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Research Paper

Systemic RANK-Fc protein therapy is efficacious against primary osteosarcoma growth in a murine model via activity against osteoclasts

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Abstract

Objectives Osteosarcoma (OS) is the most common primary malignant bone tumour, and mainly affects adolescents and young adults. Although there has been substantial improvement in management of OS with surgery and chemotherapy, further survival increase has not been achieved over the past two decades.

Methods We focused on the receptor activator of nuclear factor κB ligand (RANKL)–osteoclast (OCL) system as a biological target for OS. RANKL is a critical factor for OCL formation and bone resorption activity. The primary lesion in bone and ensuing metastasis in OS both require the induction of OCLs. RANK-Fc is a potent RANKL antagonist and inhibitor of OCL formation and activity.

Key findings In an orthotopic model in Balb/c *nu/nu* mice, a twice weekly dosing regimen of 350 μg of RANK-Fc per mouse subcutaneously ($n = 5$) reduced lung metastasis ($P > 0.05$), preserved bone structure and reduced tartrate-resistant acid phosphatase $(TRAP)^+$ OCLs ($P < 0.005$) in OS-bearing bone. *In vitro*, RANK-Fc suppressed OCL formation ($P < 0.005$), bone resorption activity ($P < 0.005$) and RANKL-induced anti-apoptosis ($P < 0.5$) of OCLs.

Keywords osteoclast; osteosarcoma; RANK; RANKL; tumour

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour, which mainly affects adolescents and young adults.[1] Current OS therapies are mainly focused on targeting the OS cell itself. We focused on the osteoclast (OCL) instead, as a potent candidate for OS therapy. An OCL is a multinucleated terminally differentiated cell, which undergoes apoptosis in the absence of factors such as macrophage colony-stimulating factor (M-CSF) or receptor activator of nuclear factor κB ligand (RANKL).[2,3] Physiologically, the OCL is the sole cell that can resorb and destroy bone directly in physiological remodelling and disease.[2–4] Bone destruction facilitates tumour progression.

Bone tumour cytokines, such as parathyroid hormone-related protein (PTHrP) and interleukins (IL-1, -6 and -11), stimulate osteoblasts and/or stromal cells to produce RANKL. RANKL is a pivotal regulator of OCL differentiation, survival and bone resorption activity. In turn, tumour growth can be supported by the normally sequestered bone matrix factors now released as a result of osteolysis. This accelerating cycle constitutes the so-called 'vicious cycle' hypothesis of bone tumour progression.[4] As such, we hypothesised that treatment with RANK-Fc would lead to inhibition of bone resorption, leading to a delay in the onset of skeletal complications and downregulation of tumour growth and development of metastases *via* OCL inhibition.

Several forms of DNA vector-based RANKL antagonists have been reported,[5–7] although they are not applicable clinically.[8] In one practical application of a RANKL antagonist, the Fc portion of the immunoglobulin heavy chain is fused to RANK (RANK-Fc) or osteoprotegerin (OPG, giving OPG-Fc) to generate effective recombinant proteins. Previously, the therapeutic effects of OPG-Fc and RANK-Fc adenovirus application on murine OS models have been reported, although they are not safe for humans.[9,10]

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OPG binds the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).[11] TRAIL deficiency facilitates tumour metastasis and carcinogen sensitivity. Thus, OPG and OPG-Fc may neutralise the anti-tumour effects of TRAIL. Aside from this, OPG and OPG-Fc application can elicit anti-OPG antibodies, which may cause early onset of osteoporosis and arterial calcification.[12,13] Against a backdrop of these agents, AMG 162, a fully human monoclonal antibody (IgG_2), has undergone testing in 21 clinical trials, with a proven safety and efficacy profile.[10] However, AMG 162 was not used in our study because AMG 162 cannot recognise murine RANKL, but human RANKL.[14] Recombinant RANK-Fc (rRANK-Fc), which recognises murine RANKL, was employed.

Materials and Methods

Chemicals

RANK-Fc, provided by Amgen Inc. (Seattle, WA, USA), contains the murine extracellular domain of RANK (through Pro213) fused to human immunoglobulin G_1 (IgG_1) Fc.[15] Recombinant human M-CSF and soluble RANKL were from Sigma. (St Louis, MO, USA); α-modified minimum essential medium (αMEM), fetal bovine serum (FBS) and dispase were purchased from Gibco-BRL, Life Technologies Inc. (Rockville, MD, USA). Bacterial collagenase, $1\alpha,25(OH)_2D_3$, and prostaglandin E2 were purchased from Sigma. The broad-spectrum caspase inhibitor zVAD-FMK was from R&D systems (Minneapolis, MN, USA).

In-vivo study

All animal experimentation was approved by the St Vincent's Health Animal Ethics Committee, and performed essentially as before.[16] From week 1 post-tumour-cell-inoculation, Balb/c *nu/nu* mice were injected twice weekly at a dose of 350 μg per mouse, subcutaneously in the mid-dorsum region with RANK-Fc. Placebo groups received the same volume of normal saline. Mice were checked daily, weighed and tumours measured with digital calipers. Tumour volume was determined using the following formula: $1/6\pi\ ab^2$ (a = long axis and b = short axis of the tumour).[17] Two groups of animals were tested: one for 28 days since cell injection, and another for 35 days. Tissues were harvested at these time-points.

X-ray and histology

Digital shots of both limbs were obtained prior to excision of left tibiae for X-ray at 35 kV, 30 s, using a cabinet system. The harvested lungs and the limbs were fixed in 4% *p*-formaldehyde for 24 h. The limbs were decalcified for 3 weeks, and all tissues were embedded in paraffin for histological analysis according to standard conditions.[16] These two analyses were used to deduce a series of clinicopathological parameters for assessment of RANK-Fc efficacy compared to the control cohort of animals.

Cell culture

The human OS cell line, SaOS-2, was obtained from ATCC (code HTB-85) and utilised as previously described.[16] Newborn and 5-week-old male Balb/c mice were used to obtain large numbers of OCLs for biochemical analyses, and a co-culture system was used.[18] Mouse bone marrow cells (2×10^5/well) were co-cultured with osteoblastic cells (1×10^4/well) for 5 days in the presence of 10 nM $1\alpha,25(OH)_2D_3$ and 1 μM prostaglandin E2 in 48-well plates. The cells were then stained for tartrate-resistant acid phosphatase (TRAP), and the number of OCL-like $TRAP^+$ multinucleated (>3 nuclei) cells was counted. The crude OCL preparation was placed on 48-well plastic plates, washed with PBS, and treated with 200 μl of PBS containing 0.1% collagenase and 0.2% dispase for 10 min to remove osteoblastic cells.[19]

Survival of osteoclasts

OCLs were purified 24 h after treatment and some of the cultures were subjected to TRAP staining. OCLs adhere to the bone surface through specialised discrete structures called 'podosomes', which consist mainly of dots containing F-actin. The rounded and sporadic appearance with ringed structure of podosomes (actin ring) is a characteristic of polarised OCLs. Cell viability was expressed as morphologically intact $TRAP^+$ multinucleated cells. Other cultures were further incubated for the indicated times, and then the number of living OCLs was counted. The number of viable cells remaining at the different time points is shown as a percentage of total cells at time zero.

Pit formation assay

The pit formation assay was performed according to a protocol previously described[20] with modification for osteologic slides. Briefly, mature OCLs were seeded onto 16-well BD BioCoat Osteologic calcium phosphate-coated quartz slides. Forty-eight hours after seeding mature OCLs on osteologic slides (Bedford, MA, USA), slides were treated with NH_4OH for 10 min. The resorbed area was measured using an image analysis system, Image J linked to a light microscope (Nikon, Tokyo, Japan).

Statistical analyses

Data were analyzed for statistical significance using the ANOVA test, followed by the Student's *t*-test (two-tailed) for comparison between two groups. A P value less than or equal to 0.05 was considered significant unless otherwise indicated.

Results

RANK-Fc-suppressed tumour growth and bone destruction at the primary site in an orthotopic model of osteosarcoma

Mice were intratibially injected with SaOS-2 cells and treated with 350 μg of RANK-Fc twice per week for 5 weeks. Treatment resulted in a 37% reduction in tumour volume ($P < 0.01$; Figures 1a and b). Radiographically, cortical bone erosion and growth plate preservation were observed in control tibia at day 21. At day 28, tumour breached the growth plate and intruded into the soft tissue in control tibia with periosteal reaction. At day 35, tumour completely lysed not only the distal third of the tibia but also the fibula (Figure 2a). In contrast, the RANK-Fc treatment group showed complete preservation of tibia even at day 28. At day 35, partial lytic lesion in the proximal tibia was

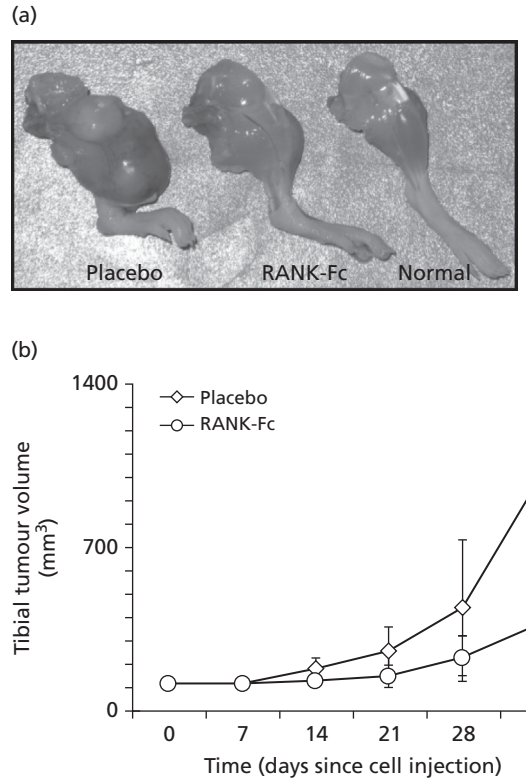

Figure 1 Effect of RANK-Fc treatment. RANK-Fc treatment decreased tumour burden and bone destruction effectively. 2×10^4 SaOS-2 osteosarcoma cells were injected into the proximal tibia of nude mice and the dimensions of the leg (including the tumour) were measured every week and the volume calculated. A non-tumour-bearing (normal) leg is shown for comparison. (a), Exposed tumour-bearing limb of representative animals at 5 weeks. (b), Tumour growth was effectively suppressed by RANK-Fc treatment. The mean tumour volume of RANK-Fc treatment group was around one third of placebo group at 5 weeks ($n = 5$, $P < 001$)

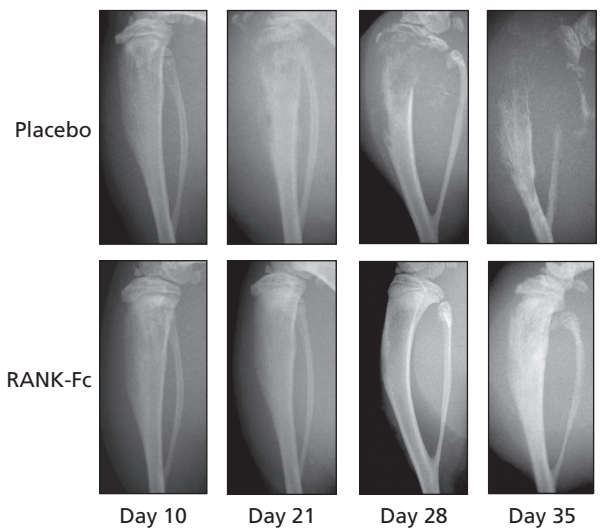

Figure 2 Chronological X-rays of limbs of mice in RANK-Fc and placebo groups. Representative X-rays of the limbs of mice both in the placebo group (upper lane) and in the RANK-Fc group (lower lane) are shown at day 10 to day 35 post-tumour-inoculation

shown, although the entire bone was slightly sclerotic and bone structure was preserved (Figure 2). Histology confirmed the X-ray data showing that RANK-Fc was beneficial in reducing osteolysis and preservation of bone structure at the site of tumour cell inoculation (data not shown).

RANK-Fc-inhibited tumour-induced osteoclasts

Tumour-inoculated tibiae treated with RANK-Fc contained significantly fewer OCLs than did tibiae taken from control mice (Figures 3a and 3b). Non-tumour-bearing tibiae treated with RANK-Fc contained fewer OCLs than did those in the control cohort of mice, although the difference was more modest than that in tumour-containing bone (not significant).

RANK-Fc-suppressed pulmonary metastasis formation

In OS, patients with numerous lung nodules fare the worst.[21] Thus, prevention of metastasis is one of the most important objectives of OS treatment. Histological examination of representative lung sections revealed numerous secondary growth pockets within the lung parenchyma in untreated mice at 4 weeks, and reduced numbers of metastases were seen in the RANK-Fc cohort at the same time-point (Figures 4a and 4b).

These data reveal that RANK-Fc could suppress tumour-induced bone destruction, local tumour growth and pulmonary metastasis. To determine whether these effects could be mediated by an effect on OCLs, we studied the effects of RANK-Fc on primary OCLs *in vitro*.

RANK-Fc-suppressed osteclast formation, survival and bone resorption activity

Interaction between tumour cells and OCLs via RANKL produced by osteoblasts is central to the vicious cycle of tumour progression in bone.[4] RANKL is not only a critical OCL differentiation factor but also stimulator of survival and bone resorption activity. We studied the activity of RANK-Fc on OCL differentiation, survival and bone resorption activity separately. RANK-Fc significantly reduced the OCL formation rate *in vitro* in a dose-dependent manner at all concentrations tested (Figure 5a) and also in the presence of osteoblasts in a co-culture system (Figure 5b).

In the absence of trophic factors such as RANKL, OCLs are readily channeled towards apoptosis. We studied OCL survival in the presence of RANKL, RANK-Fc and z-VAD-fmk. RANK-Fc did not affect OCL survival directly, although RANKL-mediated OCL survival was significantly reduced in a dose-dependent manner by RANK-Fc (Figure 6). The RANK-Fc anti-RANKL effects on OCL survival were abrogated by pan-caspase inhibitor z-VAD-fmk. Thus, RANK-Fc inhibited anti-apoptotic effects of RANKL on OCLs.

To determine RANK-Fc activity on OCL bone resorption activity, mature OCLs were plated on the BioCoat Osteologic slides and cultured for 48 h in the presence of varying doses of RANK-Fc. BioCoat Osteologic slides are coated with calcium phosphate and are useful in the analysis of OCL function.[22,23] As shown in Figure 7, RANK-Fc significantly reduces bone resorption activity of mature OCLs co-cultured with primary osteoblasts, which are stimulated by prostaglandin-E_2 and

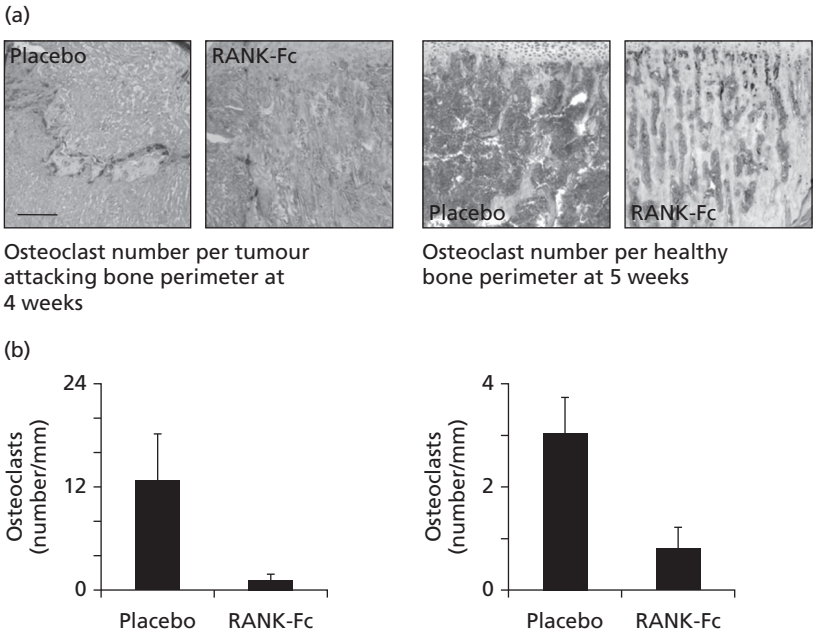

Figure 3 Effect of RANK-Fc treatment on osteoclast number per tumour perimeter. (a) Representative images of tibial sections with TRAP staining from mice with or without intratibial injection of SaOS-2 tumour cells at 4 weeks, scale bar is 10 μm. (b) Osteoclast number per trabecular bone perimeter of tumour-bearing tibia or healthy bone. Bars represent mean ± SD, $n = 5$

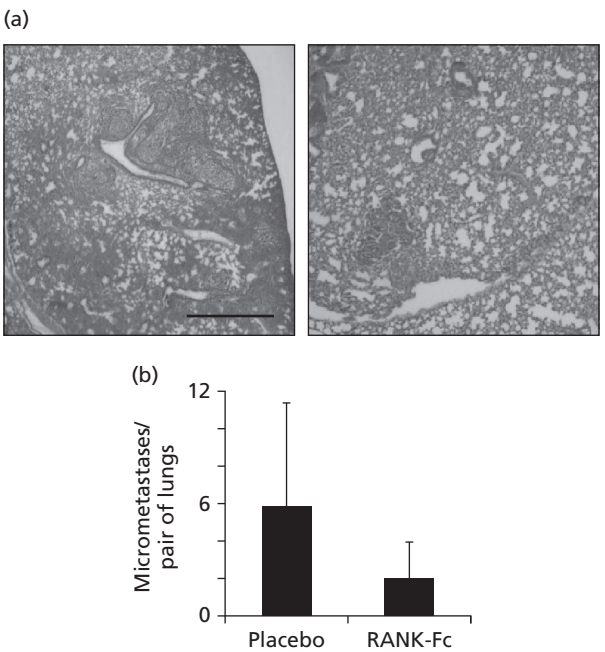

Figure 4 Effect of RANK-Fc on the lungs. (a) Representative haematoxylin and eosin section of mouse lungs from the placebo and RANK-Fc cohorts at 4 weeks after tumour inoculation, scale bar is 125 μm. (b) Number of micrometastases in the lungs (nodules per pair of lungs) of mice in the placebo and RANK-Fc-treated groups at 4 weeks. Bars represent mean ± SD, $n = 5$

vitamin-D_3 to express RANKL, at 1 μg/ml. This co-culture system mimics bone tumour-induced OCL activity prevalent during the vicious cycle. The threshold dose is between 1 and 0.1 μg/ml. These is consistent with the in-vivo findings that RANK-Fc suppresses OCLs and tumour induced-bone destruction.

Discussion

Current treatment of OS requires multidisciplinary therapy composed of surgery and systemic chemotherapy.[24,25] Without any chemotherapy, the 5-year survival rate of OS patients is less than 20%.[26] However, for the past 20 years, further increment in the survival rate has not been achieved.

One important challenge is to target not only the tumour directly but also the biological systems that support the tumour. Since primary OS is an intraosseous tumour, tumour-induced bone resorption is required for progression. However, the tumour cell itself cannot resorb surrounding bone directly. Bone resorption is the removal of existing bone by OCLs, large multinucleated cells of the monocyte–macrophage lineage, in various bone-associated pathologies, including bone tumour growth.[4] Bone tumour factors such as parathyroid hormone (PTH) or PTHrP, IL-1, -6 and -11 stimulate expression of RANKL by osteoblasts and stromal cells, and decrease the production of OPG – a decoy receptor preventing RANKL from binding to its receptor (RANK) on the OCL precursor.[4]

RANKL is not only a pivotal regulator of OCL differentiation but also promotes OCL survival and bone resorption activity. The OCL is involved in several pathological conditions of bone, including not only bone tumour but also osteoporosis, rheumatoid arthritis, periodontal diseases, periprosthetic implant loosening and Paget's disease, including familial expansile osteolysis and idiopathic hyperphosphatasia.[3] Several OCL-targeting agents have therefore been developed and some of them are in clinical trials or on the market.

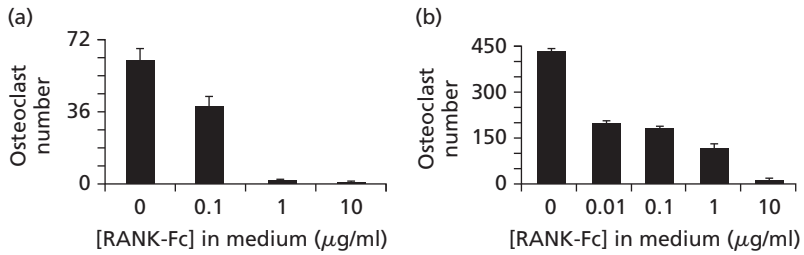

Figure 5 RANK-Fc suppressed osteoclast formation, survival and bone resorption activity *in vitro*. (a) In purified osteoclast cultures in M-CSF and RANKL system at 48 h; osteoclast formation was completely inhibited by RANK-Fc 1 μg/ml. (b) In a co-culture system at 48 h, osteoclast formation was inhibited in a dose-dependent manner, $n = 5$

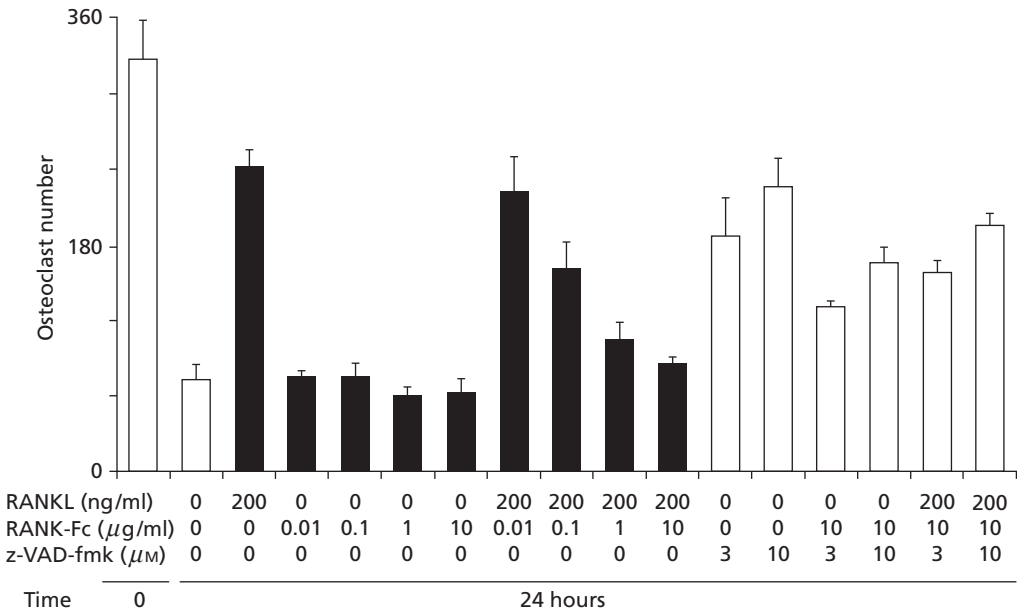

Figure 6 RANK-Fc abrogated RANKL anti-apoptotic effects on osteoclasts. Purified osteoclasts were cultured for 24 h with or without RANK-Fc and z-VAD-fmk. Cells were stained for TRAP and TRAP-positive osteoclasts were enumerated. The rate of survival of osteoclasts was calculated as the percentage of the number of osteoclasts at time 0, $n = 5$

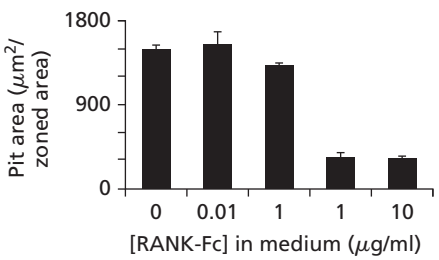

Figure 7 Effect of RANK-Fc on OCL activity. Bone resorption area was calculated as described in the Materials and Methods section. For numerical data, bars represent mean ± SD, $n = 5$

Such strategies include the use of OPG, RANK-Fc and anti-RANKL antibody. OPG-Fc or RANK-Fc gene therapies of bone tumour, including OS, are potential therapeutic candidates for the future, although a practical gene therapy system is unavailable at present.[8–10,27] OPG is a soluble RANKL decoy receptor that binds RANKL. AMGN-0007, a recombinant OPG-Fc, showed efficacy in patients of postmenopausal osteoporosis, bone metastasis from breast cancer and multiple myeloma.[3]

However, administration of OPG-Fc comes with a risk: anti-OPG antibodies were observed in one subject in a phase 1 study with OPG-Fc. OPG is an endogenous key regulator of bone homeostasis. *OPG –/–* mice showed early onset of osteoporosis and arterial calcification.[12] Thus OPG and OPG-Fc therapies are not recommended for OS patients because approximately 75% of OS patients are between 15 and 25 years of age.[28] Furthermore, OPG binds to TNF-related apoptosis-inducing ligand (TRAIL) and inhibits TRAIL-mediated apoptosis.[11] *TRAIL –/–* mice are more sensitive to chemical carcinogenesis and more susceptible to tumour metastasis, highlighting the critical role of TRAIL against tumourigenesis.[29] This again highlights that OPG is not a proper candidate for OS treatment.

AMG 162, an anti-RANKL antibody, is a fully human monoclonal antibody (IgG_2) that binds to RANKL with high

affinity and specificity and blocks the interaction of RANKL with RANK. RANKL-inhibition by AMG 162 mimics that of OPG.[30] Since AMG 162 does not resemble OPG structurally, the risks attached to OPG are avoided.[13] AMG 162 is not only safe in humans, but exhibits superior efficacy over both OPG and OPG-Fc.[13] AMG 162 is therefore a promising candidate for the future management of OS patients. One disadvantage of AMG 162 is its extraordinary stability. While a RANKL inhibitor with a longer half-life is desirable because of convenience to the patient, leading to better compliance, a shorter half-life is more desirable for young OS patients because their bones are still developing.

Monoclonal antibodies, such as AMG 162, have significantly longer half-lives than Fc fusion proteins, such as OPG-Fc and RANK-Fc. The duration of OPG-Fc efficacy is up to 45 days, while a similar dose of AMG 162 maintains its efficacy for more than 6 months in humans.[31] RANK-Fc can maintain suppression of OCL markers for less than 30 days.[32] Thus, we decided rRANK-Fc was the most suitable candidate for our study of OS treatment. rRANK-Fc prevents arthritic bone destruction, wear debris-induced osteolysis, metastatic bone tumour from prostate cancer and myeloma-associated bone pathology.[3,15]

RANK-Fc may possess a similar capacity as OPG-Fc to elicit an immunological response. *RANK –/–* mice exhibit not only profound osteopetrosis because of OCL absence but also deficiency of peripheral lymph nodes except for the Peyer's patches.[33] However, RANK-Fc does not impair host immune response and cannot perturb the TRAIL pathway.[32] At present, side-effects of RANK-Fc are not known, but need to be determined. Notwithstanding, our report is the first report of rRANK-Fc therapeutic effects in OS. RANK-Fc is a promising drug candidate for OS.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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