGF-β is the bone matrix, and this factor is released locally into the microenvironment as a consequence of osteoclastic bone resorption (21). One study showed that plasma TGF-β1 levels are elevated in more than half of the cancer patients with bone metastasis (22). The histopathological classification of a destructive invasion type correlated with TβRI accumulation, suggesting that TβRI may be considered as a histological marker for oral SCC-induced mandible destruction and that overexpression of TβRI in tumour cells induced the observed osteoclastogenesis. TGF-β released from the bone causes a further increase in the expression of the TGF-β-responsive osteoclast-inducing genes, such as *PTHrP* and *CCN2*, in cancer cells, thus establishing a composite positive-feedback cycle of metastasis (14, 23). In the present study, treatment of HSC-2 cells with TGF-β did not increase PTHrP expression; however, it up-regulated CCN2 expression in the cells significantly. In fact, the distribution of proliferating CCN2-positive oral SCC cells in the resected mandible correlates with that of the bone-destructive invasion type (24). A recent study showed that *CCN2* antisense S-oligodeoxynucleotide inhibits the induction of osteoclast formation by total mouse bone marrow cells and in a co-culture system that consisted of mouse spleen and ST2 cells, a clonal line of stromal cells from bone marrow (14). Importantly, rCCN2 stimulates osteoclastogenesis by mouse total bone marrow cells in the presence of RANKL (24) and by $CD14^+$ monocytes, with macrophage colony-stimulating factor and RANKL (25) representing a direct or indirect effect of CCN2 on osteoclastogenesis. RANKL- and TNF-α-producing cancer cells were also observed in the invasive-type resected mandible (data not shown). These data agreed with those of a previous study showing that RANKL expression is up-regulated in bone metastasis of prostate cancer and that blocking the RANK/RANKL interaction prevents the progression of prostatic carcinoma in bone (26, 27). TNF-α is also central, *via* NF-κB, to the interactions between tumour cells and macrophages that result in increased invasive capacity of malignant cells (28). Stromal/osteoblastic cells are essential for *in vitro* osteoclastogenesis through cell-to-cell interactions (29). Therefore, it is hypothesised that tumour cell-produced CCN2 and TNF-α may facilitate cell-to-cell signalling by interacting with multiple molecules on the surface of these cells.

The final decision on conservative mandibular resection is usually taken during surgery, based on pre-operative X-ray imaging, laboratory examination data and on-site histological examination, as seems appropriate to the surgeon at the time. If a rapid and automated processing device could be developed, the immunohistochemical detection of TβRI would be useful in deciding whether mandibular resection should be performed. In addition, if such a TβRI examination could be applied in advance by biopsy, the data would be useful in the discussion of treatment options with the patient. Further research needs to be done to confirm these observations in larger samples and with molecular biology techniques such as microarrays, RT-PCR and protein studies.

The results of this study indicate for the first time that inhibition of TGF-β signalling by systemic administration of TβRI-I could effectively suppress the invasion and bone destruction by human oral SCC HSC-2 cells. Clearly, more studies are needed for the determination of whether TGF-β signalling antagonists, including TβRI-I, can be suitable as therapeutic agents for the treatment and prevention of oral SCC-induced bone destruction. In conclusion, TβRI may be a diagnostic marker and potential target for the treatment of oral squamous cell carcinoma-induced destruction of the mandible. Further investigation on the molecular mechanisms of TGF-β signalling and the TGF-β-inducible genes responsible for mandibular destruction is crucial to the goal of finding an effective therapy for bone-destructive oral squamous cell carcinoma.

Acknowledgements

This work was supported, in part, by grants from the WESCO Scientific Promotion Foundation (to TS) and the programmes Grants-in-Aid for Young Scientists (A) (to TS) and Exploratory Research (to AS) from the Ministry of Education, Culture, Sports, Science and Technology.

References

1 Brown JS, Lowe D, Kalavrezos N, D'Souza J, Magennis P and Woolgar J: Patterns of invasion and routes of tumor entry into the mandible by oral squamous cell carcinoma. Head Neck *24*: 370-383, 2002.

2 Shaw RJ, Brown JS, Woolgar JA, Lowe D, Rogers SN and Vaughan ED: The influence of the pattern of mandibular invasion on recurrence and survival in oral squamous cell carcinoma. Head Neck *26*: 861-869, 2004.

3 Massague J: TGFbeta in Cancer. Cell *134*: 215-230, 2008.

4 Leask A and Abraham DJ: TGF-beta signalling and the fibrotic response. FASEB J *18*: 816-827, 2004.

5 Bierie B and Moses HL: Transforming growth factor beta (TGF-beta) and inflammation in cancer. Cytokine Growth Factor Rev, 2009.

6 Massague J, Blain SW and Lo RS: TGFbeta signalling in growth control, cancer, and heritable disorders. Cell *103*: 295-309, 2000.

7 Tan AR, Alexe G and Reiss M: Transforming growth factor-beta signalling: emerging stem cell target in metastatic breast cancer? Breast Cancer Res Treat *115*: 453-495, 2009.
8 Mundy GR: Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer *2*: 584-593, 2002.
9 Ge R, Rajeev V, Ray P, Lattime E, Rittling S, Medicherla S, Protter A, Murphy A, Chakravarty J, Dugar S, Schreiner G, Barnard N and Reiss M: Inhibition of growth and metastasis of mouse mammary carcinoma by selective inhibitor of transforming growth factor-beta type I receptor kinase *in vivo*. Clin Cancer Res *12*: 4315-4330, 2006.
10 Petersen M, Pardali E, van der Horst G, Cheung H, van den Hoogen C, van der Pluijm G and Ten Dijke P: Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. Oncogene *29*: 1351-1361, 2010.
11 Shinto O, Yashiro M, Kawajiri H, Shimizu K, Shimizu T, Miwa A and Hirakawa K: Inhibitory effect of a TGFbeta receptor type-I inhibitor, Ki26894, on invasiveness of scirrhous gastric cancer cells. Br J Cancer, 2010.
12 Brown JS, Griffith JF, Phelps PD and Browne RM: A comparison of different imaging modalities and direct inspection after periosteal stripping in predicting the invasion of the mandible by oral squamous cell carcinoma. Br J Oral Maxillofac Surg *32*: 347-359., 1994.
13 Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR and Yoneda T: Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res *55*: 3551-3557, 1995.
14 Shimo T, Kubota S, Yoshioka N, Ibaragi S, Isowa S, Eguchi T, Sasaki A and Takigawa M: Pathogenic role of connective tissue growth factor (CTGF/CCN2) in osteolytic metastasis of breast cancer. J Bone Miner Res *21*: 1045-1059, 2006.
15 Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, Cornell JE, Pollock BH, Mundy GR and Sun LZ: Inhibition of pulmonary and skeletal metastasis by a transforming growth factor-beta type I receptor kinase inhibitor. Cancer Res *66*: 6714-6721, 2006.
16 Ehata S, Hanyu A, Fujime M, Katsuno Y, Fukunaga E, Goto K, Ishikawa Y, Nomura K, Yokoo H, Shimizu T, Ogata E, Miyazono K, Shimizu K and Imamura T: Ki26894, a novel transforming growth factor-beta type I receptor kinase inhibitor, inhibits *in vitro* invasion and *in vivo* bone metastasis of a human breast cancer cell line. Cancer Sci *98*: 127-133, 2007.
17 Korpal M, Yan J, Lu X, Xu S, Lerit DA and Kang Y: Imaging transforming growth factor-beta signalling dynamics and therapeutic response in breast cancer bone metastasis. Nat Med *15*: 960-966, 2009.
18 Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR and Massague J: TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell *133*: 66-77, 2008.
19 Leivonen SK and Kahari VM: Transforming growth factor-beta signalling in cancer invasion and metastasis. Int J Cancer *121*: 2119-2124, 2007.
20 Yoneda T and Hiraga T: Crosstalk between cancer cells and bone microenvironment in bone metastasis. Biochem Biophys Res Commun *328*: 679-687, 2005.
21 Pfeilschifter J and Mundy GR: Modulation of transforming growth factor b activity in bone cultures by osteotrophic hormones. Proc Natl Acad Sci USA *84*: 2024-2028, 1987.
22 Baselga J, Rothenberg ML, Tabernero J, Seoane J, Daly T, Cleverly A, Berry B, Rhoades SK, Ray CA, Fill J, Farrington DL, Wallace LA, Yingling JM, Lahn M, Arteaga C and Carducci M: TGF-beta signalling-related markers in cancer patients with bone metastasis. Biomarkers *13*: 217-236, 2008.
23 Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA and Massagué J: A multigenic program mediating breast cancer metastasis to bone. Cancer Cell *3*: 537-549, 2003.
24 Shimo T, Kubota S, Goda T, Yoshihama Y, Kurio N, Nishida T, Ng PS, Endo K, Takigawa M and Sasakii A: Clinical significance and pathogenic function of connective tissue growth factor (CTGF/CCN2) in osteolytic mandibular squamous cell carcinoma. Anticancer Res *28*: 2343-2348, 2008.
25 Nozawa K, Fujishiro M, Kawasaki M, Kaneko H, Iwabuchi K, Yanagida M, Suzuki F, Miyazawa K, Takasaki Y, Ogawa H, Takamori K and Sekigawa I: Connective tissue growth factor promotes articular damage by increased osteoclastogenesis in patients with rheumatoid arthritis. Arthritis Res Ther *11*: R174, 2009.
26 Wittrant Y, Theoleyre S, Chipoy C, Padrines M, Blanchard F, Heymann D and Redini F: RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. Biochim Biophys Acta *1704*: 49-57, 2004.
27 Zhang J, Dai J, Yao Z, Lu Y, Dougall W and Keller ET: Soluble receptor activator of nuclear factor kappaB Fc diminishes prostate cancer progression in bone. Cancer Res *63*: 7883-7890, 2003.
28 Balkwill F: TNF-alpha in promotion and progression of cancer. Cancer Metastasis Rev *25*: 409-416, 2006.
29 Udagawa N, Takahashi N, Jimi E, Matsuzaki K, Tsurukai T, Itoh K, Nakagawa N, Yasuda H, Goto M, Tsuda E, Higashio K, Gillespie MT, Martin TJ and Suda T: 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 *25*: 517-523, 1999.

Received April 28, 2010
Revised May 18, 2010
Accepted May 25, 2010