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14. ABSTRACT Bone is the most common site of metastasis for human breast cancer (BCa), which results in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of BCa cells and cells in the bone microenvironment results in the activation of osteoclasts and increased osteolytic bone destruction. The major treatment to reduce the burden of bone metastasis in BCa patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial. The central hypothesis of the proposed work is bone-targeted delivery of genetically-engineered MSC, over-expressing OPG, will prevent osteolytic bone damage and restore skeletal remodeling. Further, based on the requirement of angiogenesis for tumor growth in primary and metastatic sites, in combination with a systemically stable anti-angiogenic therapy, long-term survival will significantly increase. These hypotheses will be tested in this proposal using an immunocompetent, preclinical mouse model of BCa dissemination to all major bones as in human patients.					
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INTRODUCTION

Bone is the most common site of metastasis for human breast cancer (BCa), which results in significant morbidity and mortality in patients with advanced disease. A vicious cycle, arising due to the interaction of BCa cells and cells in the bone microenvironment results in the activation of osteoclasts and increased osteolytic bone destruction. The major treatment to reduce the burden of bone metastasis in BCa patients is bisphosphonate therapy. Despite significant efforts to improve the potency of bisphosphonates, the complications are only retarded but not prevented. Thus, development of newer therapies that can both ameliorate the threshold of bone destruction and increase survival of patients with metastatic breast disease will be highly beneficial. The central hypothesis of the proposed work is bone-targeted delivery of genetically-engineered MSC, over-expressing OPG, will prevent osteolytic bone damage and restore skeletal remodeling. Further, based on the requirement of angiogenesis for tumor growth in primary and metastatic sites, in combination with a systemically stable anti-angiogenic therapy, long-term survival will significantly increase. These hypotheses will be tested in this proposal using an immunocompetent, preclinical mouse model of BCa dissemination to all major bones as in human patients.

Specific Aims:

- 1) To determine therapeutic effects of genetically-modified MSC, overexpressing OPG, for osteolytic bone damage using a bone-targeted delivery, in an immunocompetent mouse model of BCa dissemination to the bone
- 2) To determine the combined effect of MSC-OPG therapy with systemically-stable anti-angiogenic therapy for long-term survival.

BODY

Our preliminary studies with an osteolytic breast cancer model indicated that sustained expression of osteoprotegerin (OPG), a decoy receptor for Receptor Activator of Nuclear Factor (NF)-kB Ligand (RANKL), by gene transfer approach provided significant therapy effects. Despite the potential of OPG in restoring damaged bone following breast cancer bone metastasis, OPG is also known to bind to TRAIL, a molecule involved in inducing cell death of cancer cells. Thus, it was essential for us to develop a genetically-modified OPG that lacks TRAIL binding but possesses OPG binding. To this end, we created several mutant OPG constructs based on structural homology prediction between OPG, RANKL, TRAIL and its receptor DR5.

The following are highlights of studies performed:

- Characterized potential OPG mutants for RANK and TRAIL binding
- Two of the six mutants OPG developed showed binding to OPG while not having TRAIL binding. This was confirmed by osteoclast and TRAIL assays, respectively.
- These data and development of mutant OPG and the mice models will allow us to test the feasibility of this therapy in the next phase of studies.

Rationale for creating a mutant OPG retaining RANKL binding and devoid of TRAIL binding affinity: Recent breakthroughs in our understanding of osteoclast differentiation and activation have come from the analysis of a family of biologically related tumor necrosis factor (TNF) receptor (TNFR)/TNF-like proteins: Osteoprotegerin (OPG), RANK and RANKL, which together regulate osteoclast function (1). The dysregulation of the functional equilibrium in the OPG/RANK/RANKL triad is responsible for the osteolysis associated with malignant tumors. RANKL has already been detected in several tumor cells and can be considered as a key factor involved in the activation of osteoclasts associated with bone metastases (2). OPG binds RANKL to negatively regulate osteoclast differentiation (3), and has been investigated as a molecular therapeutic to inhibit bone destruction associated with osteolytic bone lesions. OPG also binds TRAIL, a critical effector molecule for tumor immunosurveillance, raising the concern that treatment with OPG might promote tumor cell survival (4). Our goal is to develop a variant of OPG that will bind to RANKL, but not to TRAIL. The specific hypothesis behind the proposed research is that structural interactions exist between TRAIL and OPG that do not take place between OPG and RANKL. In the absence of any solved structure for OPG, our hypothesis is based on the following observations. **First**, TRAIL possesses an insertion of 12 amino acids that forms an elongated loop that is structurally divergent from RANKL. Contacts between this loop and OPG would be excellent targets for this endeavor. **Second**, the residues in the TRAIL receptor Death Receptor 5 (DR5) that make contact with this loop are conserved in OPG. Conserved residues among proteins in the same family are an indication they may have functional importance, so these residues may be important in mediating the interaction of TRAIL with OPG. **Third**, deletion of this loop in TRAIL eliminated binding to DR5 (5). If this mode of interaction is conserved between OPG and TRAIL, then we believe that mutation of the residues in OPG that bind this loop will eliminate or reduce binding of OPG to TRAIL. Because this loop of TRAIL is structurally divergent from RANKL, we hoped that such mutants will retain their ability to bind RANKL. Preliminary studies using such a mutant OPG containing mutation at the amino acid 49, identified based on molecular modeling of OPG, RANKL, TRAIL and its death receptor DR5 resulted in an engineered OPG (**Figure 1**) Testing of the mutant OPG constructs indicated that a few of them retained RANKL binding but not TRAIL binding as provided in **Figure 2**. This mutant OPG was effective in inhibiting osteoclastogenesis as shown in **Figure 2B** and was devoid of significant TRAIL binding affinity, resulting in increased tumor cell apoptosis following treatment with recombinant TRAIL, as shown in **Figure 2C**.

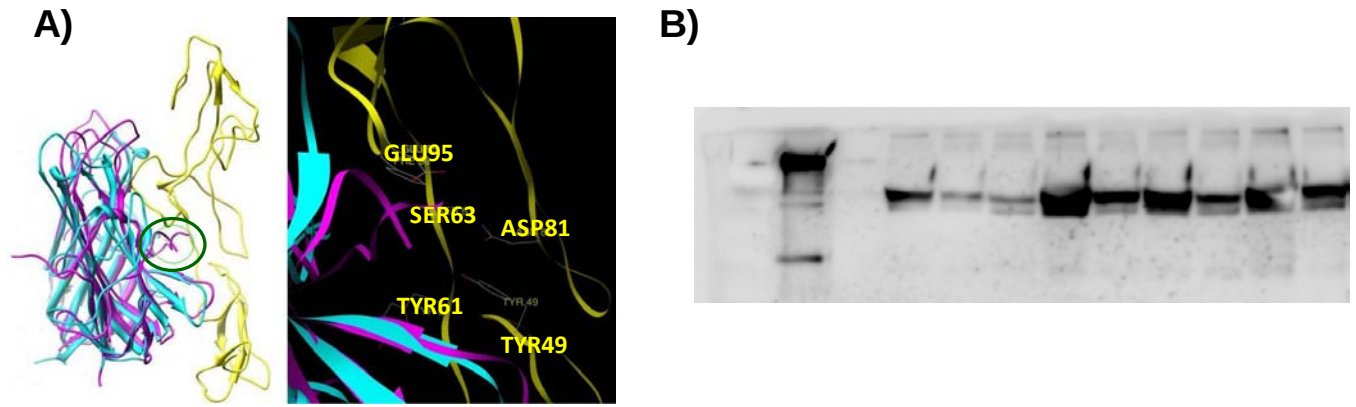


Figure 1. (A) The model of OPG complexed with TRAIL and RANKL. OPG is in yellow, RANKL is blue, and TRAIL is in purple. The AA' loop is circled in green. A close up view of the AA' loop and the interacting amino acids in the central cleft of OPG is shown on the right. **(B)** Immunoblot analysis of wild-type and mutant-OPG. 24 hours following transfection of expression plasmids containing the sequences of wild-type and mutant-OPG in 293 cells, supernatant were collected and concentrated using a 30 kDa cut off filter. Equal amounts of protein from indicated supernatant was separated in a SDS-PAGE and transferred to nitrocellulose membrane. Detection of wild-type and mutant-OPG was performed using an antibody for OPG (Jackson ImmunoResearch Laboratories Inc.). From left to right – Recombinant OPG, Negative control (Supernatant), Wild Type OPG, Y49A-OPG, Y49R-OPG, Y61E-OPG, Y81E-OPG, F96A-OPG, F96R-OPG, F107A-OPG, Y114A-OPG

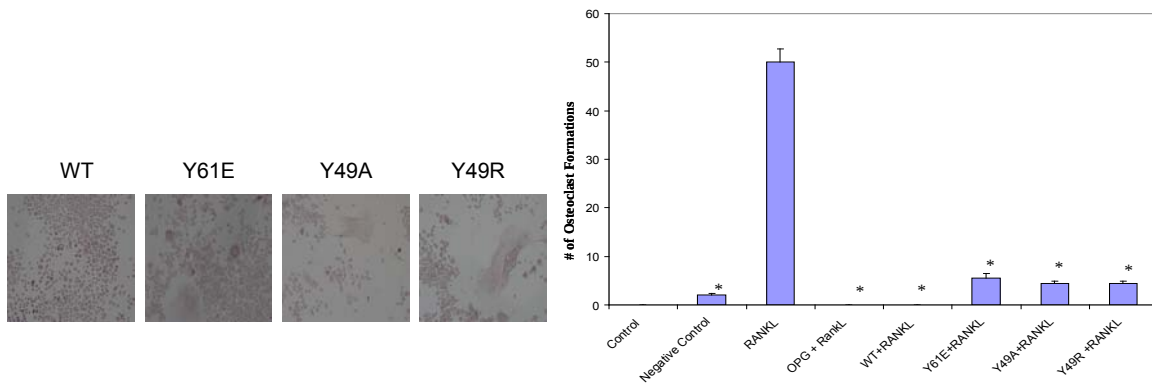


Figure 2. Osteoclast assay for wild-type and mutant-OPG. Tartrate-resistant acid phosphatase (TRAP) staining of RAW 264.7 cells was performed after 7-14 days in the presence of wild-type or mutant OPG and RANKL. As a positive control, RANKL alone was added without OPG. Recombinant protein, wild type as well as mutant OPG were all able to block osteoclastogenesis, which indicates the RANKL binding potential of wild type and mutant OPG. Quantitative analysis based on the number osteoclast seen in 10 random fields. (*p < 0.05)

In vitro TRAIL Assay: To ensure that mut-OPG do not bind to TRAIL, human BCa cell line MDA-MB 435 was cultured with recombinant, wild type and mut-OPG either alone or in combination with TRAIL. After 24 hours, MDA-MB 435 cells were fixed in 3.7% PFA and stained with 0.05% crystal violet for 30 minutes and viewed using a light microscope (100X). Cells were also cultured with 20μl of the solution 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) for 2 hours and then presence of proliferating cells was measured by absorbance at 490nm. Results are shown in Fig. 3.

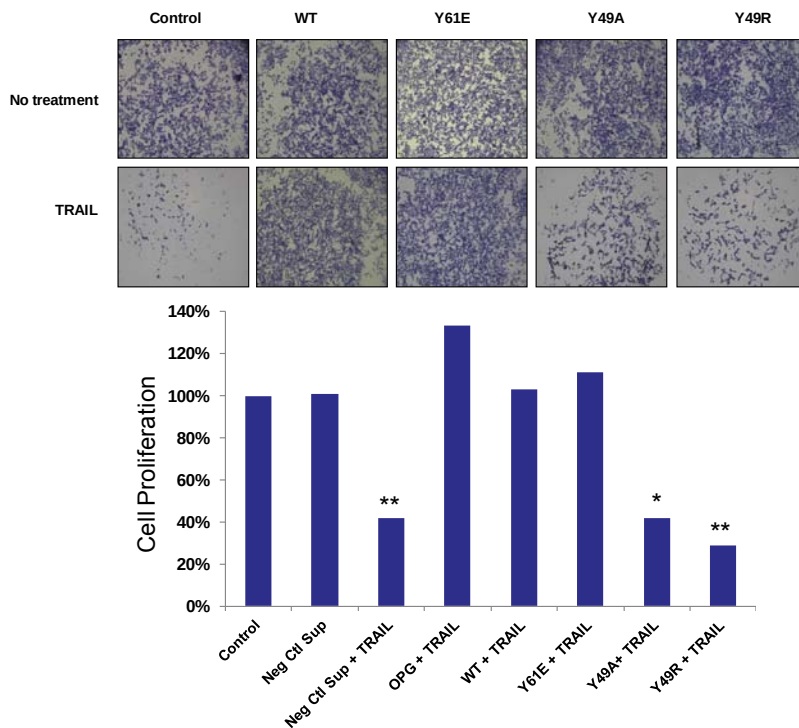


Figure 3. TRAIL assay with wild-type and mut-OPG. MDA-MB 435 cells were cultured in the presence of 100 ng of TRAIL and 400 ng of wild-type or mut-OPG. After 24 hours, MDA-MB 435 cells were either (A) fixed in 3.7% PFA and then stained with 0.05% Crystal violet for 30 minutes and viewed using a light microscope (100X) or (B) cultured with 20 μ l of the solution 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) for 2 hours and then measured at an absorbance of 490nm. (* $p < 0.05$, ** $p < 0.001$)

KEY RESEARCH ACCOMPLISHMENTS

Constructed and validated key OPG variants lacking TRAIL binding but retaining OPG binding.

REPORTABLE OUTCOMES

(Papers published or communicated)

CONCLUSIONS

Data so far indicated that recombinant and wild-type OPG bound TRAIL and inhibited its cytotoxic effects as proliferation of cancer cells still occurred despite the cells being cultured in the presence of TRAIL. However, mutant Y61E also bound TRAIL, and exhibited protection of cancer cells from the toxic effects of TRAIL. Mutants Y49A and Y49R showed significant abolished TRAIL binding as a decrease in cell proliferation and increased cell apoptosis was observed in the assays performed. Preliminary data also suggested that mutants F96R and Y107A may have a greater lack of TRAIL binding property (data not shown). Mutants Y49R, F96R and Y107A will be used for *in vivo* analysis.

PERSONNEL RECEIVING PAY FROM THIS GRANT

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APPENDICES

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