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Transforming growth factor-β synthesized by stromal cells and cancer cells participates in bone resorption induced by oral squamous cell carcinoma

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ABSTRACT

Transforming growth factor beta (TGF-β) plays a significant role in the regulation of the tumor microenvironment. To explore the role of TGF-β in oral cancer-induced bone destruction, we investigated the immunohistochemical localization of TGF-β and phosphorylated Smad2 (p-Smad2) in 12 surgical specimens of oral squamous cell carcinoma (OSCC). These studies revealed TGF-β and p-Smad2 expression in cancer cells in all tested cases. Several fibroblasts located between cancer nests and resorbing bone expressed TGF-β in 10 out of 12 cases and p-Smad2 in 11 out of 12 cases. Some osteoclasts also exhibited p ~ Smad2 expression. The OSCC cell line, HSC3, and the bone marrow-derived fibroblastic cell line, ST2, synthesized substantial levels of TGF-β. Culture media derived from HSC3 cells could stimulate *Tgf-β1* mRNA expression in ST2 cells. Recombinant TGF-β1 could stimulate osteoclast formation induced by receptor activator of nuclear factor kappa-B ligand (RANKL) in RAW264 cells. TGF-β1 could upregulate the expression of p-Smad2 in RAW264 cells, and this action was suppressed by the addition of a neutralizing antibody against TGF-β or by SB431542. Transplantation of HSC3 cells onto the calvarial region of athymic mice caused bone destruction, associated with the expression of TGF-β and p-Smad2 in both cancer cells and stromal cells. The bone destruction was substantially inhibited by the administration of SB431542. The present study demonstrated that TGF-β synthesized by both cancer cells and stromal cells participates in the OSCC-induced bone destruction.

1. Introduction

Bone destruction by oral cancer predicts poor prognosis and reduction of quality of life [1–4]; however, the molecular mechanism of bone destruction by oral cancer is poorly understood. Cancer-associated bone destruction is essentially mediated through osteoclastic bone resorption. We demonstrated that fibroblasts interspersed within the cancer and bone played a crucial role in the osteoclastic bone destruction by oral squamous cell carcinoma (OSCC) by producing receptor activator of nuclear factor-κB (RANK) ligand (RANKL) [5]. RANKL, an essential factor for osteoclastogenesis [6], is primarily synthesized by bone marrow stromal cells and osteoblast-lineage cells and it promotes hematopoietic progenitors of monocyte/macrophage lineage cells to mature osteoclasts [7]. We also reported that RANKL synthesized by both, cancer and stromal cells, was involved in the osteoclastic bone resorption in oral cancers [8].

Cancer cells express various factors that stimulate expression of RANKL. Although interleukin (IL)-8 produced by breast cancer directly enhances osteoclastogenesis independent of the RANKL pathway [9], various factors synthesized by cancer cells stimulate osteoclastogenesis via the RANK/RANKL pathway. We demonstrated that IL-6 and parathyroid hormone-related protein synthesized by OSCC induced RANKL expression in stromal cells [10]. We also reported that CXCL2 synthesized by oral cancer cells participates in bone resorption by stimulating RANKL expression and inhibiting osteoprotegerin, which is a decay receptor for RANKL [11].

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Transforming growth factor-β (TGF-β) plays a crucial role in the regulation of the tumor microenvironment, including cell proliferation, angiogenesis, and metastasis of tumors [12,13]. TGF-β binds to TGF-β receptor 2 (TGFBR2) and TGF-β receptor 1 (TGFBR1), and the TGFBR1/TGFBR2 complex in turn activates Smad2 and Smad3 by phosphorylation. Activated Smad2 and Smad3 create a Smad complex with Smad4, and translocate to the nucleus [12]. It has been reported that TGF-β derived from breast cancer [14] and prostate cancer [15] could stimulate osteoclastic bone resorption during bone metastases. Although the molecular mechanisms for TGF-β-mediated osteoclastogenesis is not clearly understood, Yasui et al. showed that TGF-β is essential for RANKL-induced osteoclastogenesis through a molecular interaction between Smad3 and Traf6 [16]. Moreover, Gingery et al. showed TGF-β could promote osteoclast survival through the TAK1/MEK/AKT/NFκB and Smad2/3 pathways [17].

Targeting the TGF-β signaling pathways in cancer-associated bone resorption may contribute to more effective treatments [18,19]. Although TGF-β is synthesized by tumor cells in various cancers, it has been reported that TGF-β produced by stromal cells is a critical factor that affects the prognosis of esophageal cancers. This led us to investigate the precise expression profile of TGF-β and its signaling molecules in OSCC; we demonstrate that TGF-β synthesized by both, cancer cells and fibroblasts, participates in osteoclastic bone resorption.

2. Materials and methods

2.1. Reagents

Recombinant human TGF-β1 and anti-TGF-β pan-specific rabbit polyclonal neutralizing antibody (AB-100-NA) were purchased from R&D Systems (Minneapolis, MN). SB431542, which is a specific and selective inhibitor of TGF-βR1, activin type-1 receptor (ACVR)-1B, and ACVR-1C, was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Smad2 and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-phospho-Smad2 (Ser465/467) rabbit polyclonal antibody (AB3849) for immunohistochemical staining and anti-phospho-Smad2 (Ser465/467) rabbit monoclonal antibody (04–953) for western blot analysis were purchased from Merck Millipore (Billerica, MA). Anti-TGF-β1/2/3 rabbit polyclonal antibody (NBP1-72193) for immunohistochemical staining was purchased from Novus Biologicals (Littleton, CO).

2.2. Cell culture

The human OSCC cell line, HSC3, was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The mouse bone marrow-derived stromal cell line, ST2, and the mouse macrophage cell line, RAW264, were purchased from the RIKEN Bioresource Center (Tsukuba, Japan). HSC3 cells were maintained in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12). The ST2 cells were cultured in RPMI 1640 medium. The RAW264 cells were cultured in α-minimum essential medium (α-MEM). All media contained 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin G, and 100 mg/ml streptomycin. To harvest conditioned medium from HSC3 cells, the cells were grown to confluence in 100 mm dishes, and cultured for an additional 48 h in 4 ml serum-free α-MEM. The collected culture media were centrifuged at 1500 rpm for 5 min and filtered through a 0.22-μm filter unit and kept at −80 °C until use.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TGF-β1 were quantified by using a human/mouse TGF-β1 ELISA Ready-SET-Go! (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.4. Reverse transcriptase-polymerase chain reaction analyses

For reverse transcriptase-polymerase chain reaction analyses, total RNA was extracted using NucleoSpin (Macherey–Nagel, Duren, Germany). RNA samples were reverse transcribed to cDNA using oligo (dT) primers (Roche Applied Science, Basel, Switzerland), M-MuLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA), and dNTP mix (Promega, Madison, WI). The mRNA expression was examined using the Light Cycler System and FastStart Essential DNA Green Master (Roche Applied Science). The relative expression level of each mRNA was normalized using the 18S rRNA expression level. The primer sequences used for mouse *Tgf-β1* were as follows: 5′-CTCCCGTGGCTTCTAGTGC-3′ (forward) and 5′-GCCTTAGTTTGGACAGGATCTG-3′ (reverse).

2.5. Osteoclast formation

RAW264 cells were cultured in 96-well plates for 24 h, the medium was subsequently changed to α-MEM containing 20 μmol/L MEK1 inhibitor (PD98059; Cell Signaling Technology) and RANKL (100 ng/ml). The cells were treated with various concentrations of TGF-β1 and SB431542. After fixing in 10% formalin, osteoclast formation was identified by tartrate-resistant phosphatase (TRAP) staining; briefly, cells were incubated with 0.1 mol/L sodium acetate buffer (pH 5.0) containing fast red violet LB salt (Sigma-Aldrich), AS-MX phosphatase (Sigma-Aldrich), and 50 mmol/L sodium tartrate (Sigma-Aldrich) as previously described [10]. TRAP-positive cells containing more than 3 nuclei were counted as osteoclasts.

2.6. Immunohistochemical staining

Surgical specimens obtained from 12 cases of surgical excisions for mandibular gingival squamous cell carcinoma were retrieved from the archives of the Department of Oral Pathology, Tokyo Medical and Dental University. They were fixed in 10% neutral buffered formalin, then decalcified in 20% ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin. For immunohistochemical staining, the sections were pretreated in 0.01 mol/L citric acid for 30 min in the microwave. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide solution for 30 min; the sections were then incubated with primary antibodies against TGF-β and phosphorylated Smad-2 (p-Smad2) overnight at 4 °C. The sections were washed with phosphate buffered saline and treated with peroxidase-conjugated secondary antibody (Envision + Dual Link System-HRP; Dako, Glostrup, Denmark) for 1 h. Diaminobenzidine was used as the chromogen. This experimental procedure was reviewed and approved by the Ethics Review Committee of the Tokyo Medical and Dental University.

2.7. Western blot analysiss

Proteins were extracted from RAW264 cells with RIPA buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 50 mM β-glycerophosphate, 150 mM NaCl, 1 mM Na_3VO_4, 1% NP-40, 25 mM NaF) containing a protease and phosphatase inhibitors cocktail (Sigma-Aldrich). The harvested proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto