Oncolytic replication-selective adenoviruses are a new class of anticancer agents with great therapeutic potential. The ability of replication-selective viruses to amplify the initial viral dose has been exploited by engineering oncolytic adenoviruses to deliver therapeutic transgenes. In this Exploration Award, we are testing the concept that an oncolytic adenovirus can be armed with a therapeutic gene that will exert a systemic effect in the treatment of breast cancer. Breast cancer most commonly metastasizes to the skeleton. Thus, a treatment that combines eradication of the primary tumor with inhibition of osteolytic bone metastases would be a highly beneficial addition to the therapeutic armamentarium. We hypothesize that an oncolytic adenovirus armed with the osteoprotegerin (OPG) gene would be able to eradicate a primary breast cancer tumor by oncolysis, and that secretion of OPG from the infected and lysed cells into the systemic circulation would inhibit osteolytic bone metastases of the breast cancer. Thus, we propose a new class of therapeutic agent for the treatment of breast cancer. To date, we have constructed a replication-defective adenoviral vector expressing human OPG fused with the Fc domain of human IgG, and have evaluated the efficacy of the armed replicating adenoviral vector in vitro.
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INTRODUCTION

Oncolytic replication-selective adenoviruses are a new class of anticancer agents with great therapeutic potential. The selective replication of the viruses in cancer cells amplifies the initial viral inoculum, leading to destruction of the infected cells by virus-mediated lysis. The viral progeny are thereby released and can spread through the tumor mass to infect neighboring cancer cells, resulting in self-perpetuating cycles of infection, replication and oncolysis. The ability of replication-selective viruses to amplify the initial viral dose has been exploited by engineering oncolytic adenoviruses to deliver therapeutic transgenes. To date, such “armed” oncolytic adenoviruses have been designed to carry therapeutic genes that will augment the virus-mediated eradication of the primary tumor mass. Replication-competent adenoviruses have been shown to yield levels of transgene expression up to three orders of magnitude greater than corresponding replication-defective vectors. In this Exploration Award, we propose to test the concept that an oncolytic adenovirus can be armed with a therapeutic gene that will exert a systemic effect in the treatment of breast cancer. Breast cancer most commonly metastasizes to the skeleton. Thus, a treatment that combines eradication of the primary tumor with inhibition of osteolytic bone metastases would be a highly beneficial addition to the therapeutic armamentarium. We have previously shown that osteoprotegerin (OPG) can inhibit osteolytic bone metastases in a murine model. We hypothesize that an oncolytic adenovirus armed with the OPG gene would be able to eradicate a primary breast cancer tumor by oncolysis, and that secretion of OPG from the infected and lysed cells into the systemic circulation would inhibit osteolytic bone metastases of the breast cancer. Thus, we propose a new class of therapeutic agent for the treatment of breast cancer.
**Task 1. Derivation of an adenoviral vector expressing OPG-Fc**

a. Construction of vector.

b. Validation of vector.

c. Propagation of vector.

A gene encoding the leader peptide and extracellular domains of human osteoprotegerin fused to the Fc domain of human IgG1 [1] was generated by overlap extension PCR using plasmid DNA templates that we already possess. A replication-defective adenoviral vector expressing OPG-Fc under the control of the constitutive CMV promoter was then constructed with the AdEasy system [2], which we use routinely in our laboratory. The recombinant Ad-OPG vector was validated by DNA sequencing. Expression and secretion of OPG-Fc was confirmed by infection of 911 cells. The presence of OPG-Fc in the infected cells and in the culture medium was detected by immunoblot analysis using an anti-OPG antibody (Fig. 1).

![Fig. 1. Expression of OPG-Fc by adenoviral vector.](image)

911 cells were mock-infected (lane 1) or infected with Ad-OPG at a multiplicity of infection of 100 (lane 2) or 1000 (lane 3) particles per cell. Forty-eight hours post-infection, conditioned media and cell lysates were subjected to immunoblot analysis using an anti-OPG antibody.

The ability of the OPG-Fc secreted by infected cells to bind its cognate ligand, receptor activator of nuclear factor kappaB ligand (RANKL) was confirmed in a pull-down assay (Fig. 2).
**Fig. 2. OPG-Fc binds RANKL.** Medium from cells infected with Ad-OPG was incubated with soluble RANKL. Fc-containing complexes were pulled down with protein G-agarose beads and then subjected to immunoblot analysis with antibodies against OPG or sRANKL.
**Task 2. Evaluation of the efficacy of the armed replicating adenoviral vector in vitro**

a. Perform in vitro experiments to determine oncolytic potency.

b. Perform in vitro experiments to determine expression of OPG.

Monolayers of MDA-MB-231 breast cancer cells were infected with a wild-type adenovirus (Ad300wt) plus the AdOPG vector, with the wild-type adenovirus alone, or with the AdOPG vector alone. We wished to confirm that expression of OPG does not inhibit the oncolytic potency of the replicating adenovirus. Hence, we measured the level of adenoviral DNA (Fig. 3), and performed both qualitative (Fig. 4) and quantitative (Fig. 5) assays of the numbers of viable cells eight days post-infection of the cells at a low multiplicity of infection (MOI). These assays confirmed that expression of OPG does not inhibit the oncolytic potency of the replicating adenovirus.

![Expression of OPG does not affect adenoviral DNA replication](image)

Fig. 3. Expression of OPG does not affect adenoviral DNA replication. Monolayers of MDA-MB-231 cells were coinfected with Ad300wt and AdOPG at the MOIs shown. Eight days later, DNA was extracted and subjected to quantitative real-time PCR to determine the copy number of the viral E1A gene, normalized for cellular actin.
Fig. 4. Expression of OPG does not affect the oncolytic potency of a replicating adenovirus. Monolayers of MDA-MB-231 cells were coinfected with Ad300wt and AdOPG at the MOIs shown. Eight days later, viable cells were stained with crystal violet.

Fig. 5. Expression of OPG does not affect the oncolytic potency of a replicating adenovirus. Monolayers of MDA-MB-231 cells were coinfected with Ad300wt and AdOPG at the MOIs shown. Eight days later, viable cells were quantified in an MS assay.

Having completed the first two specific aims of the proposal, we are now initiating the third specific aim, to evaluate the efficacy of the armed replicating adenoviral vector in vivo.
KEY RESEARCH ACCOMPLISHMENTS

- Construction of a replication-defective adenoviral vector expressing human OPG fused with the Fc domain of human IgG.
- Demonstration that expression of OPG does significantly affect the oncolytic potency of the replicating adenovirus.
- Demonstration that OPG is expressed at a higher level by the replicating virus than by the replication-defective virus.
REPORTABLE OUTCOMES

- The data generated in the work supported by this award form the basis of an application for an R01 from the NIH.
CONCLUSIONS

We have performed preliminary experiments to explore the concept that a replication-selective adenovirus armed with the OPG gene could both eradicate primary breast cancer tumors by oncolysis and inhibit osteolytic bone metastases of breast cancer. While the final form of this novel therapeutic agent will be a single virus, a breast cancer-selective replicating adenovirus carrying the OPG gene, we have employed a two-component model system in these proof-of-concept studies. In this regard, coinfection of cells with a wild-type adenovirus and a replication-defective E1-deleted adenoviral vector expressing OPG allows replication of the vector as a result of trans-complementation by the viral E1 proteins expressed by the wild-type virus.

We first constructed a replication-defective adenoviral vector expressing human OPG fused with the Fc domain of human IgG to prolong its half-life in the bloodstream. It is important that the expression of OPG should not impair the oncolytic potency of the replicating adenovirus in breast cancer cells. In addition, we wish to confirm that a greater level of OPG will be expressed by a replicating virus than by a replication-defective adenoviral vector. Hence, we perform in vitro studies to confirmed these two key indicators of the efficacy of the novel therapeutic agent. We will now determine whether the armed replicating adenoviral vector can treat primary and metastatic breast cancer in a murine model.
REFERENCES


A Dual-Action Armed Replicating Adenovirus for the Treatment of Bone Metastases of Breast Cancer

James J. Cody, Gray R. Lyons, Joanne T. Douglas Department of Pathology, University of Alabama at Birmingham, Birmingham, AL; Davidson College, Davidson, NC; Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL

The majority of patients with advanced breast cancer develop osteolytic metastases in the skeleton, which lead to such complications as pain, fractures, hypercalcemia and a general decrease in the quality of life. Current therapies for bone metastases are not curative in nature. Thus, new treatments are needed for osteolytic bone metastasis of breast cancer. Conditionally replicating adenoviruses (CRADs) are a new class of anti-cancer agents that offer the potential ability to infect and lyse all the cells of a tumor. In clinical trials, replicating adenoviruses have exhibited safety but not the desired level of efficacy, demonstrating a need for improvement. One approach to enhance the efficacy of a CRAD is to arm it with a therapeutic transgene. Osteoprotegerin (OPG), the recently described inhibitor of osteoclastic bone resorption, represents a promising candidate with which to arm a replicating adenovirus designed to treat osteolytic bone metastasis. We hypothesize that a replicating adenovirus armed with a gene for OPG will be able to inhibit breast cancer bone metastasis and reduce the tumor burden in the bone through two actions: direct lysis of the tumor cells by replication of the virus and inhibition of bone resorption by the production of OPG.

To explore this hypothesis, a two-component model system was employed in these proof-of-concept studies. Coinfection of cells with both wild-type adenovirus and a replication-defective E1-deleted adenoviral vector expressing OPG allows replication of the vector via transcomplementation of E1 proteins from the wild-type virus. Therefore, we constructed a replication-defective adenoviral vector expressing OPG fused to the Fc portion of human IgG, to prolong its half-life. It was first necessary to demonstrate that the expression of OPG does not inhibit the replication of the virus or reduce its oncolytic potency. To this end, MDA-MB-231 breast cancer cells were coinfected with both Ad-OPG-Fc and the replication-competent wild-type Ad5, Ad300wt, to allow replication. Assays for viral DNA replication as well as both qualitative and quantitative assays of cell viability showed that the expression of OPG-Fc neither inhibited viral DNA replication nor reduced the oncolytic ability of the replicating adenovirus. Having shown the potential utility of an OPG-Fc expressing adenovirus in vitro, we are currently preparing in vivo studies using a murine model of osteolytic bone metastasis.

Keywords: Adenovirus; Cancer Gene Therapy;