AWARD NUMBER:  W81XWH-04-1-0800

TITLE:  A Dual-Action Armed Replicating Adenovirus for the Treatment of Osteoblastic Bone Metastases of Prostate Cancer

PRINCIPAL INVESTIGATOR: Joanne T. Douglas, Ph.D.

CONTRACTING ORGANIZATION:  University of Alabama at Birmingham
Birmingham, Alabama  35294-0109

REPORT DATE:  March 2006

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
A Dual-Action Armed Replicating Adenovirus for the Treatment of Osteoblastic Bone Metastases of Prostate Cancer

In this Exploration – Hypothesis Development Award, we hypothesize that the efficacy of a replicating adenovirus for the treatment of bone metastases of prostate cancer could be enhanced by arming it with the therapeutic protein sOPG-Fc, which will block osteoclastic bone resorption and hence inhibit bone remodeling. Thus, the objective of this proposal is to generate a dual-action armed replicating adenovirus, which will both directly kill metastatic prostate cancer cells by oncolysis and will also secrete sOPG-Fc into the microenvironment of the bone, thereby inhibiting osteoclastic bone resorption. To date, we have constructed armed replication-selective adenoviruses expressing sOPG-Fc. We have confirmed that the sOPG-Fc gene is expressed in a similar temporal manner to the E3B genes which it replaced, and that the remaining E3 genes continue to be expressed. We have also confirmed that expression of sOPG-Fc does not impair the selectivity or oncolytic potency of the armed replication-selective adenovirus.
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. In recognition of their potential, replication-selective adenoviruses have been rapidly translated into human clinical trials in patients with advanced cancer, where the safety of these agents has been demonstrated. However, clinical studies of replicating adenoviruses have yielded disappointing results, indicating the need for new approaches to improve their efficacy. The ability of replication-selective viruses to amplify the initial viral dose has previously been exploited by engineering “armed” oncolytic adenoviruses designed to carry therapeutic genes that will augment the virus-mediated eradication of the primary tumor mass.

Prostate cancer most commonly metastasizes to the skeleton, causing significant morbidity, including intractable pain, pathological fractures and nerve compression. Bone metastases of prostate cancer are predominantly bone-forming or osteoblastic. However, it is becoming increasingly apparent that bone metastases of prostate cancer have an extensive bone resorptive component mediated by osteoclasts: resorption of the bone matrix provides space for the prostate cancer cells to occupy. The differentiation and activation of osteoclasts is regulated by RANKL (receptor activator of NF-kappaB ligand), a membrane-bound cytokine expressed in osteoblasts/stromal cells, which binds to RANK, a cell-surface protein present on osteoclast precursor cells. The biological activity of RANKL is abrogated by binding to the “decoy receptor” osteoprotegerin (OPG), which is also secreted by osteoblast lineage cells. Previous studies have shown that both recombinant OPG and a recombinant soluble RANK-Fc fusion protein can block the binding of RANKL to RANK, and consequently diminish prostate cancer progression in bone.

In this Exploration – Hypothesis Development Award, we hypothesize that the efficacy of a replicating adenovirus for the treatment of bone metastases of prostate cancer could be enhanced by arming it with a therapeutic protein, which will block osteoclastic bone resorption and hence inhibit bone remodeling. Thus, the objective of this proposal is to generate a dual-action armed replicating adenovirus, which will both directly kill metastatic prostate cancer cells by oncolysis and will also secrete the therapeutic protein into the microenvironment of the bone, thereby inhibiting osteoclastic bone resorption. This therefore represents a two-pronged approach to the reduction of the bone tumor burden.

Of the two candidate RANKL-binding therapeutic proteins, we originally proposed to arm the replicating adenovirus with sRANK-Fc, because OPG has been demonstrated to bind the receptor TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and block TRAIL-mediated apoptosis in cancer cells. In contrast, sRANK-Fc is able to bind RANKL but cannot bind TRAIL. However, the lack of a commercially available anti-RANK antibody which could reliably detect the expressed protein was considered to present an obstacle to the project. It was later reported that the TRAIL-binding activity of OPG resides in the C-terminal domains, and that the four N-terminal cysteine-rich domains are sufficient to bind RANKL and inhibit osteoclastogenesis. Hence, the dual-action armed replicating adenovirus is designed to express the four cysteine-rich domains of human OPG fused to the Fc domain of human IgG1 to prolong its half-life.
Task 1. Derivation of an armed replicating adenovirus expressing sOPG-Fc, and control viruses:

- Construction of viruses.
- Validation of viruses.
- Propagation of viruses.

The first task was to construct an armed replicating adenovirus expressing the four cysteine-rich domains of human OPG fused with the Fc domain of human IgG1 to prolong its half-life, as previously reported [1]. Selective replication of this oncolytic virus in prostate cancer cells was conferred by engineering a 24-base pair deletion into the E1A gene. This mutation renders the E1A protein unable to bind and inactivate the retinoblastoma tumor suppressor/cell cycle regulator protein, Rb, and therefore precludes efficient viral replication in cells with an intact G1-S phase checkpoint (i.e., non-neoplastic or "normal" cells) [2]. A number of studies have shown that primary cancer cells express low levels of the coxsackievirus and adenovirus receptor, CAR, and are poorly infected by adenoviruses. Hence, the RGD-4C peptide was incorporated into the HI loop of the fiber knob to allow enhanced infectivity via a CAR-independent pathway. We chose to exploit the endogenous adenovirus E3 region gene expression control signals (promoter, splicing and polyadenylation signals) to express the OPG-Fc gene from the E3B region of the genome. The E3B region encodes three proteins which protect infected cells from lysis and consequently are redundant in oncolytic adenoviruses: this region can therefore be substituted with transgenes, as previously reported [3, 4]. This strategy has the advantage of retaining the E3-11.6K protein, the so-called adenovirus death protein, which is responsible for the efficient lysis and release of progeny virus from infected cells [5].

AdΔ24-OPG-RGD, the tropism-modified, armed replication-selective adenovirus expressing sOPG-Fc was constructed by homologous recombination in E. coli. Table 1 defines the panel of adenoviruses to serve as controls in subsequent experiments. Adwt300 was purchased from the American Type Culture Collection; AdΔ24-RGD, AdΔ24 and Ad-OPG-RGD were already available in our laboratory; AdΔ24-OPG and Ad-RGD were constructed for this study by homologous recombination in E. coli.

Table 1. Adenoviruses employed in this study.

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdΔ24-OPG-RGD</td>
<td>Tropism-modified, armed replication-selective adenovirus expressing sOPG-Fc</td>
</tr>
<tr>
<td>AdΔ24-OPG</td>
<td>Armed replication-selective adenovirus expressing sOPG-Fc (native tropism)</td>
</tr>
<tr>
<td>AdΔ24-RGD</td>
<td>Tropism-modified, unarmed replication-selective adenovirus with intact E3B region</td>
</tr>
<tr>
<td>AdΔ24</td>
<td>Unarmed replication-selective adenovirus with intact E3B region (native tropism)</td>
</tr>
<tr>
<td>Ad-RGD</td>
<td>Tropism-modified, unarmed replication-competent adenovirus</td>
</tr>
<tr>
<td>Adwt300</td>
<td>Unarmed replication-competent adenovirus</td>
</tr>
<tr>
<td>Ad-OPG-RGD</td>
<td>Tropism-modified, replication-defective adenoviral vector expressing sOPG-Fc</td>
</tr>
</tbody>
</table>
Having constructed the armed replication-selective adenoviruses, we wished to confirm that sOPG-Fc was expressed by AdΔ24-OPG-RGD and AdΔ24-OPG. To this end, monolayers of C4-2B prostate cancer cells were infected with AdΔ24-OPG-RGD or AdΔ24-OPG and the culture medium harvested at 4, 8, 12, 24, 36, 48 and 60 hours post-infection. Expression and secretion of sOPG-Fc were confirmed by immunoblot analysis using an anti-OPG antibody (Fig. 1).

**AdΔ24-OPG-RGD**

<table>
<thead>
<tr>
<th>hrs P.I.</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
</table>

![Image 1](https://example.com/image1.png)

**AdΔ24-OPG**

<table>
<thead>
<tr>
<th>hrs P.I.</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
</table>

![Image 2](https://example.com/image2.png)

**Fig. 1. Expression of sOPG-Fc.** C4-2B cells were infected with AdΔ24-OPG-RGD or AdΔ24-OPG. At the indicated times post-infection, conditioned media were subjected to immunoblot analysis using an anti-OPG antibody.

**Task 2. Evaluation of the efficacy of the armed replicating adenovirus in vitro:**

b. Perform in vitro experiments to determine expression of sOPG-Fc.

We then wished to determine whether the sOPG-Fc gene was expressed by AdΔ24-OPG-RGD and AdΔ24-OPG in a similar temporal manner to the E3B genes which it replaced (14.7K and RIDβ). Monolayers of C4-2B prostate cancer cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG or Adwt300, the parental wild-type virus. Cells were harvested at 4, 8, 12, 24, 36, 48 and 60 hours post-infection. Total cellular RNA was extracted and subjected to quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using primers specific for the sOPG-Fc, 14.7K and RIDβ genes. As shown in Figs. 2A and 2B, the expression of sOPG-Fc mimics the temporal pattern of the substituted native E3B proteins.
Fig. 2. Expression of sOPG-Fc mimics the temporal pattern of the substituted native E3B proteins. C4-2B cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG or Adwt300. At the indicated times post-infection, total cellular RNA was extracted and subjected to quantitative RT-PCR using primers specific for the sOPG-Fc, 14.7K and RIDβ genes.
We next wished to demonstrate that expression of the sOPG-Fc gene by AdΔ24-OPG-RGD and AdΔ24-OPG did not have an adverse effect on expression of the remaining E3 genes, which encode the gp19K protein and adenovirus death protein (ADP). Monolayers of C4-2B prostate cancer cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG or Adwt300, the parental wild-type virus. Cells were harvested at 4, 8, 12, 24, 36, 48 and 60 hours post-infection. Total cellular RNA was extracted and subjected to quantitative RT-PCR using primers specific for the sOPG-Fc, gp19K and ADP genes. As shown in Figs. 3A and 3B, the expression of sOPG-Fc by AdΔ24-OPG-RGD and AdΔ24-OPG does not block expression of the remaining native E3 proteins.

Fig. 3. Expression of sOPG-Fc does not inhibit expression of remaining E3 proteins. C4-2B cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG or Adwt300. At the indicated times post-infection, total cellular RNA was extracted and subjected to quantitative RT-PCR using primers specific for the sOPG-Fc, gp19K and ADP genes.
Task 2. Evaluation of the efficacy of the armed replicating adenovirus in vitro:

a. Perform in vitro experiments to determine oncolytic potency.

We wished to confirm that expression of sOPG-Fc does not inhibit the replication of the armed replicating adenoviruses. Monolayers of C4-2B prostate cancer cells were infected with the armed replication-selective adenoviruses (AdΔ24-OPG-RGD and AdΔ24-OPG), the unarmed replication-selective adenoviruses with intact E3B regions (AdΔ24-RGD and Ad-OPG), the unarmed replication-competent adenoviruses (AdRGD and Adwt300) or the replication-defective adenoviral vector expressing sOPG-Fc (Ad-OPG-RGD). The conditioned culture medium was harvested at 2, 4 and 6 days post-infection. DNA was extracted and subjected to PCR using primers specific for the E4 region of the adenoviral genome, as a measure of viral DNA replication. Human beta-actin DNA was also amplified to allow normalization of the data. As shown in Fig 4, the expression of sOPG-Fc does not inhibit the replication of the armed replicating adenoviruses.

![Fig. 4. Expression of sOPG-Fc does not inhibit adenoviral replication.](image)

Having shown that expression of sOPG-Fc did not inhibit adenoviral DNA replication in C4-2B prostate cancer cells, we wished to confirm that neither did it affect the selectivity of adenoviral replication conferred by the 24-base pair deletion in the E1A gene. This mutation renders the E1A protein unable to bind and inactivate the retinoblastoma tumor suppressor/cell cycle regulator protein, Rb, and therefore precludes efficient viral replication in cells with an intact G1-S phase checkpoint (i.e., non-neoplastic or “normal” cells). Human liver slices were infected with the armed replication-selective adenoviruses (AdΔ24-OPG-RGD and AdΔ24-OPG) or Adwt300, the parental wild-type virus. The conditioned culture medium was harvested at 2 and 4 days post-infection. DNA was extracted and subjected to PCR using primers specific for the E4 region of the adenoviral genome, as a measure of viral DNA replication. Human beta-actin DNA was also amplified to allow normalization of the data. As shown in Fig 5, AdΔ24-OPG-RGD and AdΔ24-OPG did not significantly replicate in the liver cells. Hence, the
expression of sOPG-Fc does not affect the selectivity of replication of the armed replicating adenoviruses.

![Graph showing E4 copy number/ng actin DNA over days post-infection for different adenoviruses.]

**Fig. 5. Replication-selective adenoviruses armed with sOPG-Fc do not replicate in human liver cells.** Human liver slices were infected with AdΔ24-OPG-RGD, AdΔ24-OPG or Adwt300. At the indicated times post-infection, DNA was extracted from the conditioned medium and subjected to quantitative PCR using primers specific for the E4 region of the adenoviral genome. Human beta-actin DNA was also amplified to allow normalization of the data.

We performed both qualitative and quantitative assays to confirm that expression of sOPG-Fc does not inhibit the oncolytic potency of the replicating adenovirus. Monolayers of C4-2B, LNCaP and PC3 prostate cancer cells were infected with the armed replication-selective adenoviruses (AdΔ24-OPG-RGD and AdΔ24-OPG), the unarmed replication-selective adenoviruses with intact E3B regions (AdΔ24-RGD and Ad-OPG) and the unarmed replication-competent adenoviruses (AdRGD and Adwt300). In the qualitative assay, eight days post-infection, the cells were fixed and stained with crystal violet to visualize intact cells. In the quantitative assay, eight days post-infection, a commercial cell proliferation assay was employed to measure cell survival. As shown in Figs. 6 and 7, expression of sOPG-Fc does not inhibit the oncolytic potency of the replicating adenoviruses.
Fig. 6. Expression of sOPG-Fc does not inhibit oncolytic potency of the armed replicating adenoviruses. C4-2B, LNCaP and PC3 cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG and the control adenoviruses. Eight days post-infection, viable cells were fixed and stained with crystal violet.
Fig. 7. Expression of sOPG-Fc does not inhibit oncolytic potency of the armed replicating adenoviruses. C4-2B cells were infected with AdΔ24-OPG-RGD, AdΔ24-OPG and the control adenoviruses at the indicated multiplicities of infection. Eight days post-infection, viable cells were counted using a cell proliferation assay.

Having completed the first specific aim and the first part of the second specific aim of the proposal, we are now initiating experiments to confirm that the armed replicating adenovirus can simultaneously eradicate prostate cancer cells by oncolysis and inhibit osteoclast formation and activation in vitro. We will then evaluate the efficacy of the armed replicating adenovirus in a murine model of bone metastases of prostate cancer.
Key Research Accomplishments

- Construction of armed replication-selective adenoviruses expressing N-terminal cysteine-rich domains of human OPG fused with the Fc domain of human IgG (sOPG-Fc).
- Demonstration that the sOPG-Fc gene is expressed by the armed replication-selective adenoviruses in a similar temporal manner to the E3B genes which it replaced.
- Demonstration that expression of sOPG-Fc by the armed replication-selective adenoviruses does not negatively influence the expression of the remaining E3 genes.
- Demonstration that expression of sOPG-Fc by the armed replication-selective adenoviruses does not significantly affect the replication of adenoviral DNA.
- Demonstration that the armed replication-selective adenoviruses replicate in human prostate cancer cells but not in human liver cells.
- Demonstration that expression of sOPG-Fc does significantly affect the oncolytic potency of the armed replication-selective adenoviruses in human prostate cancer cells.
Reportable Outcomes

Manuscripts in press (see appendices):

Conclusions

We have performed preliminary experiments to explore the hypothesis that the efficacy of a replicating adenovirus for the treatment of bone metastases of prostate cancer could be enhanced by arming it with sOPG-Fc which will block osteoclastic bone resorption and hence inhibit bone remodeling.

We first constructed armed replication-selective adenoviruses expressing sOPG-Fc. We confirmed that the sOPG-Fc gene is expressed in a similar temporal manner to the E3B genes which it replaced (14.7K and RIDβ), and that the remaining E3 genes, in particular the ADP gene, continue to be expressed. It is important that expression of sOPG-Fc should not impair the selectivity or oncolytic potency of the armed replication-selective adenovirus. Hence, we performed in vitro studies to confirm these two key indicators of the efficacy of the novel therapeutic agent. We are now initiating experiments to confirm that the armed replicating adenovirus can simultaneously eradicate prostate cancer cells by oncolysis and inhibit osteoclast formation and activation in vitro. We will then evaluate the efficacy of the armed replicating adenovirus in a murine model of bone metastases of prostate cancer.
References


Adenoviral vectors for Gene Therapy

Running Title: Adenoviral Vectors

Joanne T. Douglas

Division of Human Gene Therapy, Departments of Medicine, Obstetrics & Gynecology, Pathology and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, 901 19th Street South, BMR2 412, Birmingham, Alabama 35294, USA

Tel: (205) 975-2897; Fax: (205) 975-8565

E-mail: Joanne.Douglas@ccc.uab.edu
Abstract

Vectors are based on human adenovirus serotypes 2 (Ad2) and 5 (Ad5) of species C possess a number of features that have favored their widespread employment for gene delivery both *in vitro* and *in vivo*. However, the use of recombinant Ad2- and Ad5-based vectors for gene therapy also suffers from a number of disadvantages. These vectors possess the tropism of the parental viruses, which infect all cells that possess the appropriate surface receptors, precluding the targeting of specific cell types. Conversely, some cell types that represent important targets for gene transfer express only low levels of the cellular receptors, which leads to inefficient infection. Another major disadvantage of Ad2- and Ad5-based vectors *in vivo* is the elicitation of both an innate and an acquired immune response. Considerable attention has therefore been focused on strategies to overcome these limitations, thereby permitting the full potential of adenoviral vectors to be realized.
1. Introduction

Adenoviruses, which were first isolated from human adenoid tissue in the 1950s, have been developed as gene delivery vehicles, or vectors, since the early 1980s (1). The adenoviruses constitute the Adenoviridae family, which was originally divided into two genera: the Aviadenovirus genus which infects only birds; and the Mastadenovirus genus which contains viruses that infect a range of mammalian species. In 2002, two additional genera were created: Atadenovirus, so-named because the genome of the first recognized members of the genus (from ruminant, avian and marsupial hosts) has an unusually high adenine and thymine content; and Siadenovirus, whose name derives from the putative sialidase homolog possessed by members of the genus (2). Adenoviruses that infect humans are classified into six species (designated A-F), based on the percentage of guanine and cytosine in the DNA molecules and the ability to agglutinate red blood cells. Human adenoviruses are further subdivided into more than fifty serotypes (designated by Arabic numerals), primarily on the basis of neutralization assays (reviewed in ref. (3)).

The majority of recombinant adenoviral vectors are based on human adenovirus serotypes 2 (Ad2) and 5 (Ad5) of species C. These serotypes cause a mild respiratory disease in humans and are non-oncogenic. These safety features, coupled with the fact that adenovirus-based vaccines have been administered to humans without ill effects, have favored the development of adenoviral vectors for in vivo gene therapy applications (4). The safety of recombinant adenoviral vectors is also enhanced by deletion of the E1 region of the genome, which renders the vectors replication-deficient and capable of propagation only in specially designed complementing cell lines. Other advantages of recombinant adenoviral vectors derived from human serotypes 2 and 5 include the ability of the vectors to be purified to high titers (up to
10^{13} virus particles per ml), which means that it is practical to employ them *in vivo*. Adenoviral vectors also possess the important attribute of stability in the bloodstream, which means that they can potentially be employed for gene delivery following intravenous administration. Adenoviruses can infect both dividing and postmitotic cells, and have evolved an extremely efficient mechanism for delivery of their genome to the nucleus. The genome remains extrachromosomal, which minimizes the risk of insertional mutagenesis. So-called “first generation” E1-deleted Ad2 and Ad5 vectors can accommodate up to 7.5 kb of foreign DNA, and the capacity of the vectors can be expanded by additional deletions of the viral genes. These characteristics of Ad2 and Ad5 vectors have spawned considerable interest in their exploitation as gene delivery vehicles, which, in turn, has led to the development of a range of techniques by which their genomes can be manipulated and recombinant vectors generated with relative ease.

However, the use of recombinant Ad2- and Ad5-based vectors for gene therapy also suffers from a number of disadvantages. These vectors possess the tropism of the parental viruses, which infect all cells that possess the appropriate surface receptors, precluding the targeting of specific cell types. Conversely, some cell types that represent important targets for gene transfer express only low levels of the cellular receptors, which leads to inefficient infection. Another major disadvantage of Ad2- and Ad5-based vectors *in vivo* is the elicitation of both an innate and an acquired immune response. Considerable attention has therefore been focused on strategies to overcome these limitations, thereby permitting the full potential of adenoviral vectors to be realized.

This article will review the biology of adenoviruses and adenoviral vectors, discuss the applications of adenovirus-mediated gene delivery and describe the strategies that are being developed to improve the utility of adenoviral vectors.
2. Structure of Adenoviruses

2.1. Capsid Structure

Adenoviruses possess a nonenveloped icosahedral protein shell or capsid of 70-100 nm in diameter surrounding an inner DNA-containing core (ref. (3) and references therein). The 20 facets of the capsid are comprised of 12 copies of the trimeric hexon protein, which is the most abundant component of the virion and performs a structural role. Each vertex of the capsid is composed of a pentameric penton base protein in association with a trimeric fiber protein that projects from the viral surface and ends with a globular knob domain. The fiber and penton base both play important roles in the initial steps of the virus-cell interaction during infection. A number of minor polypeptides are involved in stabilization of the capsid, while two additional polypeptides bridge between the capsid and core components of the virion. The capsid structure is depicted schematically in Fig. 1.

2.2. Genome Organization

The core of the adenoviral particle contains the viral genome, a linear, double-stranded DNA molecule approximately 36 kb in length (ref. (3) and references therein). The genome is highly condensed and associated with two basic proteins that organize the DNA into a nucleosome-like structure. The \textit{cis}-acting origins of replication of the viral DNA are located in the first 50 base pairs (bp) of the 100- to 140-bp inverted terminal repeat sequences (ITRs) located at each end of the genome. The ITRs play an important role in replication of the DNA. A terminal protein is covalently attached to each of the 5’ termini of the DNA and serves as a primer for DNA replication. The left end of the genome also includes a \textit{cis}-acting packaging signal that directs the interaction of the viral DNA with its encapsidating proteins.
The adenoviral genome is shown schematically in Fig. 2. By convention, it is drawn with the immediate early transcription unit (E1A) at the left end, adjacent to the packaging signal. In addition, there are four early transcription units (E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one late unit (major late) which is processed to give five families of late mRNAs (L1 to L5), all of which are transcribed by RNA polymerase II. Transcription of each of the adenovirus genes leads to multiple mRNAs.

3. The Biology of Adenoviral Infection

The rational design of adenoviral vectors is based on an understanding of the infectious cycle of the parental viruses (ref. (3) and references therein). The replication cycle is conventionally divided into two phases separated by the onset of viral DNA replication. The early phase starts as soon as the virus interacts with the host cell: entry into the cell and transport of the viral genome to the nucleus, followed by the transcription and translation of early viral genes. These events modulate the functions of the host cell to facilitate the replication of the virus DNA and the transcription and translation of the late genes. In permissive cells, the early phase takes 5-6 hours, after which time viral DNA replication is first detected. The late phase begins concomitantly with the onset of DNA replication and involves the expression of the late viral genes, leading to the assembly in the nucleus of the structural proteins and the maturation of infectious viruses. The host cells lyse to release progeny virions about 20-24 hours post-infection.

The entry of adenoviruses into susceptible cells requires two distinct, sequential steps--binding and internalization--each mediated by the interaction of a specific capsid protein with a cellular receptor (Fig. 3). The initial high affinity binding of Ad2 and Ad5 to the primary
cellular receptor (5, 6), designated CAR (for coxsackievirus and adenovirus receptor), occurs via the globular knob domain of the fiber capsid protein (7, 8). CAR appears to function purely as a docking site for the virus on the cell surface: the cytoplasmic and transmembrane domains of the molecule are not essential for adenoviral infection (9, 10). Subsequent internalization of the virus by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein (11) with secondary host cell receptors, integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) (12). The virion then escapes from the endosome, the capsid is disrupted and the virus is transported to the nuclear membrane. The genome then passages through the nuclear pore into the nucleus where the primary transcription events are initiated.

Expression of the adenoviral genes is temporally regulated (3). E1A is the first transcription unit to be expressed after the adenoviral chromosome enters the nucleus of an infected cell; its expression requires only cellular proteins. The E1A proteins activate transcription from the other adenoviral early regions and induce the host cell to enter the S phase of the cell cycle. The E1B gene encodes two proteins (E1B 19K and E1B 55K) that inhibit apoptosis and further modulate cellular metabolism to render the cell more susceptible to viral replication. The E2 transcription unit encodes three proteins involved in viral DNA replication: DNA polymerase (Pol), preterminal protein (pTP) and DNA binding protein (DBP). The E3 region encodes multiple proteins designed to inhibit pathways of cell death induced by the host innate and cellular immune response to the infected cell. The E3 proteins are dispensable for the replication of adenoviruses in tissue culture. The E4 gene products perform a range of functions, with distinct proteins playing roles in viral DNA replication, viral mRNA transport and splicing, shut-off of host protein synthesis and regulation of apoptosis.
The expression of the early adenoviral genes sets the stage for replication of the viral DNA. Replication of the adenoviral DNA starts at the origins of replication in the ITRs at either end of the chromosome, with the terminal protein serving as a primer. The expression of the late adenoviral genes commences with the onset of DNA replication. The late gene products are expressed after processing a 20 kb transcript from the major late promoter, which is attenuated during transcription of the early genes. This primary transcript undergoes multiple splicing events to generate five families of late mRNAs encoding proteins that are part of the viral capsid or are involved in the encapsidation and assembly of viral particles in the host cell nucleus. Encapsulation of the viral DNA is directed by the packaging signal at the left end of the chromosome. This process is accompanied by alterations in the nuclear infrastructure and the permeabilization of the nuclear membrane, facilitating the egress of the progeny viruses into the cytoplasm. The plasma membrane subsequently disintegrates and the progeny are released from the cell.

4. Adenoviral Vectors

4.1. Design of Adenoviral Vectors

The most widely used adenoviral vectors for gene therapy are the so-called “first generation” replication-deficient vectors based on human Ad2 and Ad5, in which the E1 region of the genome is deleted (1, 4). Deletion of the E1 region, while retaining the ITR and packaging signal, is designed to prevent expression of the E2 genes and thus block viral DNA replication and the synthesis of late structural proteins. E1-deleted adenoviral vectors are therefore propagated in complementing human cell lines that provide the E1 proteins in trans. In order to provide additional cloning space in the vector, the E3 region, which is not necessary for
viral replication in culture, is also commonly deleted. Since adenoviruses can encapsidate DNA ranging from 75 to 105% of the length of the wild-type viral genome, these modifications allow up to 7.5 kb of foreign DNA to be accommodated.

Although E1-deleted adenoviral vectors are in theory replication-defective, in practice many cells possess E1-like proteins that can activate the E2 genes, leading to viral DNA replication and the expression of the late structural proteins. It has also become clear that the E1-dependence of E2, E3 and E4 gene transcription can be circumvented at high multiplicities of infection. Newly synthesized adenoviral peptides displayed on the surface of infected cells are recognized and destroyed by cytotoxic T lymphocyte and natural killer cell-mediated responses. In many cases the expressed transgene product has also been shown to be immunogenic. As a consequence of the elimination of infected cells by the cellular immune response, transgene expression mediated by first generation adenoviral vectors in vivo is only transient, lasting 2-3 weeks.

In an attempt to reduce immunogenicity, subsequent generations of adenoviral vectors have been designed to be defective for multiple viral genes, in addition to E1. The removal of genes encoding proteins essential for DNA replication (the DNA binding protein, DNA polymerase and terminal protein), or key regulatory functions (the E4 proteins) has led to vectors which in some studies have been reported to be less immunogenic than first generation E1-deleted vectors and to mediate longer term gene expression. However, in other studies these vectors have shown minimal or no advantage over first generation vectors. The production of these multiply deleted vectors has necessitated the construction of novel complementing cell lines that provide the missing function in trans.
The strategy of deleting regions of the viral genome has met its ultimate realization with the so-called “gutted vectors” which retain only the ITRs and packaging signals. The gutted vectors can accommodate up to 36 kb of foreign DNA, and can therefore carry large cDNAs together with appropriate regulatory elements. Production of these vectors requires the use of helper viruses, from which the gutted vectors must be separated and purified, a process which has been simplified by the development of packaging-defective helper viruses. Compared to first generation vectors, gutted vectors have shown reduced immunogenicity and more persistent gene expression in vivo.

4.2. Construction of Adenoviral Vectors

A number of different approaches have been used to construct adenoviral vectors (reviewed in (13)). The classical method for construction of vectors with the E1 region substituted with the transgene of interest employs homologous recombination in an E1-complementing human cell line between two DNA molecules, one carrying sequences mapping to the left end of the adenoviral genome and the gene of interest, and one carrying the adenoviral genome with the left end deleted but retaining some sequences that partially overlap the 3’ end of the first molecule. This second molecule can be either a linearized partial viral genome purified from virions (14) or a plasmid (15, 16). This technique suffers from the inefficiency of homologous recombination in mammalian cells, and the need for purification of individual viral plaques, which means it is both labor-intensive and time-consuming. Another big disadvantage is that if no recombinants are generated, the researcher is unable to determine whether the problem is technical or biological.

The past few years have seen the development of new methods to facilitate the generation of adenoviral vectors by constructing the recombinant vector genome prior to transfection of the
E1-complementing mammalian cells, thereby avoiding multiple rounds of plaque purification. One approach which has found widespread use exploits the highly efficient homologous recombination machinery in bacteria to generate a recombinant adenoviral vector by homologous recombination in *Escherichia coli* between a large plasmid containing most of the adenoviral genome and a small shuttle plasmid containing the expression cassette flanked by sequences homologous to the region to be targeted in the viral genome (17-19). The recombinant adenoviral genome is then linearized by restriction digestion and used to transfect E1-complementing mammalian cells to produce viral particles and propagate the vector.

### 4.3. Production and Purification of Adenoviral Vectors

The original E1-complementing cell line, designated 293, was generated by transformation of human embryonic kidney cells with sheared Ad5 DNA (20). The cells constitutively express the left 11% of the Ad5 genome and can be used to produce E1-deleted vectors at high titers of up to $10^{13}$ particles per ml. However, a disadvantage of the 293 cell line is that it allows the emergence of replication-competent adenovirus (RCA) as a result of homologous recombination between the host cell genome and the vector (21). This has led to strategies to avoid RCA by creating rationally designed E1-complementing helper cell lines with minimal or no homologous sequences between the transfected E1 DNA and E1-deleted vector (22, 23).

The classical method for purification of adenoviral vectors is cesium chloride density gradient ultracentrifugation. This is an efficient technique that can yield highly purified viral particles, although it is time-consuming, rather expensive and is not amenable to large-scale purification of adenoviral vectors. More recently, adenoviral vectors have been purified by column chromatography using resins originally developed for protein purification (24).
exchange chromatography is commonly used in an initial purification step, followed by immobilized metal affinity chromatography or reversed phase high-performance liquid chromatography as the second step. Column chromatography offers the ability to rapidly purify large amounts of virus to a highly pure state without compromising the viability of the viral particles.

After purification, the concentration of the adenoviral vector preparation is determined by physical and/or biological methods (25). The most common physical method for calculating the number of viral particles is to disrupt the particles with sodium dodecyl sulfate (SDS) and determine the optical absorbance of the virion DNA at 260 nm, using the conversion factor $1.1 \times 10^{12}$ particles per absorbance unit (26). Biological methods involve the infection of cells in culture followed by the determination of infectious adenoviral vector particles, either by counting visible plaques in a monolayer of cells which support replication of the vector, or by histochemical or immunohistochemical staining of cells to detect expression of a viral structural protein or a reporter gene delivered by the vector. The biological titer of the vector is then expressed in terms of plaque-forming units (PFU), infectious units (IU) or transducing units (TU).

5. Applications of Adenoviral Vectors

Adenoviral vectors can mediate high, albeit transient, levels of expression of the transgene in mammalian cells, resulting in yields of the recombinant protein of up to 30% of total cellular protein. The expressed proteins are subject to the full range of complex post-translational modifications that might be necessary for their appropriate folding and function. Recombinant viral and mammalian proteins are therefore identical to the native proteins, thereby
avoiding the disadvantages associated with expression of these proteins in prokaryotes, lower eukaryotes and insect cells.

Based on these favorable characteristics, E1-deleted adenoviral vectors have been employed for expression of recombinant proteins in cultured mammalian cells in vitro (1). Since adenoviral vectors can infect a range of dividing and nondividing mammalian cells, they have also been widely used in gene transfer experiments and gene therapy applications in vitro and in vivo, in both preclinical studies in animal models and in clinical trials in human patients (4, 27). As of July 2006, more gene therapy clinical trials (305 or 26%) have employed adenoviral vectors than any other vector, viral or nonviral (28). These clinical trials have been designed to exploit the ability of adenoviral vectors to accomplish in vivo gene delivery.

In the field of cancer gene therapy, adenoviral vectors have been widely employed in mutation compensation and molecular chemotherapy approaches where the goal is to eradicate the transduced cell. Adenoviral vectors have delivered a variety of therapeutic genes for cancer, including the tumor suppressor genes p53 (29) and p16 (30), antisense DNA, ribozymes and single-chain antibodies (31, 32), and the suicide genes herpes simplex virus thymidine kinase and cytosine deaminase. In the absence of a vector capable of targeted, tumor cell-specific gene delivery upon systemic administration, clinical trials involving adenovirus-mediated gene transfer have concentrated on those cancers that would benefit from improved local or regional control of tumor growth, including head and neck squamous cell carcinoma, brain, bladder and ovarian cancers, locally advanced prostate cancer, and non-metastatic stages of non-small cell lung cancer and breast cancer. The first commercially approved gene therapy product is based on a human adenovirus serotype 5 vector engineered to express the p53 tumor suppressor gene (33). This product, designated Gendicine (distributed by the Chinese company Shenzhen
SiBiono GeneTech, Shenzhen, China), has been approved by the State Food and Drug Administration of China for treatment of patients with head and neck squamous cell carcinoma and is in late-stage clinical trials for a variety of other malignancies (34). In the United States and Europe, an Ad5 vector carrying the p53 gene is in phase II/III and phase III clinical trials for stage III ovarian and primary peritoneal cancer, locally advanced unresectable non-small cell lung cancer and head and neck squamous cell carcinoma, both as monotherapy and in combination with radiation and/or chemotherapy agents (28, 35). However, for reasons that will be discussed below, the results of adenovirus-mediated cancer gene therapy have, in general, been disappointing, with only limited efficacy being observed in preclinical and clinical studies.

Adenoviral vectors have advanced to late stage clinical trials in patients with coronary artery disease (28), in which intracoronary administration of an Ad5 vector carrying the fibroblast growth factor 4 gene is designed to achieve therapeutic angiogenesis (36). The favorable properties of adenoviral vectors for gene therapy could also be rationally exploited in the treatment of numerous other diseases or conditions requiring short-term, high-level gene expression. There is also considerable interest in developing adenovirus-based vaccines for infectious and acquired diseases, including AIDS (37), Ebola virus (38), pulmonary tuberculosis (39) and cancer (40).

6. **Limitations of Adenoviral Vectors and Strategies to Improve the Vectors**

6.1. **Targeting**

A number of limitations of adenoviral vectors have been identified in the course of preclinical and clinical studies. In general, the results of adenovirus-mediated cancer gene therapy have been disappointing, with only limited efficacy being observed in preclinical and
clinical studies. A number of studies have shown that primary cancer cells express only low levels of CAR (41), the primary cellular receptor for Ad5, and it has been demonstrated that the therapeutic efficacy of Ad5 vectors is restricted by the inability of the vectors to infect tumor cells expressing low levels of CAR (42). A number of other cells and tissues that represent potentially important targets for gene therapy, including airway epithelium (43), mature skeletal muscle (44) and monocyte-derived dendritic cells (45), have similarly been shown to be CAR-deficient, resulting in a low efficiency of Ad2- and Ad5-mediated gene delivery. This therefore suggests that the efficacy of Ad5 vectors for many gene therapy applications could be improved by modifying the viruses to allow efficient infection via a CAR-independent pathway. Moreover, the CAR-dependence of transduction by Ad2 and Ad5 will result in sequestration of recombinant vectors by non-target, yet high-CAR-expressing cells. Hence, targeting of Ad2 and Ad5 vectors to alternate cellular receptors is mandated for specificity of gene delivery, with the ultimate goal of developing a targeted, injectable vector capable of cell-specific gene delivery upon systemic administration.

Modification of adenoviral tropism is accomplished by alteration of the knob domain of the fiber capsid protein to redirect binding to an alternative cellular receptor. Since adenoviruses use two distinct capsid proteins for cell binding and entry, modifications to the fiber protein, which is responsible for binding to the primary cellular receptor, will not adversely affect internalization, which is mediated by binding of the viral penton base protein to cellular integrins.

Targeted adenoviral vectors have been constructed by two general strategies (reviewed in ref. (46)). In one approach, the vector is complexed with molecular bridges, either chemical conjugates (47) or recombinant fusion proteins (48), with specificity for both the vector and a
cellular receptor. A truly targeted vector can be generated by designing the vector-specific component of the bispecific molecule to ablate native viral tropism -- for example, a neutralizing anti-fiber antibody (47) or a soluble form of CAR (48). This approach has the advantage that a single targeting moiety can be employed with different vectors, but suffers from the problem that the two components, vector and targeting molecule, must be generated separately, limiting its attractiveness for clinical application.

An alternative approach to targeting involves the genetic modification of the vector, thus forming a single-component system (reviewed in ref. (49)). While the most commonly used adenoviral vectors for gene therapy are based on species C serotypes 2 and 5, which recognize CAR, other adenoviral serotypes recognize a different primary cellular receptor. This has led to the hypothesis that CAR-independent gene transfer could be accomplished by substituting fiber genes from the Ad2 or Ad5 backbone with genes encoding homologous fiber proteins from alternate adenoviral serotypes, a process known as “pseudotyping”. While pseudotyping an Ad5-based vector with fiber proteins from serotypes such as Ad3 (50) and Ad35 (51) of species B, has allowed efficient, CAR-independent gene transfer to cancer cells, the approach is still limited by its reliance on the expression of a native adenoviral receptor by the target cells. This limitation can be overcome by incorporating cell-specific targeting ligands into the fiber to redirect adenoviral infection.

To date, the majority of genetically modified adenoviral vectors incorporating targeting peptide ligands possess expanded tropism – they retain the ability to recognize the native primary receptor, CAR. Adenoviral vectors containing the αv integrin-specific Arg-Gly-Asp (RGD) peptide motif have been shown to increase the efficiency of gene delivery by up to 3 orders of magnitude to a variety of CAR-deficient primary human cancer cells in vitro, without increasing
gene transfer to normal, CAR-positive cells (52). A major advantage of such vectors with enhanced infectivity is that a given level of gene transfer can be achieved with a lower viral dose, compared with the untargeted vector. Since the viral dose is directly related to toxicity, this has important implications for safety. The improvement in infectivity that is observed with these vectors translates into an enhanced therapeutic benefit in preclinical animal models, supporting their evaluation in human clinical trials.

Now that the amino acid residues responsible for binding CAR have been identified, site-directed mutagenesis of the fiber protein will permit the engineering of vectors lacking native tropism but possessing specificity for target receptors. A more radical approach to the construction of truly targeted adenoviral vectors involves the replacement of the knob domain of the fiber with a targeting moiety. The technical challenge is to retain trimerization of the modified fiber protein, so that mature viral particles can be assembled. This has been achieved by replacing the fiber with the trimeric fibritin protein from bacteriophage T4 (53), a maneuver which has allowed the trimeric CD40L protein to be incorporated as a targeting motif (54, 55).

An additional level of specificity for the target cancer cell can be achieved by placing the therapeutic gene under the transcriptional control of a tissue- or tumor-selective promoter. Since both transductional and transcriptional targeting approaches by themselves tend to be “leaky”, the combination of two complementary targeting approaches leads to enhanced specificity for the target cells (56).

It is anticipated that the further improvements in the area of transductionally targeted adenoviral vectors will ultimately lead to a targeted, injectable vector that will be capable of transducing target cells upon vascular administration. This will require additional obstacles to be overcome, which will necessitate a better understanding of adenovirus-host interactions,
including sequestration of adenoviral vectors by blood cells (57) and adenoviral binding to blood factors leading to infection of hepatic cells (58). Furthermore, a better understanding of the role of both hepatocytes and Kupffer cells in hepatic sequestration of systemically administered vectors should lead to rational strategies by which this can be overcome. It is also recognized that there are physical barriers to adenoviral transduction of target cells and tissues (59, 60).

6.2. Immune Response

The use of first generation adenoviral vectors in vivo is associated with the induction of both an innate and an acquired immune response (reviewed in (61, 62)). Studies in mice and primates have indicated that within the first few hours of administration of adenoviral vectors by the intravenous route, the viral capsid proteins trigger an acute inflammatory response characterized by the rapid release of inflammatory cytokines, including interleukin-6 (IL-6) and IL-8, and the recruitment of immune effector cells, such as neutrophils, into the liver. This acute-phase toxicity does not require expression of viral genes but is dependent on the dose of vector: minimal toxicity has been shown to result from administration of low doses of E1-deleted vectors to mice. Since the acute inflammatory response is directly related to the vector dose, toxicity could be reduced by lowering the number of viral particles necessary for a given level of gene transfer, as discussed above.

Over the next 24 to 96 hours, toxicity associated with first generation Adenoviral vectors results from an acquired cellular immune response. In those instances where the goal of gene delivery by a first generation adenoviral vector is the elimination of the transduced cell, for example in cancer gene therapy, the induction of a cytodestructive immune response is beneficial. However, in many cases the eradication of the transduced cell would be a serious problem. This has led to strategies to overcome cellular immunity by modifying either the vector
(by deleting the E2 and E4 regions or creating gutted vectors for long-term gene expression) or the host (by means of various immunosuppressive regimens (63) or by blocking the co-stimulatory molecules necessary for T-cell activation (64)).

In addition to cellular immunity, a humoral immune response is generated to the adenoviral vector. This leads to a reduction in Adenoviral-mediated gene delivery upon repeat vector administration. Moreover, even the initial vector dose may be inefficient in human patients who possess neutralizing antibodies to the commonly used Ad2 or Ad5 vectors, as a result of prior exposure to the parental viruses. Strategies to circumvent pre-existing immunity to Ad2 or Ad5 vectors would therefore have obvious practical implications for vaccination and gene therapy. One approach involves the development of vectors based on alternate human or animal adenoviral serotypes to which the seroprevalence of neutralizing antibodies is low, such as human Adenoviral serotype 49 (65) and chimpanzee adenovirus (66). It has been demonstrated that Ad5-specific neutralizing antibodies are directed against epitopes located within short hypervariable regions (HVRs) on the Ad5 hexon capsid protein (67). Substitution of these HVRs in Ad5 with the corresponding HVRs from the rare adenovirus serotype Ad48 allowed the resultant. HVR-chimeric Ad5 vectors to circumvent pre-existing anti-vector immunity (67). Although the host might not have pre-existing immunity to a given vector, it can be envisioned that the subsequent development of neutralizing antibodies would mean that repeat administration would necessitate the use of a distinct vector.

7. Summary

Adenoviral vectors possess a number of features that have favored their widespread employment both in vitro and in vivo. In fact, the use of Adenoviral vectors is increasing as
technologies to facilitate their construction are being developed and refined. First generation, E1-deleted Adenoviral vectors have been shown to be associated with limitations that are being addressed by rational strategies based on the biology of the virus. These advances should allow the realization of the full potential of Adenoviral vectors for \textit{in vivo} gene delivery upon systemic administration.
Acknowledgements

Research in the author’s laboratory is supported by NIH grant R01 CA 108585.
References


**Figure Legends**

Fig. 1. Schematic diagram of Ad5 virion. The double-stranded DNA genome is packaged within an icosahedral protein capsid. The major structural protein of the capsid is the hexon. Penton capsomers, formed by association of the penton base and fiber, are localized at each of the twelve vertices of the Adenoviral capsid.

Fig. 2. Schematic diagram of the structure of the Ad5 genome. The Ad5 genome is approximately 36 kb long, divided into 100 map units. The direction of transcription is indicated by arrows. Closed arrows represent early transcripts; open arrows represent late transcripts.

Fig. 3. The pathway of adenoviral infection. The entry of Adenoviral into susceptible cells involves two distinct, sequential steps. The initial high affinity binding of Ad5 to the primary cellular receptor, CAR, occurs via the globular knob domain of the trimeric fiber capsid protein. Subsequent internalization of the virus by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base protein with secondary host cell receptors, integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \). The virion then escapes from the endosome and localizes to the nuclear pore whereupon its genome is translocated to the nucleus.
Research Paper

Tissue Inhibitor of Metalloproteinases-2 Improves Antitumor Efficacy of a Replicating Adenovirus In Vivo

Myung-hee Kim¹
Thomas M. Bodenstine²
Lucretia A. Sumerel¹
Angel A. Rivera¹
Andrew H. Baker³
Joanne T. Douglas¹

¹Division of Human Gene Therapy, Departments of Medicine, Obstetrics & Gynecology, Pathology and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham; Birmingham, Alabama USA
²Department of Pathology, University of Alabama at Birmingham; Birmingham, Alabama USA
³Division of Cardiovascular and Medical Sciences, University of Glasgow; Glasgow, UK

Correspondence to: Joanne T. Douglas; Gene Therapy Center; University of Alabama at Birmingham; 901 19th Street South; BMR2 412; Birmingham, Alabama 35294; Tel: 205.975.2897; Fax 205.975.8565 Email: Joanne.Douglas@cc.uab.edu

Original manuscript submitted: 08/21/06
Manuscript accepted: 09/07/06

This manuscript has been published online, prior to printing for Cancer Biology & Therapy, Volume 5, Issue 12. Definitive page numbers have not been assigned. The current citation is: Cancer Biol Ther 2006; 5(12): e1-e7, Ahead of Print: http://www.landesbioscience.com/journals/cbt/abstract.php?id=3374

ABSTRACT

Clinical studies of replicating adenoviruses for the treatment of cancer have demonstrated their safety but have yielded disappointing results, indicating the need for new strategies to improve their efficacy. We hypothesized that the efficacy of a replicating adenovirus could be improved by expression of tissue inhibitor of metalloproteinases-2 (TIMP-2), a 21-kDa unglycosylated secretory protein. TIMP-2 specifically inhibits the active forms of a number of matrix metalloproteinases (MMPs) that play a role in the degradation of basement membranes and the extracellular matrix and are therefore involved in the control of the growth, invasion and metastasis of tumor cells, as well as angiogenesis. In addition, TIMP-2 can abrogate tumor growth and angiogenesis by a variety of mechanisms independent of MMP inhibition. In this study, we demonstrate that expression of TIMP-2 enhanced the antitumor efficacy of a replicating adenovirus in vivo, by reducing both tumor growth and angiogenesis.

INTRODUCTION

Oncolytic viruses tailored to replicate selectively within tumor cells are novel 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. Replication-selective viruses therefore overcome a major limitation of replication-defective vectors for cancer gene therapy, which are unable to infect all, or even most, cells within a solid three-dimensional tumor mass. The safety of oncolytic viruses derives from the restriction of their replication to tumor cells, while sparing normal cells.

While several oncolytic viruses have been identified to date, replication-selective adenoviruses possess a number of advantages. Human serotype 5 adenoviruses are associated with relatively mild diseases, their biology is relatively well characterized and their genomes can be manipulated with relative ease. Moreover, adenoviruses can be purified to high titer or tissue sparing normal cells.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant R01 CA108585 and DOD grant W81XWH-04-1-0800.
TIMP-2 is a 21-kDa unglycosylated protein that is secreted in a soluble form by endothelial cells and fibroblasts. TIMP-2 binds in a 1:1 stoichiometric ratio to the active forms of a number of MMPs, including membrane type 1 MMP (MT1-MMP), MMP-2 and MMP-9, thereby specifically abrogating the MMP activity associated with tumor growth and angiogenesis. In addition to its antitumor activity as an inhibitor of MMPs, TIMP-2 can also inhibit tumor growth and angiogenesis by a variety of mechanisms independent of MMP-inhibition. TIMP-2 is unique among the members of the TIMP family or synthetic MMP inhibitors in being able to directly inhibit the proliferation of endothelial cells in response to angiogenic stimuli such as fibroblast growth factor 2 or vascular endothelial growth factor A. Seo et al. have shown that the growth-inhibitory activity of TIMP-2 for human microvascular endothelial cells is mediated through binding to α3β1 integrin and induction of protein tyrosine phosphatase activity. Oh et al have recently demonstrated that TIMP-2 inhibits endothelial cell migration through an indirect MMP-inhibitor effect that requires transcription, synthesis, and cell surface localization of the RECK gene product. Furthermore, Feldman et al have reported that upregulation of mitogen-activated protein kinase phosphatase 1 in tumors overexpressing TIMP-2 results in dephosphorylation of p38 mitogen-activated protein kinase, leading to inhibition of tumor growth and angiogenesis.

A number of early studies using TIMP-2 delivered as a recombinant protein or via plasmid-mediated expression demonstrated the feasibility of employing TIMP-2 for anticancer therapy. To this end, TIMP-2 was shown to block both tumor growth and local invasion through extracellular matrices. Subsequent studies demonstrated that delivery of TIMP-2 by replication-defective adenoviral vectors results in the reduction of tumor growth, angiogenesis and metastasis.

Moreover, in addition to having a direct effect on cancer growth, adeno-virus-mediated delivery has demonstrated the potential of TIMP-2 in protection of normal organs against metastatic cancer cells.

Based on this biology, we hypothesized that the efficacy of a replicating adenovirus could be enhanced by expression of TIMP-2. In this strategy, the replicating adenovirus would kill cancer cells directly from within by oncolysis, while secretion of TIMP-2 by the infected cells would complement this therapeutic effect by restricting tumor growth and angiogenesis via both MMP-dependent and -independent mechanisms.

**MATERIALS AND METHODS**

**Viruses.** Adw300, a wild-type human adenovirus serotype 5, was obtained from the American Type Culture Collection (Manassas, VA). Ad-TIMP-2, an E1-, E3-deleted replication-deficient Ad5 vector which expresses TIMP-2 under the control of the CMV promoter, has been described previously. AdLacZ is an E1-, E3-deleted adenovirus that expresses β-galactosidase under the control of the CMV promoter. The wild-type adenovirus and the vectors were propagated in the permissive 293 cell line and purified by two rounds of cesium chloride density centrifugation. To determine the viral particle concentration, the virus was diluted in 10 mM Tris (pH 8.0)-1 mM EDTA-0.1% SDS, incubated at 56°C for 10 min, and the absorbance at 260 nm was measured. Under these conditions, an absorbance of 1 corresponds to $1.1 \times 10^{12}$ particles/ml.

**Cell lines.** MDA-MB-231 human breast cancer cells and LNCaP human prostate cancer cells were acquired from the American Type Culture Collection. SKOV3.ip1 human ovarian cancer cells were obtained from Janet Price (Baylor University, Houston, TX). 293 cells were purchased from Microbix (Toronto, Ontario, Canada). MDA-MB-231, SKOV3.ip1 and 293 cells were maintained in DMEM/Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 µg/ml). LNCaP cells were maintained in RPMI 1640 medium containing 10% FCS, penicillin (100 units/ml), streptomycin (100 µg/ml), sodium pyruvate (1 µM) and sodium bicarbonate (1.5 g/l). All cells were propagated at 37°C in a humidified 5% CO$_2$ atmosphere.

**FCs** was purchased from Gibco-BRL, Grand Island, NY and media and supplements were from Mediatech, Herndon, VA.

**Quantification of adenoviral DNA replication.** Monolayers of MDA-MB-231, SKOV3.ip1 or LNCaP cells in 6-well plates were coinfected with Adw300 at a multiplicity of infection (MOI) of 1 viral particle per cell and Ad-TIMP-2 at MOIs of 0, 0.1, 1 and 10 viral particles per cell. Ten days post-infection, attached and detached cells were harvested and DNA extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The DNA was subjected to quantitative real-time PCR using primers and a probe specific for the adenoviral E1A region (forward primer: 5'-AACAGTTGCGCTGAGTTG-3'; reverse primer: 5'-CTCGTTAACGAATCTCGATA-3'; probe: 5'-CACAGGCTGGCGAGGCCA-3'). Human β-actin DNA was also amplified to allow normalization of the data (forward primer: 5'-TAAGTAGGCGCACAGTAGGTCTGA-3'; reverse primer: 5'-AAAGTGCAAAGAACAGCGTAA-3'; probe: 5'-CAGACTCCCATCCCCAAGCCCA-3').

**Adenovirus yield assay.** Monolayers of MDA-MB-231, SKOV3.ip1 or LNCaP cells in 6-well plates were coinfected with Adw300 at an MOI of 1 viral particle per cell and Ad-TIMP-2 at MOIs of 0, 0.1, 1 and 10 viral particles per cell. Ten days post-infection, the cells and media were harvested and the number of infectious particles determined by titering on 293 cells. Forty hours post-infection, 293 cells were fixed with methanol and infected cells were identified in an immunoassay using polyclonal rabbit anti-Ad5 antiserum (Cocalico, Reamstown, PA) as the primary antibody with a horse-radish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). DAB (Sigma, St Louis, MO) was employed as the chromogenic substrate and brown cells were counted using a light microscope.

**CPE assay.** Monolayers of MDA-MB-231, SKOV3.ip1 or LNCaP cells in 24-well plates were coinfected with Adw300 at an MOI of 1 viral particle per cell and Ad-TIMP-2 at MOIs of 0, 0.1, 1 and 10 viral particles per cell. Ten days post-infection, the cells were fixed and stained with crystal violet.

**In vitro cytotoxicity assay.** Monolayers of MDA-MB-231, SKOV3.ip1 or LNCaP cells in 96-well plates were coinfected with Adw300 at an MOI of 1 viral particle per cell and Ad-TIMP-2 at MOIs of 0, 0.1, 1, 10 and 100 viral particles per cell. Ten days post-infection, a commercial cell proliferation assay (Promega, Madison, WI) was used to measure cell survival according to the manufacturer’s instructions.

**Animal experiments.** Animal experiments were performed in accordance with federal and institutional guidelines for animal care. MDA-MB-231 tumor xenografts were established by subcutaneous injection of 4 x 10^6 cells into the flank of 8–10 week-old female NGC athymic nude mice (*nu/nu*; Taconic, Germantown, NY). On reaching 80–100 mm^3, the tumor nodules were injected with 50 µl PBS or with a single dose of the following virus treatments in 50 µl PBS: 10^6 particles of Adw300 plus 10^6 particles of Ad-TIMP-2;
10^6 particles of Ad-TIMP-2 alone; 10^6 particles of Adwt300 plus 10^6 particles of AdLacZ, as a control (8 mice per group). Bidimensional tumor measurements were taken twice a week with calipers and the tumor volume was calculated using the simplified formula for a rotational ellipsoid: 0.5 x length x width^2 (see ref. 20). Animals were followed for 28 days, until the tumor burden in some of the groups became excessive, whereupon the mice were sacrificed and tumors excised.

**Immunohistochemistry.** Tumors excised from the treated mice were snap frozen in liquid nitrogen and cut into 50 μm sections. The sections were fixed with acetone and endogenous peroxidase activity was quenched by incubation in 0.3% (v/v) H_2O_2 in methanol for 30 min. Blood vessels were then visualized via a three-step staining procedure using a rat anti-mouse CD31 monoclonal antibody (BD Biosciences, San Jose, CA), followed by a biotin-conjugated goat anti-rat Ig-specific polyclonal antibody (BD Biosciences) and then an avidin: biotinylated HRP complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). DAB was employed as the chromogenic substrate. The number of brown-stained blood vessels was counted in three microscopic fields with magnification x10 in tumor sections from three mice per group.

**Statistical analysis.** Differences between groups were analyzed using the Student-Fisher t-test. p < 0.05 was considered statistically significant.

**RESULTS**

In this proof-of-concept study, we employed a two-component model system to investigate the hypothesis that the efficacy of a replicating adenovirus could be enhanced by arming it with TIMP-2. In this regard, coinfection of cells with a wild-type adenovirus and a replication-defective E1-deleted adenoviral vector expressing TIMP-2 allows replication of the vector as a result of trans-complementation by the E1 proteins expressed by the wild-type virus.

**Expression of TIMP-2 does not inhibit the oncolytic potency of a replicating adenovirus in vitro.** Monolayers of MDA-MB-231 human breast cancer cells, SKOV3.ip1 human ovarian cancer cells and LNCaP human prostate cancer cells (which express MMP-2 and MMP-9 (see refs. 21–24) were coinfected with a replicating human serotype 5 adenovirus, Adwt300, at a multiplicity of infection (MOI) of 1 viral particle per cell, and a previously described replication-defective Ad5 vector expressing TIMP-2, Ad-TIMP-2 (see ref. 17), at MOIs of 0.1, 1 and 10 viral particles per cell. As a control, the cells were infected with Adwt300 alone. Ten days post-infection, the cells were harvested and DNA was extracted and subjected to quantitative real-time PCR analysis to determine the number of copies of the adenoviral E1 region, which is indicative of the synthesis of viral DNA by the replicating adenovirus. As shown in (Fig. 1), the number of copies of the E1 gene produced by Adwt300 was not significantly affected by coinfection with Ad-TIMP-2 at MOIs of 0.1, 1 or 10 viral particles per cell (p < 0.04 for all cell lines and MOIs tested).

We next sought to confirm that expression of TIMP-2 did not interfere with the ability of the replicating adenovirus to produce infectious progeny. To this end, monolayers of MDA-MB-231, SKOV3.ip1 and LNCaP cells were coinfected with a replicating adenovirus, Adwt300, at an MOI of 1 viral particle per cell, and Ad-TIMP-2 at MOIs of 0.1, 1 and 10 viral particles per cell. As a control, cells were infected with Adwt300 alone. Ten days post-infection, the cells and media were harvested and the number of infectious particles was determined by titrating on 293 cells.19 As shown in Figure 2, the number of viral progeny was not significantly affected by coinfection of Adwt300 with Ad-TIMP-2 at MOIs of 0.1 or 10 viral particles per cell (p < 0.05 for all cell lines and MOIs of 0.1 and 10; p = 0.06, 0.08 and 0.07 for MDA-MB-231, SKOV3.ip1 and LNCaP cells, respectively, at an MOI of 1).

We then examined whether expression of TIMP-2 would impair the oncolytic potency of the replicating adenovirus. Monolayers of MDA-MB-231, SKOV3.ip1 and LNCaP cells were coinfected with
We hypothesized that the level of expression of TIMP-2 that could be achieved by coinfection of the replicating adenovirus and Ad-TIMP-2 would be significantly greater than could be achieved by the replication-defective adenoviral vector alone. To investigate this, monolayers of MDA-MB-231, SKOV3.ip1 and LNCaP cells were coinfected with a replicating adenovirus, Adwt300, at an MOI of 1 viral particle per cell, and Ad-TIMP-2 at MOIs of 1 and 10 viral particles per cell. As a control, cells were infected with Ad-TIMP-2 alone at MOIs of 1 and 10 viral particles per cell. Expression and secretion of TIMP-2 were assayed by harvesting the culture medium at 3, 6, 9 and 12 days post-infection and subjecting it to immunoblot analysis using an anti-TIMP-2 monoclonal antibody. As shown in Figure 4A, at each MOI tested, higher levels of TIMP-2 were secreted by cells coinfectected with Ad-TIMP-2 and the replicating adenovirus, than cells infected with the replication-defective Ad-TIMP-2 alone. This observation was confirmed by an enzyme-linked immunosorbent assay in which the levels of TIMP-2 in the culture medium of infected MDA-MB-231 cells were quantified at various time-points post-infection (Fig. 4B).

Expression of TIMP-2 enhances antitumor efficacy of a replicating adenovirus in vivo. We next wished to determine whether expression of TIMP-2 would enhance the antitumor efficacy of the replicating adenovirus in vivo. Female athymic nude mice bearing subcutaneous MDA-MB-231 xenografts on the flank were given a single intratumoral injection of one of the following treatments in 50 μl PBS: $10^6$ particles of Adwt300 plus $10^6$ particles of Ad-TIMP-2; $10^6$ particles of Ad-TIMP-2 alone; $10^6$ particles of Adwt300 plus $10^6$ particles of AdLacZ, a control replication-defective adenoviral vector expressing β-galactosidase; or PBS alone (8 mice per group). Bidimensional tumor measurements were taken twice a week with calipers, and the tumor volume was calculated using the simplified formula for a rotational ellipsoid: $0.5 \times \text{length} \times \text{width}^2$ (see ref. 20). Tumor growth kinetics are shown in Figure 5. Twenty-eight days post-injection of the virus, tumors treated with the replicating adenovirus plus Ad-TIMP-2 were significantly smaller than tumors injected with the replicating adenovirus plus the irrelevant vector, AdLacZ (p = 0.04), with Ad-TIMP-2 alone (p = 0.03), or with PBS (p = 0.006). Hence, expression of TIMP-2 by a replicating adenovirus enhanced the inhibition of tumor growth in vivo.

In addition to playing a role in tumor growth, MMP-dependent matrix proteolysis is also involved in the process of angiogenesis. To analyze angiogenesis, tumor sections from these mice were subjected to immunohistochemical staining using a primary antibody against mouse CD31 (also known as PECAM-1, platelet endothelial cell adhesion molecule) to visualize endothelial cells. As shown in Figure 6A and B, significantly fewer blood vessels were observed in sections of tumors treated with the replicating adenovirus plus Ad-TIMP-2 compared to tumors injected with the replicating adenovirus plus the irrelevant vector, AdLacZ, with Ad-TIMP-2 alone, or with PBS (p ≤ 0.01 for all groups tested). Hence, expression of TIMP-2 by a replicating adenovirus reduced angiogenesis in vivo.

**Discussion**

In recognition of their potential, replication-selective adenoviruses have been rapidly translated into human clinical trials in patients with advanced cancer, where the safety of these agents has been demonstrated. However, clinical studies of replicating adenoviruses have yielded disappointing results, indicating the need for new strategies to improve their efficacy. In one approach to improve the efficacy of replication-selective adenoviruses, they have been engineered to

![Image 329x305 to 558x737]
TIMP-2 Enhances Antitumor Efficacy of Replicating Adenovirus

In most cases, such “armed” oncolytic adenoviruses have been designed to carry therapeutic genes, such as the suicide genes cytosine deaminase and herpes simplex virus thymidine kinase, that will augment the virus-mediated eradication of the infected tumor cells. However, rather than arm a replicating adenovirus with a protein which would merely act by killing the infected cancer cells, we hypothesized that it would be rational to arm the replicating adenovirus with a secreted protein with a distinct mechanism of action within the microenvironment of the cancer cells.

To this end, we propose to enhance the efficacy of a replicating adenovirus by arming it with TIMP-2. TIMP-2 possesses a number of attractive features that favor its use in this therapeutic strategy. It is a relatively small (21 kDa) unglycosylated protein that is naturally secreted in a soluble form.

Figure 3. Expression of TIMP-2 does not inhibit the oncolytic potency of the replicating adenovirus. Monolayers of MDA-MB-231, SKOV3.ip1 and LNCaP cells were coinfected with Adwt300, at an MOI of 1 viral particle per cell, and Ad-TIMP-2 at MOIs of 0, 0.1, 1 and 10 viral particles per cell. (A) Ten days post-infection, the cytopathic effect was monitored by staining proteins in the viable cells with crystal violet. (B) Ten days post-infection, a cell proliferation assay was performed to count viable cells. Representative results with MDA-MB-231 cells are shown.

deliver therapeutic transgenes. In most cases, such “armed” oncolytic adenoviruses have been designed to carry therapeutic genes, such as the suicide genes cytosine deaminase and herpes simplex virus thymidine kinase, that will augment the virus-mediated eradication of the infected tumor cells. However, rather than arm a replicating adenovirus with a protein which would merely act by killing the infected cancer cells, we hypothesized that it would be rational to arm the replicating adenovirus with a secreted protein with a distinct mechanism of action within the microenvironment of the cancer cells.

To this end, we propose to enhance the efficacy of a replicating adenovirus by arming it with TIMP-2. TIMP-2 possesses a number of attractive features that favor its use in this therapeutic strategy.

Figure 4. A replicating adenovirus expresses a greater level of TIMP-2 than a replication-defective adenoviral vector. Monolayers of MDA-MB-231, SKOV3.ip1 and LNCaP cells were coinfected with Adwt300 at an MOI of 1 viral particle per cell and Ad-TIMP-2 at MOIs of 1 and 10 viral particles per cell. As a control, cells were infected with Ad-TIMP-2 alone at MOIs of 1 and 10 viral particles per cell. (A) Expression and secretion of TIMP-2 were assayed by harvesting the culture medium at 3, 6, 9 and 12 days post-infection and subjecting it to immunoblot analysis using an anti-TIMP-2 monoclonal antibody. (B) The levels of TIMP-2 in the culture medium of MDA-MB-231 cells at various time-points post-infection were quantified in an ELISA (Fig. 4b). Hence, a greater level of TIMP-2 was expressed by a replicating adenovirus than by a replication-defective adenoviral vector.

It is a relatively small (21 kDa) unglycosylated protein that is naturally secreted in a soluble form.
TIMP-2 Enhances Antitumor Efficacy of Replicating Adenovirus

Oh et al have recently demonstrated that TIMP-2 inhibits endothelial cell migration through an indirect MMP-inhibitor effect that requires transcription, synthesis, and cell surface localization of the RECK gene product. Furthermore, Feldman et al have reported that upregulation of mitogen-activated protein kinase phosphatase 1 in tumors overexpressing TIMP-2 results in dephosphorylation of p38 mitogen-activated protein kinase, leading to inhibition of tumor growth and angiogenesis. Thus, there is a clear rationale for a therapeutic strategy exploiting the localized overexpression of TIMP-2 in the tumor microenvironment.

We hypothesized that the replicating adenovirus would kill cancer cells directly from within by oncolysis, while secretion of TIMP-2 by the infected cells would complement this therapeutic effect by restricting tumor growth and angiogenesis via both MMP-dependent and -independent mechanisms. In the proof-of-concept study described in this manuscript, we employed a two-component model system to investigate this hypothesis. In this regard, coinfection of cells with a wild-type adenovirus and a replication-defective E1-deleted adenoviral vector expressing TIMP-2 allows replication of the vector as a result of trans-complementation by the E1 proteins expressed by the wild-type virus.

For such a dual-action, armed replicating adenovirus to be of utility, it is important that the expression of TIMP-2 should not impair its oncolytic potency. Accordingly, we demonstrated that the oncolytic potency of a replicating adenovirus in MMP-2- and MMP-9-positive MDA-MB-231 human breast cancer cells was not inhibited by expression of TIMP-2. Moreover, we showed that a greater level of TIMP-2 was expressed by a replicating adenovirus than by a replication-defective adenoviral vector. Having performed in vitro studies to establish these two key indicators of efficacy, we evaluated the expression of TIMP-2 by a replicating adenovirus in the treatment of solid tumors in vivo. Expression of TIMP-2 by the replicating adenovirus was shown both to enhance the inhibition of tumor growth and to reduce angiogenesis in vivo. We have therefore shown that the therapeutic efficacy of a replicating adenovirus can be enhanced expression of TIMP-2.

TIMP-2 plays a central role in many physiological processes by limiting MMP activity and through other non-MMP inhibitory functions. As such it will be important to limit TIMP-2 transgene expression locally within the tumor. Now that we have proof-of-concept for usage of TIMP-2 in the oncolytic virus setting, we can develop a single-agent armed replicating adenovirus using both transcriptional and transductional targeting approaches to limit transgene expression to the tumor without compromising efficacy.
References