

The RANKL/RANK system as a therapeutic target for bone invasion by oral squamous cell carcinoma (Review)

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Abstract. Squamous cell carcinomas (SCCs) of the gingiva frequently invade the mandible or maxilla; this invasion is associated with a worse prognosis. The bone destruction associated with carcinomal invasion is mediated by osteoclasts rather than directly by the carcinoma. Therefore, if the cellular and molecular mechanisms by which oral SCC regulates bone invasion were known, it could inform the development of new therapeutic targets. Recently, dysregulation of the functional equilibrium in the receptor activator of NF- κ B ligand (RANKL)/RANK/osteoprotegerin (OPG) triad has been shown to be responsible for osteolysis associated with the development of malignant tumors in bone sites. Furthermore, the administration of OPG or soluble RANK prevents bone metastasis by cancer cells. In this review, we discuss recent findings indicating that bone invasion by oral SCC is mediated via RANKL/RANK and may be successfully prevented by RANKL inhibition.

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1. Introduction

Malignant tumors of the oral cavity, 95% of which are squamous cell carcinoma (SCCs), account for approximately 30% of all head and neck cancers (1). Although oral SCC represents 1-2% of all human malignancies in Japan, it is the sixth most common cancer worldwide, with more than 500,000 new cases diagnosed each year. The most common sites for SCCs are the tongue and gingiva (1-3).

Carcinoma of the mandibular gingiva, in particular, is associated with bone invasiveness in many patients (Fig. 1A and B) (4-6). Gingival SCC may eventually directly invade the mandible, a feature associated with a worse prognosis. The presence of mandibular invasion is an important criterion for deciding whether surgical intervention is necessary (7). According to the American Joint Committee on Cancer Classification, mandibular invasion is the most advanced primary stage (T4) and overall stage (IV) for these tumors. The 5-year survival of patients with stage IV oral lesions has been demonstrated to be 39%, as compared with 53, 68 and 70% for stages III, II and I, respectively (8).

The invasion of bone by oral SCCs may be associated with an increase in both osteoclastic and osteoblastic activity. Before invasion of the mandible, SCCs in close proximity to bone initially induce deposition of new bone, especially on the periosteal surface. Two distinct types of invasion of the mandible by oral SCCs have been described (6,8,9). In the erosive pattern the tumor advances on a broad front separated from bone by a layer of connective tissue and osteoclasts are present in the region between bone and stromal tissue. In contrast, the infiltrative pattern of bone invasion is associated with finger and islands of tumor tissue, which invades cancellous spaces with higher osteoclastic activity (Fig. 1C) (10,11). Cases exhibiting features of both patterns are designated as having a mixed pattern. The infiltrative type showed significantly higher rates of positive bone margin and primary site recurrence than the erosive types. In fact, the 3-year disease-free survival of patients with infiltrative and erosive patterns was 30 and 73%, respectively (12). Since these clinicopathological studies indicate that bone invasion by gingival SCC is a critical event that determines prognosis, it is important to develop a therapeutic approach to prevent the bone invasion process.

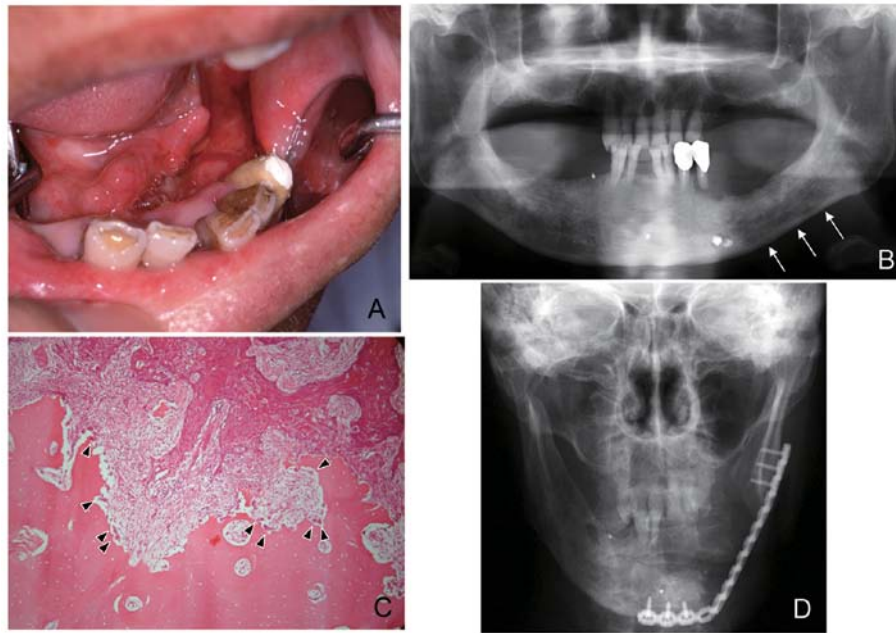


Figure 1. Clinical features of bone invasion by gingival SCCs. (A) Intraoral view of gingival SCCs. (B) Radiographic evidence of bone invasion of the mandible. Arrows outline the lesion. (C) Surgically resected samples were decalcified and stained with Hematoxylin and eosin (H&E). The infiltrative pattern of bone invasion is characterized by nests and projections of tumor cells along an irregular front, residual bone islands within the tumor, and Haversian system penetration (x100). Arrowheads indicate osteoclasts. (D) Postoperative panoramic radiograph of a patient showing the resected mandible with a free fibula microvascular transfer in place.

Mandibulectomy, if necessary, has a major influence on the patient's quality of life (QOL) and is a critical determinant of postoperative functional outcome (Fig. 1D). Patients with advanced oral SCC have a high mortality, but treatment is complicated by the disruption of speech and swallowing after surgical resection (6-11). It is generally agreed that patients with mandibular invasion should be treated surgically, although the extent of mandibular resection required remains controversial. Resection of the mandibular bone leads to physical damage and frequently also to psychological problems for patients (6-11).

The ability of oral SCCs to invade the maxilla or mandibular bone is a critical factor, which, because it leads to metastasis, affects the prognosis of patients (Fig. 1C) (3). Although controversial, bone destruction that occurs with oral SCC invasion is thought to be mediated by osteoclasts rather than by the carcinoma itself (5). Recent studies have established that bone resorption by osteoclasts is an important step in the process of bone invasion and metastasis in several types of malignancy, indicating that a full understanding of the regulation of osteoclastogenesis by oral SCC cells is necessary to prevent bone invasion by oral SCC cells. Several *in vitro* and animal experiments using human OSCC cells have shown that tumor cells produce prostaglandins and several cytokines, including interleukin-6 (IL-6), IL-11, TNF α and parathyroid hormone-related protein (PTHrP) (9,13). Indeed, bone is also a storehouse for a variety of cytokines and growth factors and thus provides an extremely fertile environment for cell growth once cancer cells arrest there (Fig. 2) (13). Shibahara *et al* have reported that the invasive tumors showed a high level of expression of IL-6, IL-11, TNF α and PTHrP (14). In contrast, expression of TGF- β , IL-1 α/β and IL-18 were not different between invasive and non-invasive tumors,

suggesting that various cancer-derived cytokines, such as IL-6, IL-11, TNF α and PTHrP, play important roles in bone invasion by oral SCC (14).

Three proteins crucial for osteoclast development and activation are the receptor activator of NF- κ B ligand (RANKL), its receptor, RANK and its decoy receptor, osteoprotegerin (OPG) (15-19). *In vitro*, the RANKL/RANK signaling pathway, together with macrophage colony-stimulation factor (M-CSF), regulates osteoclast differentiation from monocyte/macrophage progenitors (15,16), whereas the addition of OPG into these culture systems prevents osteoclastogenesis (17,18). Furthermore, these proteins are known to be involved in both normal and pathological bone metabolism. IL-6, IL-11, TNF α and PTHrP released from oral SCCs also induce RANKL expression in osteoblasts or bone marrow stromal cells (19). Thus, RANKL levels are increased in osteolytic lesions associated with malignant tumors, whereas OPG levels are increased in osteoblastic lesions (20). This suggests that blocking RANKL/RANK with soluble RANK (sRANK) or OPG inhibits osteoclastogenesis and successfully prevents bone destruction by cancers (21-23). Therefore, in this review, we first summarize the RANKL/RANK signaling pathway and its effects on osteoclastogenesis. We also describe recent discoveries on the role of RANKL/RANK in bone invasion by oral SCC, based on recent findings from our lab and others. Finally, we discuss the possibility that RANKL inhibition might successfully prevent bone invasion by oral SCC.

2. RANKL/RANK system

In the past decade, great progress has been made in understanding bone biology and, in particular, the molecular

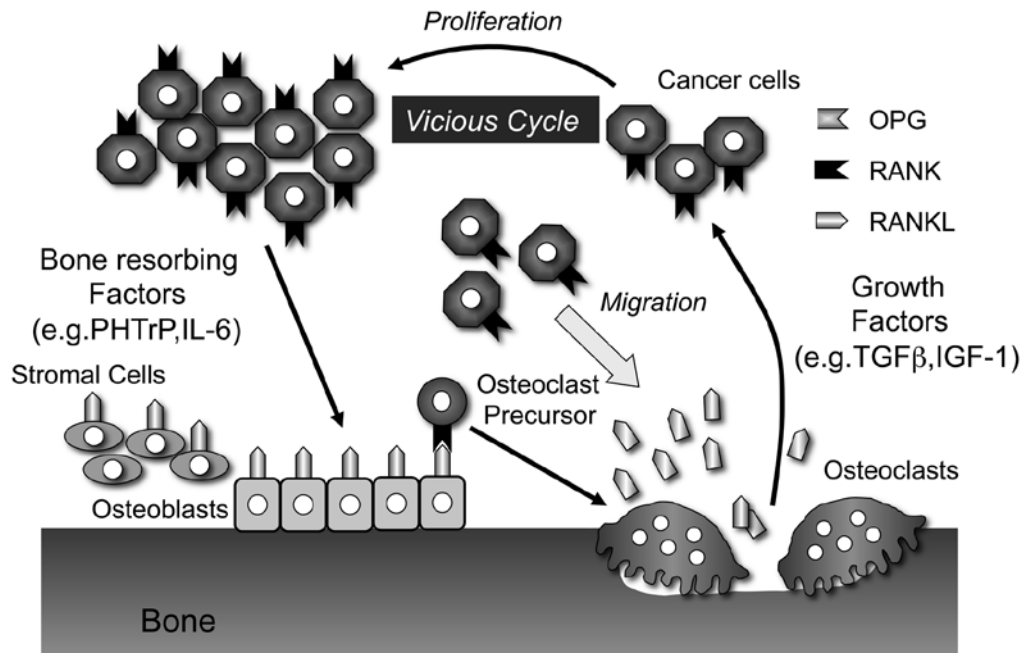


Figure 2. The vicious cycle accelerates bone invasion and cancer cell growth. Bone is a storehouse for various growth factors including insulin-like growth factor and transforming growth factor β . As a consequence of osteoclastic bone resorption, these growth factors are released into the bone microenvironment in active forms where they stimulate cancer cell proliferation. RANKL released from bone by osteoclasts also induces migration of RANK-expressing SCC cells. The bone resorption factors, such as IL-6 and PTHrP, produced by oral SCC cells induce fibroblast stromal cells/osteoblasts to synthesize RANKL or suppress OPG expression. This subsequently induces osteoclast formation and bone resorption activity.

mechanisms of osteoclast development. Osteoclasts, which are present only in bone, are large, multinucleate cells with the capacity to resorb mineralized tissue (19,24,25). Osteoclastic bone resorption consists of multiple steps: the proliferation of osteoclast precursors belong to hematopoietic cells, differentiation of progenitors into mononuclear preosteoclasts (pOCs), fusion of pOCs into multinucleate osteoclasts, clear zone (actin ring) and ruffled border formation (activation), and apoptosis. It has been proposed that osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast precursors. This hypothesis was proven by the discovery of RANKL, a member of TNF ligand family (19,24,25).

In 1997, Simonet *et al* reported the discovery of OPG, an inhibitor of bone resorption (18). OPG is a member of the TNF receptor family but, unlike all other members of the family, lacks a transmembrane domain and represents a secreted TNF receptor (Fig. 3). Tsuda *et al* independently isolated the same protein as 'osteoclast inhibitory factor (OCIF)', a heparin-binding molecule from the conditioned media of human fibroblast cultures, and showed that its cDNA sequence is identical to that of OPG (17). OPG strongly inhibits osteoclast formation in cocultures of mouse bone marrow cells and primary osteoblasts (POBs) induced by osteotropic factors such as 1α dihydroxyvitamin D_3 or PGE_2 . The administration of OPG to ovariectomized rats causes an increase in bone volume and mineral density associated with a decreased number of osteoclasts (18). Furthermore, OPG-deficient mice exhibit severe osteoporosis caused by enhanced osteoclast formation, suggesting that OPG is a physiological regulator of osteoclast development (26).

A recombinant soluble form of RANKL, together with M-CSF, induces osteoclastogenesis from mouse bone marrow cells or spleen cells in the absence of osteoblasts (15,16). RANKL was first cloned during a search for apoptosis regulatory genes in mouse T cell hybridoma and was named TNF-related activation-induced cytokine (TRANCE) (27). Two other groups also isolated ligands of OCIF and OPG, respectively, which turned out to be identical to TNF-related activation-induced cytokine (TRANCE) (15,16). RANKL is a type II transmembrane protein of the TNF ligand family that is expressed in cells such as osteoblasts and T cells (Fig. 3). RANKL-deficient mice exhibit typical osteopetrosis due to lack of osteoclasts, suggesting that RANKL is an absolute requirement for osteoclast development (28).

Anderson *et al* cloned a new member of the TNF receptor family, termed 'RANK', from a cDNA library of human dendritic cells. The mouse homolog was also isolated from the fetal mouse liver cDNA library (29). RANK is a transmembrane heterotrimer on the surface of hematopoietic osteoclast progenitors, mature osteoclasts, dendritic cells, and mammary gland epithelial cells (Fig. 3). It fails to bind other members of the TNF ligand family, such as Fas ligand, CD40 ligand, TNF α or TRAIL (19). A soluble form of RANK prevents RANKL-induced osteoclastogenesis (30). RANK-deficient mice have a complete block in osteoclast development that can be rescued by transplantation of bone marrow cells from wild-type mice, indicating that they have an intrinsic defect in osteoclast function (31).

RANK, RANKL and OPG are involved in not only physiological but also pathological conditions of bone metabolism. Bone resorption is a major pathological factor in chronic

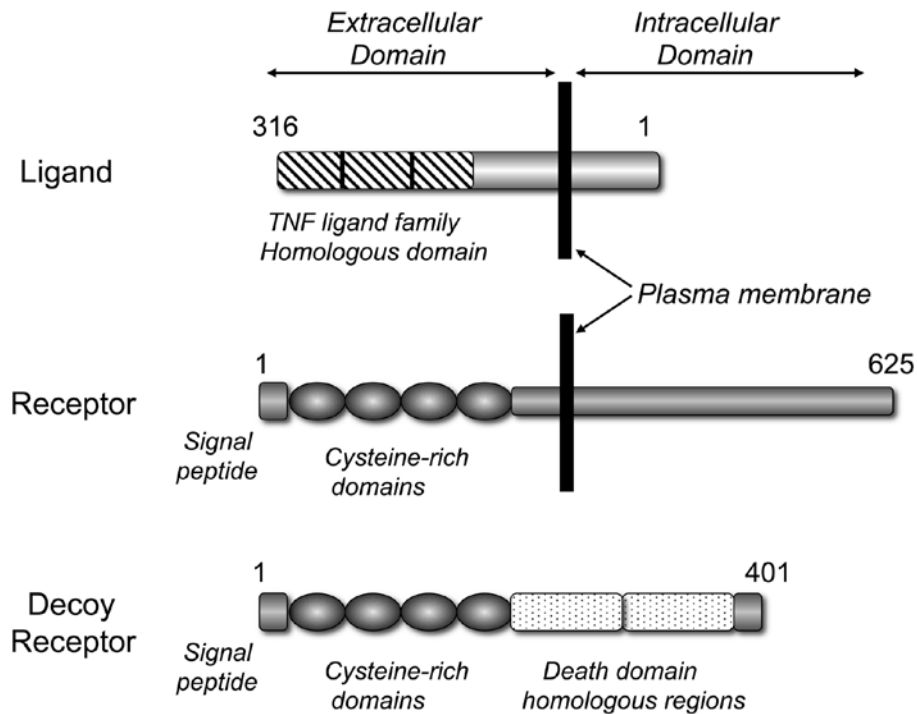


Figure 3. Molecular characteristics of RANKL, RANK and OPG. RANKL belongs to the TNF superfamily. Membrane-bound RANK and OPG belong to the TNF receptor family.

inflammatory diseases such as periodontitis, osteoporosis and arthritis. It is now clear that an imbalance in the RANKL-OPG ratio is crucial for initiating the bone loss associated with these conditions (24,25). Therefore, inhibition of RANKL/RANK signaling might be effective for preventing inflammatory bone destruction. In fact, denosumab, a human monoclonal antibody against RANKL, has been shown to effectively reduce RANK signaling and thus osteoclast activity. In large, randomized, phase III studies, it has been demonstrated to prevent fractures and bone loss and improve the bone mineral density in various cancerous and non-cancerous settings (32).

3. RANKL/RANK signaling is involved in oral SCC cell-induced osteoclastogenesis

The dysregulation of functional equilibrium in the RANKL/RANK/OPG triad is responsible for osteolysis associated with the development of malignant tumors in bone sites. Recent studies have shown that administration of OPG or sRANK prevents bone metastasis by cancer cells *in vivo* (21-23). These findings suggest that the RANKL/RANK system contributes to bone metastasis by cancer cells. However, it remains controversial whether cancer cells directly express RANKL on their surface and to what extent this expression contributes to osteoclast formation.

The expression of RANKL has already been detected in several tumor cell types and can be considered a key factor in bone remodeling associated with bone metastases (33-37). Several oral SCC cells in patients and oral SCC cell lines express RANKL (38-40). Recently, Chuang *et al* compared RANKL expression between buccal SCC without bone invasion (25 cases) and gingival SCC with invasion (15 cases).

There were no differences between the immunohistochemical expression of RANKL in cases of buccal and gingival SCC (41). This suggests that, in cases of human buccal SCC without bone invasion, tumor cells do possess the potential to induce osteoclastogenesis through the RANKL/RANK pathway if triggered under appropriate conditions. It is possible that close proximity of the cancer to the jawbone may be a prerequisite.

It has been reported that cancer cells expressing RANKL are able to induce osteoclastogenesis even in the absence of other accessory cells (38,42). On the other hand, not all cancers express RANKL, and cell-to-cell contact between cancer cells and host cells does not always lead to RANKL expression (43,44). We showed in a previous study that BHY cells, which were highly invasive to the mandibular bone when inoculated into the masseter of nude mice, expressed RANKL on their cell surface but failed to induce osteoclastogenesis in cocultures of mouse bone marrow cells (BMCs) and BHY cells (39). However, adding BHY cells to a coculture of mouse POBs and BMCs markedly induced osteoclastogenesis in the absence of osteotropic factors. Consistent with these results, HSC-2 cells, which do not express RANKL, also induced osteoclast formation in cocultures of mouse BMCs and POBs without any osteotropic factors (39). The addition of BHY cells suppressed mouse OPG mRNA expression and protein production by POBs. This finding is consistent with the observation that BHY cells did not enhance osteoclastogenesis in cocultures of BMCs and POBs from OPG-deficient mice. Furthermore, immunohistochemical analysis showed a reduction of OPG expression in stromal cells from osteolytic lesions as compared to normal lesions. Therefore, oral SCC-induced suppression of OPG expression in POBs, rather than expression of RANKL in oral SCC cells, appears critical for osteoclastogenesis (Fig. 4A) (39).

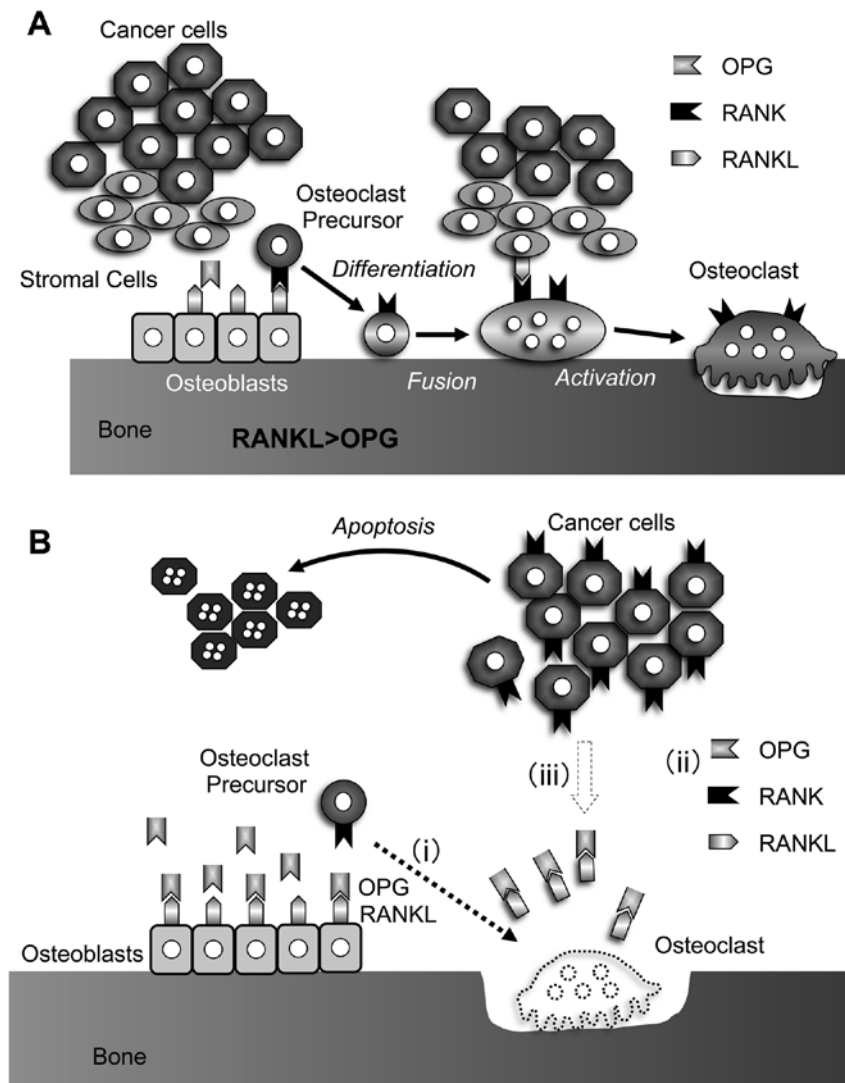


Figure 4. Possible mechanisms of OPG action on bone invasion by oral SCC. (A) Oral SCC cells induce osteoclastogenesis and bone resorption activity. (B) It is suspected that OPG suppresses mandibular bone invasion by three mechanisms; (i) OPG inhibits osteoclast differentiation; (ii) OPG also limits the supply of bone-derived growth factors, which facilitate the proliferation and survival of cancer cells that colonize bone by inhibiting osteoclastic bone resorption; (iii) furthermore, OPG suppresses RANKL-induced oral SCC cell migration.

4. Osteoclast function regulated by RANKL/RANK signaling

Despite the importance of oral SCC for osteoclastogenesis, its roles in osteoclast function, such as multinucleation, survival and pit-forming activity, is not fully understood. Therefore, we also examined the effect of oral SCCs on osteoclast function using BHY cells (45). Osteoclasts are terminally differentiated cells, with a short life span, that undergo rapid apoptosis in the absence of cytokines such as M-CSF, IL-1, TNF α or RANKL. Both BHY cells and CM from BHY cells support osteoclast survival by suppressing expression of Bim, a member of the BH (Bcl-2 homology) 3-only family of pro-apoptotic proteins (45). Soluble factors, such as IL-1 β , TNF α or RANKL, from BHY cells might contribute to the survival of osteoclasts. M-CSF, IL-1 and RANKL induced both the survival and multinucleation of prefusion osteoclasts (46). BHY cells induced not only the survival but also the multinucleation of prefusion osteoclasts.

Although the CM from BHY cells also induced multinucleation of prefusion osteoclasts, it did so less efficiently. Furthermore, adding BHY cells, but not the CM of BHY cells, induced pit-forming activity of osteoclasts. Adding OPG abrogated the activity (45). Thus, oral SCC cells regulate not only osteoclast formation but also function (Fig. 4A).

5. The role of RANKL/RANK signaling on bone invasion by oral SCCs

The addition of OPG dramatically suppresses oral SCC regulation of both osteoclast differentiation and function *in vitro*, suggesting that RANKL/RANK inhibition might be an effective therapeutic approach for inhibiting oral SCC-induced bone invasion. In support of this notion, recent studies in rodent models of breast and prostate cancer have established that inhibition of RANKL/RANK signal decreases bone lesion development and tumor growth in bone (21-23). Alternatively,

it has been reported that functional RANK is expressed on some bone-associated tumor cells (21-23,54). Indeed, the migration of RANK-positive tumor cells is induced by RANKL stimulation. Thus, these observations suggest that increased RANKL expression in the tumor-bone environment is a promoting factor for bone tumor development (Fig. 2) (21-23,47).

We injected B88 human oral SCC cells into the masseter region of nude mice to establish an animal model of oral SCC bone invasion and then determine whether OPG prevents bone invasion by oral SCC *in vivo*. Treatment with OPG for 3 weeks decreased bone invasion by B88 cells and reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts. OPG decreased tumor burden and increased cell death in B88 cells, whereas B88 cell mitosis was unchanged. Thus, the suppressive effect of OPG on B88 tumor burden is attributed to increased cell death rather than inhibition of proliferation. However, OPG did not affect apoptosis and proliferation of B88 cells *in vitro*, suggesting that effects of OPG on apoptosis in B88 cells are restricted to the bone environment (48).

Consistent with other types of cancer, tumor tissue from oral SCC patients and oral SCC cell lines, including BHY and B88, express RANK. Chuang *et al* reported that RANK expression was observed in oral SCC, but not normal mucosal tissue (41). RANK might become upregulated in cancer cells. Although tumor cells were closely associated with bone in controls, tumors were farther from the bone in mice treated with OPG. To support these results, RANKL enhanced B88 cell migration in a modified chemotaxis chamber equipped with a gelatin-coated filter. This effect was inhibited by OPG (48).

Taken together, RANKL/RANK inhibition suppresses bone invasion by inhibiting osteoclastogenesis and cancer cell migration and by inducing apoptosis of cancer cells via indirect anticancer action *in vivo* (Fig. 4B).

6. Conclusions and perspectives

Bone is a good environment for the progression of bone invasion. It has been suggested that oral SCC cells release soluble factors that activate osteoclast differentiation and function directly or indirectly via osteoblasts (Fig. 2) (13). During bone destruction, osteoclasts release various growth factors, including insulin-like growth factor and transforming growth factor β (Fig. 2). This cycle has been proposed to explain tumor development in bone. The inhibition of osteoclast differentiation and function by blocking RANKL/RANK constitutes a potentially novel approach to maintaining skeletal integrity. Indeed, blocking RANKL/RANK with sRANK or OPG successfully prevents the development of bone invasion (Fig. 4).

A phase I study testing recombinant OPG in patients with multiple myeloma- or breast carcinoma-related bone metastases is currently in progress (49). Thus far, OPG has no side-effects when administered as a single subcutaneous injection to patients (50), and it has been used subcutaneously to treat bone metastases from multiple myeloma and breast cancer cells (23,51). The RANKL inhibitor denosumab, a human monoclonal antibody against human RANKL, has also developed and is tested in the clinic. Denosumab was generally well tolerated throughout clinical trials without the patients having detectable anti-denosumab antibodies (32). In the case of mandibular bone invasion, it is possible to treat the cancer with OPG locally

because oral SCC cells invade from cortical bone, and the tumor and defect area are at the surface of the body. However, recent reports showed that the observed incidence of osteonecrosis of the jaw was comparable to that with bisphosphonates (52,53). Therefore, it is important to carefully consider the dosage and schedule of administration to avoid any side-effects of local injection of OPG or denosumab before the realization of the clinical application.

A great deal of progress has been made in understanding the pathogenesis of oral SCC. In addition, new approaches have been developed in the fields of molecular biology, cancer genetics, and cancer biology to examine the cellular and molecular mechanisms of bone invasion by oral SCCs (54). In particular, the discovery of the RANKL/RANK/OPG triad was a breakthrough in our knowledge of osteoclast biology. The development of targeted approaches against the RANKL/RANK system will contribute to preventing bone invasion by oral SCCs.

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