Novel therapeutic strategy for osteosarcoma targeting osteoclast differentiation, bone-resorbing activity, and apoptosis pathway

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Abstract

Osteosarcoma is the most common bone sarcoma, which mainly affects adolescents and young adults. Although the combination of modern surgery and systemic chemotherapy has improved osteosarcoma treatment dramatically, no substantial change in survival has been seen over the past 20 years. Therefore, novel therapeutic strategies for osteosarcoma are required if the 35% of patients with fatal metastases are to be successfully treated. Recently, osteoclasts have drawn attention as a therapeutic target in various bone disorders including osteosarcoma. The osteoclast is the sole cell that resorbs bone and is central in pathologic situations, where bone destruction is intricately involved. Osteosarcoma cells are of the osteoblastic lineage, the latter of which is characterized by cells secreting the osteoclast-inducing factor, receptor activator of nuclear factor-κB ligand. Hence, osteosarcoma is a better candidate for osteoclast-targeted therapy than other primary and metastatic bone tumors. The rapid progress on the molecular mechanism regulating osteoclast has propelled a development of new therapeutic approaches. In this review article, we present the prospects of osteoclast-targeted therapy as a novel treatment strategy for osteosarcoma. Receptor activator of nuclear factor-κB-Fc, osteoprotegerin, bisphosphonates, and Src inhibitor are shown as positive candidates and can control various aspects of osteoclast function. This review article will attempt to discuss these issues in term. [Mol Cancer Ther 2008; 7(11):3461–9]

Osteosarcoma

Osteosarcoma is the most commonly diagnosed primary malignant tumor of bone (1, 2). Contemporary treatment of osteosarcoma requires multidisciplinary therapy incorporating surgery and systemic chemotherapy (1, 3). The prognosis of osteosarcoma patients significantly improved with the advent of chemotherapy. In the pre-chemotherapy era, when patients only received surgery, the survival rate was <20% (4). In the 1970s, the role of chemotherapy before surgery (neoadjuvant chemotherapy) was introduced offering the opportunity for evaluating chemotherapy-induced tumor necrosis by histologic examination of the resected surgical specimen (5). Adoption of chemotherapy saw an increase in patient longevity that approached 65% to 70% at 5 years.

In addition, developments in imaging techniques have contributed to more effective management of osteosarcoma (6). At the end of the 19th century, it was reported that the majority of these tumors were inoperable either because of their stage, from insufficient recognition, or even from neglect (7). In other words, a large part of osteosarcoma cases were invisible because of the below par imaging techniques. Imaging techniques, including computed tomography and magnetic resonance evaluation of bone and soft-tissue involvement, was a tremendous boon to surgical planning and, when combined with functional nuclear imaging and computed tomography of the chest for detection of metastatic disease, decreased the local recurrence rate and improved patients’ survival rates dramatically. Furthermore, advances in limb-salvage surgery and reconstructive techniques in selected patients, aside from the advancement of imaging techniques, have enabled preservation of limb function without compromising oncologic outcomes (6, 8).

In the post-chemotherapy era, patient outcomes have significantly improved (2). With these various forms of advance of osteosarcoma therapy, a 5-year survival rate of 70% is expected for nonmetastatic osteosarcoma of the extremity ages <40 years. However, for these 20 years, a plateau in the survival rate seems to have been reached after this dramatic improvement. Some reasons for this stagnation have been pointed out. It is reported that dose intensification alone is not able to improve prognosis of localized osteosarcoma patients (3); thus, simple augmentation of existing chemotherapy is not effective. Even today, metastatic or multifocal osteosarcoma is a severe problem.
Clinically detectable metastatic disease at initial diagnosis in patients with high-grade osteosarcoma predicts a poor outcome, with long-term survival rates between 10% and 40% (9). The prognosis of patients with synchronous multifocal osteosarcoma is particularly poor. These reports suggested that even potent conventional chemotherapy is inadequate to address all metastases. Therefore, to improve the survival of osteosarcoma patients, there is an imperative to develop novel tumor therapies.

One important challenge is to target not only the tumor directly but also the biological systems that support it. In the case of osteosarcoma, two points are significant. One is the osteosarcoma cell lineage and the other is the primary site of osteosarcoma. Although there are three major subtypes of conventional osteosarcoma: osteoblastic osteosarcoma, chondroblastic osteosarcoma, and fibroblastic osteosarcoma. The definition of osteosarcoma is a primary intramedullary high-grade malignant tumor in which the neoplastic cells produce osteoid (1). Osteoid is secreted by osteoblasts or bone-forming cells (10). Thus, conventional osteosarcoma possesses osteoblastic features.

Under normal conditions, the skeletal system undergoes replacement continuously. Bone resorption, where the existing bone is removed by osteoclasts, large multinucleated cells of monocyte-macrophage lineage, is coupled to subsequent formation of new bone synthesized by osteoblasts. The balance of these two processes is disturbed in pathologic states such as the development and progression of primary or metastatic bone tumor. The majority of bone tumor lesions are predominantly osteolytic, biased toward excessive destruction of the bone. These primary and metastatic bone tumors secrete osteoclast-stimulating cytokines, which stimulate bone resorption by osteoclasts. In turn, tumor growth can be supported by the bone matrix-releasing factors during osteolysis. Thus, these accelerating events constitute the so-called “vicious cycle” hypothesis of bone tumor progression. In osteoblastic tumor lesions, an excess of bone is deposited, although this bone is weak with a disorganized woven structure (11). Some allude to the important role of osteoclast in the formation of osteoblastic lesion (12, 13). In most cases, osteosarcoma is a mixed osteolytic and osteoblastic lesion. This situation may be attributed to the fact that, being of the osteoblast cell lineage, osteosarcoma forms bone as well as supports osteoclastogenesis. Thus, osteoclast-targeted therapy is potentially an important avenue for addressing the progression of local and systemic disease (Fig. 1).

In this review article, we discuss the possibility of osteoclast-targeted therapy as a new therapeutic strategy for osteosarcoma.

Osteoclast Biology

Osteoclastogenesis

Bone integrity is maintained through a balance between bone formation and resorption. Osteoclast is primarily involved in bone resorption (14). It is a multinucleated cell created by the differentiation of monocyte/macrophage precursor cells. The close interaction between osteoblasts/osteoclasts and bone marrow-type cells is essential for osteoclastogenesis. Recently, it was recognized that two hematopoietic factors, tumor necrosis factor (TNF)-related cytokine receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), were critical for osteoclastogenesis (1) and the subsequent activation of RANK on the surface of osteoclast precursor cells (15, 16) that leads to the development of mature osteoclasts (ref. 17; Fig. 2). RANKL expression is induced in osteoblastic cells and bone marrow stromal cells in response to osteotropic factors such as 1,25(OH)2 vitamin D3, parathyroid hormone (PTH), and prostaglandins (14).

Binding of RANKL to RANK induces various intracellular signaling cascades including TNF receptor-associated factor 6 (TRAF6) and nuclear factor of activated T cells c1 (NFATc1) activation. TRAF family proteins are adapter molecules. They mediate intracellular signaling of various cytokine receptors including TNF receptor superfamily and Toll/interleukin (IL)-1 receptor family members (18, 19). Although RANK recruits several members of TRAF family, only TRAF6 is indispensable for osteoclastogenesis signaling capability (18, 20). TRAF6 is pivotal not only in the formation of osteoclast but also in the bone-resorbing function of mature osteoclasts (20–22). TRAF6-induced intracellular signaling includes NF-κB, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase, which are all critically involved in osteoclast development (14). Moreover, the TRAF6-induced intracellular signaling is activated by not only RANK but also CD40, although only RANK signal can induce osteoclastogenesis.

NFATc1 is a master switch in osteoclastogenesis. RANK signal is indispensable but insufficient for osteoclast differentiation alone and requires transcriptional up-regulation of NFATc1 (14) for osteoclastogenesis to occur. Several groups have used gene expression arrays to map changes in osteoclast cell culture systems. It is reported that NFATc1 is induced in response to RANKL stimulation of the macrophage cell line, RAW264.7 cells, or osteoclast precursors and that inhibiting NFAT activity by either calcineurin/NFATc1 inhibitor cyclosporine A and FK506 or antisense oligonucleotides suppressed osteoclast differentiation in vitro (23, 24). It is also shown that embryonic stem cells deficient in NFATc1 were unable to differentiate into osteoclasts in vitro and retroviral transduction of NFATc1 causes osteoclast precursor cells to engage in differentiation without RANKL stimulation. Thus, NFATc1 is regard as a terminal master regulator of osteoclastogenesis (24). It was recently revealed that intracellular calcium (Ca2+) oscillation is an important stimulus to activate NFATc1, and molecules containing immunoreceptor tyrosine-based activation motif, such as DNAX-activating protein 12 and Fc receptor common γ chain, facilitate the Ca2+-mobilizing mechanism during osteoclastogenesis (25).

RANKL not only induces the differentiation of osteoclasts from their precursor cells but also promotes the

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bone-resorbing activity of osteoclasts and prolongs their survival (26). The other important component in this signaling system is osteoprotegerin (OPG), which also belongs to the TNF receptor superfamily. OPG is a soluble decoy receptor of RANKL named for its effects to protect against bone loss. OPG specifically binds to RANKL and inhibits osteoclastogenesis induced by RANKL and bone resorption promoted by treatment with Ca2+-inducing factors (26). Thus, these events indicate that the RANK signaling pathway regulates bone resorption and Ca2+ homeostasis.

**Osteoclast Activity**

Osteoclast is a terminally differentiated cell and primarily responsible for bone resorption (27). The key to its resorptive capacity is that the osteoclast itself is able to form a microenvironment between itself and the adjacent bone. This event mainly consists of migration and adhesion to the bone surface, synthesis and directional secretion of hydrolytic enzymes, and acidification of the bone-resorbing compartment directly beneath the osteoclast. During this process, the highly polarized osteoclasts form the sealing zone. The sealing zone is formed by numerous actin filaments surrounded by a ring containing the adhesion-related cytoskeletal molecules and the structure is referred to as the “actin ring.”

Osteoclasts secrete several proteolytic enzymes into the sealed compartment. These enzymes contain cathepsin K and matrix metalloproteinase-9. Cathepsin K is an
osteoclast-specific protease and one of the main enzymes responsible for the bone resorption (17, 26). Their roles in bone mineral degradation are quite important. The proton pump ATP6i and chloride channel CIC7 are key regulator of osteoclast activity. Their function to keep the low pH (pH 3-4) of the compartment is important for demineralization of the bone matrix (ref. 26; Fig. 1).

Src, a protein tyrosine kinase and the first described proto-oncogene, is another key molecule in osteoclast biology. Although Src is expressed ubiquitously, a unique feature of osteoclasts is the requirement of Src for their function. Unexpectedly, osteopetrosis is the sole phenotype of Src knockout mice, where impaired breakdown of bone is described as a direct result of a functional defect in osteoclasts. Osteoclasts express high levels of Src. Src knockout mice form increased numbers of osteoclasts, although the cells are morphologically abnormal and their bone resorption functions are impaired. They show defective phosphorylation of cellular proteins in response to extracellular stimuli, such as M-CSF and engagement of integrin receptors (28, 29). RANKL-RANK signal activates c-Src, causing it to cross-talk with TRAF6 (30). TRAF6 is involved not only in the formation of osteoclast but also in the bone-resorbing function of mature osteoclasts (20–22).

**Osteoclast Apoptosis**

Osteoclast numbers decline dramatically in the absence of trophic factors such as M-CSF and RANKL. This rapid cell death of osteoclasts is caused by apoptosis, the programmed cell death in vitro and in vivo (27). Increasing research is being dedicated toward the examination of signal transduction pathways regulating osteoclast apoptosis. It is well known that antiapoptotic factors such as RANKL, IL-1, and M-CSF induce extracellular signal-regulated kinase activation in osteoclasts.

Adenovirus infection systems used to transduce constitutively active mutant form of mitogen-activated protein kinase/extracellular signal-regulated kinase 1 and dominant-negative form of Ras showed that the activation of Ras/extracellular signal-regulated kinase pathway supported osteoclast survival and inhibited its apoptosis; hence, Ras/extracellular signal-regulated kinase pathway has an essential role in osteoclast survival (31). The critical role of phosphatidylinositol 3-kinase/Akt pathways in the survival of osteoclasts has also been suggested. Other reports, supporting the importance of Akt for osteoclast survival, showed that the survival of osteoclasts is elongated by the adenovirus vector-mediated overexpression of constitutively active Akt and that the small G protein Rac1 is critically involved in this process (32). Moreover, a study using Src251 transgenic mice revealed that AKT activation by the c-Src family was important for osteoclast survival induced by RANKL (30). Src251 is a truncated Src molecule that lacks the kinase domain and is under the control of the TRAP promoter in transgenic mice. The mice showed osteopetrosis and a reduced number of osteoclasts. Some reports showed that osteoclast survival is supported by the mammalian target of rapamycin/S6K pathways not by phosphatidylinositol 3-kinase/Akt pathways. The central role of mammalian target of rapamycin/S6K pathways in M-CSF- and/or RANKL-induced osteoclast survival was pointed out by rapamycin-inducing osteoclast apoptosis (33, 34).

The features of apoptosis result from controlled cellular degradation precipitated by intracellular cysteine proteases called caspases. In response to apoptotic signals, these enzymes are activated in a pathway-specific manner and the classic caspase activation chain reaction is set in motion (35). The apoptotic pathway can be divided into two major cascades, extrinsic and intrinsic (35). The extrinsic pathway is primed by the binding of extracellular death ligands to their transmembrane receptors.

The mitochondrial pathway, one of the intrinsic pathways, is reported as a major pathway of osteoclast apoptosis. Depolarization of the mitochondrial transmembrane potential, chromatin condensation, and cytochrome c release from mitochondria into cytoplasm has been observed in the apoptotic osteoclasts (36). Antia apoptotic Bcl-2 family members have potentially important roles in not only osteoclast survival but also osteoclast activity. In support of this hypothesis, Bcl-2 mRNA expression in osteoclasts is increased by M-CSF treatment, and adenovirus vector-mediated overexpression of Bcl-xL markedly prolonged osteoclast survival in vitro (27, 37). TRAP promoter-controlled Bcl-xL and SV40 large T antigen transgenic mice showed increased numbers of osteoclast precursors in vivo, and their osteoclast precursors showed resistance to apoptosis in vitro, although their bone showed mild osteopetrosis (38).

The BCL-2 family of proteins has a crucial role in the regulation of apoptosis through the intrinsic pathway by their ability to regulate mitochondrial cytochrome c release. They contain between one and four BCL-2 homology domains. The BCL-2 family comprises three subfamilies: an antiapoptotic family, proapoptotic multidomain family, and BH3-only protein family.

The activities of BH3-only proteins are strictly regulated in programmed cell death through both transcription and post-transcription mechanisms (39). Bim, one of the members of BH3-only proteins, has an important role in osteoclast apoptosis. Bim is expressed in hematopoietic, epithelial, neuronal, and germ cells (40). Bim plays essential roles in the apoptosis of T and B lymphocytes, neurons, and osteoclasts (27, 41–43).

During osteoclast apoptosis, activation of Bim is controlled by a novel system, ubiquitylation-proteasome degradation. In the presence of M-CSF, Bim is constitutively degraded by ubiquitylation, and cytokine deprivation induced a rapid up-regulation of Bim due to the inhibition of its ubiquitylation (27). Bim ubiquitylation is reduced in c-Cbl knockout osteoclasts; hence, c-Cbl is a putative candidate for E3 ubiquitin ligase activity on Bim in osteoclasts (27). Bim knockout osteoclasts exhibit prolonged survival both in vitro and in vivo (27). Another group confirmed these data with the study that silencing the Bim gene by small interfering RNA prolonged the survival of osteoclasts (33). However, their bone-resorbing
activity was significantly impaired, consistent with the in vivo observation that Bim knockout mice showed mild osteosclerosis, just like Bcl-XL/SV40T antigen transgenic mice (27). The homology of both types of apoptosis-impaired osteoclasts, Bim knockout and Bcl-XL/SV40T antigen transgenic cells, suggests that osteoclast apoptosis is closely related to cellular activity and does not always equate to reduction of bone resorption. Further studies are required to elucidate the role of osteoclast apoptosis in bone resorption regulatory mechanism.

Bone Tumor Progression and Osteoclast

Certain primary tumors, including osteosarcoma, and metastatic malignant tumors, such as breast, lung, and prostate cancer, necessarily induce and stimulate osteoclasts to invade bone. There are different patterns of bone tumor lesions, ranging from destructive or osteolytic to bone-forming or osteoblastic, and the osteoclast has an important role in progression of both types of bone tumor lesions. In osteolytic lesions, bone destruction is not caused by the direct effects of cancer cells on bone. It is promoted by osteoclast stimulation (11). Tumors produce many factors that stimulate osteolysis: PTHrP, IL-1, IL-6, IL-8, IL-11, and transforming growth factor-β, which induce RANKL expression in osteoblasts or bone marrow stromal cells (11, 14). Interactions between tumor cells and osteoclasts through these factors can induce the vicious cycle of osteolytic lesions. They cause not only osteoclast induction and subsequent bone destruction but also aggressive growth and proliferation of the bone lesion.

PTHrP is able to activate osteoblasts to produce RANKL, reduce OPG secretion, and stimulate osteoclast precursors, leading to osteolysis. Briefly, PTHrP not only stimulates osteoclast inducing factor, RANKL secretion but also reduces production of RANKL-specific inhibitor, OPG. Osteolysis leads to the release of bone-derived growth factors, such as transforming growth factor-β, and increases extracellular Ca²⁺ concentrations. These factors bind to receptors on the tumor cell surface and activate autophosphorylation and signaling through pathways that involve SMAD (cytoplasmic mediators of most transforming growth factor-β signals) and mitogen-activated protein kinase. Extracellular Ca²⁺ binds and activates a Ca²⁺ pump. Signaling through these pathways promotes tumor cell proliferation and production of PTHrP (11). Thus, this constitutes the hypothesis of the vicious cycle and signifies that therapies targeting osteoclasts and their regulatory processes are potential therapeutic strategies for osteolytic lesions (Fig. 1).

Despite a central role in bone destruction, osteoclasts have also been implicated in the development of osteosclerotic bone lesions. Prostate cancer is well known as a cancer forming osteoblastic bone metastasis. As a representative osteoblastic skeletal tumor, the possibilities of relationship between the prostate cancer cell and the osteoclast are intriguing. Increased OPG levels have been reported in both histologic specimens and the serum of patients with bone lesions due to primary prostate cancer. The serum levels of soluble RANKL in such patients were not increased above that of nonmetastatic controls (13, 44, 45). On the contrary, OPG inhibits prostate tumor growth in the bone in vivo and possesses little contribution to tumor cell proliferation (13). In both osteoblasts and prostate cancer cells, increased levels of protein and mRNA of OPG and RANKL were described in histologic sections of bone metastatic lesions of prostate cancer (13, 44, 45). As a result of pathologically increased bone turnover, a heterogeneous pattern of woven bone formation close to areas of osteolytic activity is observed in prostate cancer metastatic to bone (13). The evidence of increased bone resorption markers suggests that RANKL signaling is upregulated and that the vicious cycle promotes subsequent tumor growth in bone (13).

Therefore, for the reasons stated above, osteoclast-targeted therapies may well deliver on the promise of being a potent breakthrough for the treatment of both types of bone tumors, including osteosarcoma. Osteosarcoma has a unique and special feature; direct RANKL expression by the osteosarcoma cell itself. Hence, osteoclast-targeted therapy is possibly a more suitable therapeutic option for osteosarcoma than for other primary and metastatic bone tumors (46).

Osteoclast-Targeted Treatments

There are three strategies for osteoclast-targeted therapy: inhibiting tumor-induced osteoclastogenesis, suppressing osteoclast bone resorption activity, and inducing osteoclast-specific apoptosis. Some agents have combined effects on osteoclasts. From this viewpoint, and based on several supporting reports in the literature, four possible therapeutic model agents are discussed (Table 1).

**OPG and RANK-Fc**

RANKL has an important role in osteoclast biology, specifically in osteoclastogenesis, osteoclast activity, and osteoclast survival (Fig. 2). Therefore, it is quite expected to target RANKL directly. There are two major candidates: OPG and RANK-Fc. OPG is a soluble decoy receptor for RANKL and strongly suppressed osteoclast differentiation in vitro and in vivo (26, 47). On the other hand, RANK-Fc is a recombinant RANKL antagonist that is formed by fusing the extracellular domain of RANK to the Fc portion of human IgG1 (48). The experimental effect of OPG on bone metastasis model has been reported previously (49, 50). These tumors included breast cancer, lung cancer, colon cancer, prostate cancer, and osteosarcoma.

The therapeutic effect of OPG in the mouse and rat osteosarcoma model has been shown (51). OPG gene therapy was effective in suppression of the osteolytic lesion formation, as reflected by a reduction in the number of osteoclasts associated with tumor development, hence reducing the tumor incidence and the local tumor growth and leading to prolonged survival. On the contrary, OPG did not inhibit the development of pulmonary metastatic lesion alone. OPG therapy was only effective when the
and this phenomenon was shown both in vitro (cell binding, cell proliferation, apoptosis, or cell cycle distribution). The effects of recombinant OPG-Fc on bone metastasis models of breast and prostate cancer are reported as well (49, 50). In both cases, OPG-Fc therapy was effective and skeletal tumor progression was inhibited. Although the RANK-Fc therapeutic effect on osteosarcoma has never been reported, positive effects of RANK-Fc on other malignant bone tumor models have been observed (52). Thus, RANK-Fc is a potent candidate as a new osteosarcoma therapeutic agent, one capable of targeting osteoclasts.

### Bisphosphonates

Bisphosphonates are potent inhibitors of bone resorption. Therefore, they are clinically applied for the treatment and prevention of osteoporosis, Paget’s disease, tumor metastases in bone, and other skeletal disorders (53). They are pyrophosphate analogues in which the oxygen bridge between the two phosphorus atoms is replaced by a carbon with various side chains (53). One of their therapeutic effects depends on strong induction of osteoclast apoptosis and this phenomenon was shown both in vitro and in vivo (54). Although the precise osteoclast apoptosis induction system of bisphosphonates has not been revealed yet, it is speculated that the mevalonate pathways and small G proteins are involved (55). Interestingly, although bisphosphonates needed osteoclast apoptosis to suppress bone resorption, the nitrogen-containing bisphosphonates and alendronate- and risedronate-induced inhibition of bone resorption do not require osteoclast apoptosis. The pan-caspase inhibitor, zVAD-fmk, suppressed osteoclast apoptosis and maintained the osteoclast number but did not affect nitrogen-containing bisphosphonates activity, whereas the activity of non-nitrogen-containing bisphosphonates was inhibited (56).

The third-generation bisphosphonates such as zoledronic acid (ZOL) are clinically applied as novel therapeutic agents reducing skeletal-related events of cancer patients and recognized as generally safe and well tolerated (57). ZOL has been shown to prevent or inhibit skeletal-related events, to possess direct antitumor effects, and to synergistically augment the effects of anticancer agents in various cancer cell lines (58, 59). The reported side effects of oral bisphosphonates are gastrointestinal disorders including indigestion, abdominal pain, and diarrhea. The mild to moderate flu-like symptoms and osteonecrosis of the jaw are recognized as side effects of i.v. bisphosphonate administration (57). Although osteonecrosis of the jaw is an uncommon condition and the etiology is still unclear, a dental examination and preventative dentistry before beginning bisphosphonate therapy and regular dental care (every 6 months) and good oral hygiene thereafter are recommended in the recently published guidelines to reduce the risk of osteonecrosis of the jaw (57).

ZOL inhibits not only osteolysis but also lung metastasis in vivo (60, 61). ZOL or other new bisphosphonates are anticipated to inhibit osteolysis and lung metastasis in osteosarcoma patients through the suppression of osteoclast function or other unknown mechanisms. Recently, the antiosteosarcoma effects of novel bisphosphonates have been reported (60, 62). The direct effects of ZOL on tumor cells contain the induction of osteosarcoma cell apoptosis, inhibition of cell proliferation due to cell cycle arrest (60, 61), and suppression of PTHrP expression (63). Moreover, ZOL inhibits cell migration and caused an increased mRNA expression of osteocalcin and decreased expression of alkaline phosphatase, osteopontin, osteonectin, and vascular endothelial growth factor, with no change in expression of OPG. These data suggest its ability to directly halt tumor cell growth and metastasis via its effects on viability, invasion, differentiation, and angiogenesis in osteosarcoma management (60). Hence, bisphosphonates, particularly ZOL, appear applicable in a clinical setting of osteosarcoma therapy.

### c-Src Inhibition

c-Src is a tyrosine kinase that is indispensable for osteoclast bone resorption activity. c-Src is required for the formation of the ruffled border phenomenon in osteoclasts. This is revealed by the Src knockout mice, which develop osteopetrosis because of the lack of bone resorption due to osteoclast activity impairment. Consistent reports suggest the involvement of c-Src in various human cancer development and progression (64, 65). Therefore, c-Src is one of a potential target candidates important to both osteoclast and tumor cell biologies.

Metastases to bone and lung were more severe in mice injected with wild-type or constitutively active c-Src-transfected MDA-MB-231 breast cancer cells, whereas transfection in injected cells of a c-Src kinase-dead dominant-negative construct resulted in reduced morbidity, lethality, and incidence of metastases (66, 67). The growth in vitro and in vivo and production of PTHrP were promoted by transfection of constitutively active c-Src and diminished in dominant-negative c-Src (66). CGP76030, Src

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**Table 1. Advantages and disadvantages of osteoclast-targeting agents**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>OPG RANK-Fc</td>
<td>Targeting osteoclast and possibly tumor cells as well</td>
<td>Possibility of hindrance of skeletal development and immune system impairment</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>Commonly used drugs; hence, side effects and safety are known</td>
<td>Possibility of impaired skeletal development, mild to moderate flu-like symptoms, and osteonecrosis of the jaw</td>
</tr>
<tr>
<td>c-Src inhibitor</td>
<td>Targeting both osteoclast and tumor progression</td>
<td>Possibility of skeletal development impairment</td>
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tyrosine kinase inhibitor, decreased morbidity and lethality and suppressed metastasis-induced osteolysis of the mice inoculated with MDA-MB-231 breast cancer cells as well (67). The relationship between c-Src activity and osteosarcoma progression was reported as well. The Src kinase is activated in human sarcomas and sarcoma cell lines including osteosarcoma.

Inhibition of c-Src activity by dasatinib, a Src inhibitor, or small interfering RNA induced osteosarcoma cell line apoptosis and inhibited cell motility and invasion in vitro (68). Novel Src inhibitor SI-83 induced apoptosis in SaOS-2 cells and had a far lower effect in primary human osteoblasts in vitro. It decreased in vivo osteosarcoma tumor mass in a mouse model as well (69). Src targeting therapy means osteoclast and Src-related tumor-targeted therapy, because Src deficiency affects only osteoclasts in the healthy body and Src promotes tumor progression as a proto-oncogene (28, 29, 64–67). Thus, Src targeting therapy is a plausible treatment for osteosarcoma.

Future Directions
This review has considered the possibility of osteoclast-targeted therapy as a novel management option for osteosarcoma. To establish this novel therapy, intense basic and clinical studies to uncover the potential advantages and disadvantages of such therapy are needed. To date, osteoclast-targeted therapy has focused on pathologies occurring after completion of skeletal formation, such as osteoporosis. Osteosarcoma commonly occurs in the immature skeleton and the repeat administrations of osteoclast-targeted agents may increase risks of skeletal abnormalities, such as osteopetrosis-like abnormalities or earlier termination of bone growth. Alternatively, possible disadvantages of osteoclast-targeted therapy may result in events not directly related to the osteoclast itself.

For instance, RANKL promotes not only osteoclast but also dendritic cell survival with induction of Bcl-xL expression. Therefore, anti-RANKL therapy can potentially impair the function of dendritic cells. RANKL or RANK knockout mice show complete defect of lymph nodes and RANK signaling is indispensable for the lymph node formation in the developmental stage but not for adult lymph node function (70). OPG binds to TNF-related apoptosis-inducing ligand (TRAIL) and inhibits TNF-related apoptosis-inducing ligand-mediated apoptosis (71). Therefore, OPG treatment may inhibit the function of TNF-related apoptosis-inducing ligand. TNF-related apoptosis-inducing ligand knockout mice were more susceptible to experimental and spontaneous tumor metastasis and more sensitive to chemical carcinogenesis as well. Hence, TNF-related apoptosis-inducing ligand is an important molecule for host defense against tumor (72).

However, no abnormality in the dendritic cell function has yet been reported in anti-RANKL clinical studies. Neither the induction of immune abnormalities nor the exacerbation of tumor progression has been reported in the clinical trials of anti-RANKL antibody or OPG. However, the mechanisms of avoiding possible side effects have not been elucidated as yet.

Targeting RANKL may produce anticancer actions with fewer side effects. RANKL is not only osteoclast differentiation factor but also possible key regulator of tumor cells. For example, RANKL stimulates migration and metastasis of human epithelial cancer cells and melanoma cells that express RANK (73). Human osteosarcoma biopsies and cell lines showed functional RANK expression (74). RANKL induces the phosphorylation of extracellular signal-regulated kinase 1/2, p38, and IκB and directly modulates the gene expression profile of RANK-positive human osteosarcoma cells (74, 75); thus, RANK-Fc or OPG could be a potent therapeutic target.

In its infancy, osteoclast-targeted strategies for osteosarcoma have tremendous potential. Referring other osteolytic bone metastasis therapeutic reports, cathepsin K inhibitor and PTHrP monoclonal antibody are possible candidate for osteoclast targeting osteosarcoma therapy, although the targets of reports are not osteosarcoma-induced osteoclasts. Therefore, cautious and steady approaches are needed for testing in a rigorous manner using reliable cell culture and in vivo model systems centering on bone biology.

Summary
Osteosarcoma is the most common skeletal sarcoma, which appears more commonly in the second to third decades of life. Although the outcome of osteosarcoma treatment has been improved by the chemotherapy-based combination therapy, progress has been painfully slow for the past 20 years. Therefore, novel treatment concepts are required to generate further improvement. Recently, osteoclast-targeted therapy has commanded considerable attention for skeletal disorders. The osteoclast is a unique cell that resorbs bone and is central in skeletal pathologic situations, where bone destruction is evident. Osteosarcoma cells are of the osteoblastic lineage, cells secreting the osteoclast-inducing factor, RANKL and able to induce osteoclastogenesis. Hence, osteosarcoma is a better candidate for osteoclast-targeted therapy than many other bone tumors. The rapid progress on the molecular mechanisms regulating osteoclast has propelled a development of new therapeutic approaches against bone tumors including osteosarcoma. RANK-Fc, OPG, bisphosphonates, and Src inhibitors are shown as positive candidates and can control all or some aspects of osteoclast function: osteoclast differentiation, bone resorbing activity, and cell survival. To establish this novel therapy, intense basic and clinical studies to uncover the potential advantages and disadvantages of such therapy are needed. Several possible side effects were expected, although none have been reported yet. Interestingly, possible beneficial effects have been reported recently in preclinical studies. As future challenges, lower toxicity therapy, such as the tumor site-specific osteoclast-targeted therapy, or development of
combination therapies with therapies in existence remain to be developed. Osteoclast-targeted strategy for osteosarcoma may represent the next phase of research and development in osteosarcoma treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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