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# Treatment of Primary and Metastatic Breast Cancer by An Armed Replicating Adenoviral Vector

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14. ABSTRACT
In this Exploration Award, we were testing the concept 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. 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. We have demonstrated that sCAR-ligand fusion proteins targeted to CEA, erbB-2 and the EGFR can mediate CAR-independent adenoviral infection of MDA-MB-231 breast cancer cells. These studies provided preliminary data for a funded NIH R01 grant to develop an armed replicating adenovirus for the treatment of bone metastases of breast cancer. To this end, we hypothesize that a replication-selective adenovirus armed with OPG would eradicate bone metastases of breast cancer both directly, by oncolysis, and indirectly, by inhibiting osteoclastic bone resorption and thus reducing the tumor burden.

15. SUBJECT TERMS
Adenoviral vector; bone metastases; oncolysis; replicating adenovirus
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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.

![Fig. 3. Expression of OPG does not affect adenoviral DNA replication.](image-url)

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.
Task 3. Evaluation of the efficacy of the armed replicating adenoviral vector in vivo

The goal of this Exploration Award was to determine the feasibility of employing a replicating adenoviral vector armed with OPG to simultaneously treat primary breast tumors and bone metastases of breast cancer. To this end, we proposed to evaluate the efficacy of the armed replicating adenoviral vector in vivo in a mouse model with both subcutaneous breast cancer tumors and bone metastases. As we sought to develop this model, it became apparent both from the literature and as a result of input from colleagues and reviewers of a submitted grant proposal, that it lacked clinical relevance. In this regard, in the clinical situation, a primary breast tumor will be treated surgically rather than by a replicating adenovirus. Moreover, bone metastases will usually need to be treated after the primary tumor has been removed, rather than existing simultaneously.

However, the proposed armed replicating adenoviral vector would be highly relevant to the treatment of bone metastases of breast cancer. To this end, we hypothesize that a replication-selective adenovirus armed with OPG would eradicate bone metastases of breast cancer both directly, by oncolysis, and indirectly, by inhibiting osteoclastic bone resorption and thus reducing the tumor burden. Intrinsic to this is the need to selectively deliver a systemically administered adenovirus to the target bone metastases.

In order to restrict adenoviral infection exclusively to the desired target cells, it is necessary to simultaneously ablate native tropism, by preventing the interaction between the knob domain of the fiber capsid protein and its cellular receptor, the coxsackievirus and adenovirus receptor (CAR), and to introduce novel tropism. We propose to retarget the initial inoculum of the systemically administered armed replicating adenovirus by means of a fusion protein comprising the soluble extracellular domain of CAR (sCAR) fused to a ligand for a receptor that is overexpressed on bone metastases of breast cancer.

To this end, we evaluated the ability of a panel of sCAR-ligand fusion proteins to mediate efficient, CAR-independent adenoviral infection of MDA-MB-231 breast cancer cells, the cell line to be employed in our proposed studies. Based on our knowledge of the receptors expressed by the target cells, we compared sCAR-ligand fusion proteins targeted to CEA, erbB-2 and the epidermal growth factor receptor (EGFR).

All three sCAR-ligand fusion proteins were capable of mediating targeted, CAR-independent infection of MDA-MB-231 breast cancer cells by an adenovirus vector with wild-type fiber proteins (not shown). We then confirmed that the three sCAR-ligand fusion proteins were capable of mediating targeted, CAR-independent gene transfer by an adenovirus vector containing the RGD-4C fiber modification that allows efficient infection of CAR-negative cancer cells (Fig. 6). An RGD-modified adenovirus carrying the luciferase reporter gene, Ad5LucRGD, was complexed with sCAR-ligand fusion proteins targeted to CEA, erbB-2 and EGFR prior to infection of MDA-MB-231 breast cancer cells preincubated with Ad5 knob to block CAR-dependent gene transfer. The optimal dose of sCAR-ligand fusion protein had been determined in a pilot experiment. Each of the sCAR-ligand fusion proteins was capable of mediating targeted, CAR-independent gene transfer. The ability of the Ad5 knob to block CAR-mediated infection was determined in a separate experiment. The specificity of each ligand for
its cognate receptor was confirmed by infection of receptor-positive and -negative cells, as shown in Fig. 7 for sCAR-MFE, the CEA-specific sCAR fusion protein.

**Fig. 6. Targeted, CAR-Independent Infection of MDA-MB-231 Cells By Ad5LucRGD Complexed With sCAR-Ligand Fusion Proteins.** A luciferase-expressing adenoviral vector with the RGD-4C fiber modification was preincubated with sCAR-EGF (anti-EGFR), sCAR-MFE (anti-CEA) or sCAR-C6.5 (anti-erbB-2) prior to infection of MDA-MB-231 cells in the presence of Ad5 knob to block CAR-mediated entry. Thirty hours post-infection, a luciferase assay was performed.

**Fig. 7. The sCAR-MFE Fusion Protein Mediates CAR-Independent, CEA-Specific Adenoviral Infection.** A luciferase-expressing adenoviral vector with the RGD-4C fiber modification was preincubated with sCAR-MFE (anti-CEA) prior to infection of CAR-negative, CEA-negative MC38 cells or CEA-positive MC38-CEA cells. Thirty hours post-infection, a luciferase assay was performed.

After consideration of a number of biological factors, we have selected the sCAR fusion protein targeted to CEA as the most suitable candidate for initial targeting of the systemically administered adenovirus to metastatic breast cancer cells. In this regard, the fusion protein consisting of sCAR fused with an scFv against CEA has already been shown to be capable of retargeting a systemically administered adenovirus to CEA-expressing cells [3]. Hence, this protein has been demonstrated to possess the requisite specificity and affinity for systemic targeting of an adenovirus. There are many reports in the literature documenting the expression of CEA by breast cancer cells, supporting the clinical relevance of this target [4, 5]. Moreover, CEA is selectively expressed by cancer cells but absent from normal cells in the adult, which means that a systemically administered adenovirus targeted to metastatic breast cancer cells will not be exposed to CEA-expressing non-target cells.

These research completed through this Exploration Award has provided sufficient preliminary data to enable the investigator to prepare a hypothesis-based proposal for further research. The PI was awarded NIH/NCI R01 CA 108585, Armed replicating Ad for breast cancer bone metastasis.
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 not 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.
- Demonstration that scAR-ligand fusion proteins targeted to CEA, erbB-2 and EGFR were capable of mediating targeted, CAR-independent adenovirus infection of MDA-MB-231 breast cancer cells.
REPORTABLE OUTCOMES

- Construction of a replication-defective adenoviral vector expressing human OPG fused with the Fc domain of human IgG.
- Research completed through this Exploration Award provided sufficient preliminary data to enable the investigator to prepare a hypothesis-based proposal for further research. The PI was awarded NIH/NCI R01 CA 108585, Armed replicating Ad for breast cancer bone metastasis.
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 wished 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 confirm these two key indicators of the efficacy of the novel therapeutic agent.

We originally proposed to determine the feasibility of employing a replicating adenoviral vector armed with OPG to simultaneously treat primary breast tumors and bone metastases of breast cancer. However, it became apparent that this proposal lacked clinical relevance. However, the proposed armed replicating adenoviral vector would be highly relevant to the treatment of bone metastases of breast cancer. To this end, we hypothesize that a replication-selective adenovirus armed with OPG would eradicate bone metastases of breast cancer both directly, by oncolysis, and indirectly, by inhibiting osteoclastic bone resorption and thus reducing the tumor burden. Intrinsic to this is the need to selectively deliver a systemically administered adenovirus to the target bone metastases. We demonstrated that sCAR-ligand fusion proteins targeted to CEA, erbB-2 and EGFR were capable of mediating targeted, CAR-independent adenovirus infection of MDA-MB-231 breast cancer cells.

The studies conducted in this Exploration Award provided the preliminary data for a funded R01 to establish the therapeutic potential of an armed replicating adenovirus for the treatment of bone metastases of breast cancer in humans.
REFERENCES


