Retargeting recombinant adenoviral vectors expressing bone morphogenic protein 2 (BMP2) to improve the efficacy of bone formation in mice with applications in spinal fusion

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Abstract

Spinal fusion is a common medical procedure with potential for improvement. Bone deossification and pathologic fracture often occur in patients after surgery, so there is a need for improved therapeutic approaches to bone healing. One promising approach to improve this procedure is gene therapy mediated by adenoviral (Ad) vectors. These vectors can be armed with bone morphogenic protein-2 (BMP2) and used to infect human mesenchymal stem cells (MSCs) in vitro. In this model, the infected cells are then applied to the area of injury during surgery, thereby promoting bone healing. Unfortunately, MSCs express low levels of the primary Ad receptor, CAR, which limits the efficiency of gene delivery. To circumvent this problem, it is necessary to develop and characterize an Ad vector that bypasses the native CAR infection pathway for another receptor highly expressed in MSCs. To this end, we have constructed a tropism-modified vector targeted to \( \alpha_v \beta_3 \) integrins and expressing BMP2. We are in the process of determining the ability of the vector to deliver
BMP2 and stimulate bone formation in vitro. Successful completion of this study should provide guidance for further experiments in vivo.

Introduction

**Spinal Fusion.** Spinal fusion is a procedure that involves fusing together vertebrae in the spine. In the United States, this procedure is performed over 980,000 times per year (Katz, 1995). Spinal fusion is commonly used in those who require spine stabilization due to vertebral damage from ruptured discs, fractures, osteoarthritis, tumors, or scoliosis. The procedure involves the surgical fastening of vertebrae with pins and wires, although autogenous bone graft is often additionally used to enhance spine fusion (Lieberman et al., 2002).

Pseudarthrosis, a condition characterized by bone deossification and pathologic fracture, occurs after almost 40% of all spine fusions (Boden et al., 2000), and even more frequently after revisions (Hannallah et al., 2002). Since complications are frequent after spinal fusion, there is a need for improved therapeutic approaches to bone repair in addition to the common surgical techniques.

**Bone Morphogenic Proteins.** BMPs are naturally occurring bone growth factors which have been shown to induce bone formation when injected in rats (Alden et al., J Neuro 1999; Wozney et al., 1988). BMPs are soluble signaling proteins which bind to specific serine/threonine kinase cell surface receptors. BMP signaling is transduced by a group of proteins called Smads and results in the activation of certain genes that promote bone formation (Kusanagi et al., 2000). In experimental applications, BMPs are often used to induce
differentiation and can effectively turn mesenchymal stem cells (MSC) into bone-forming osteoblasts (Ebara and Nakayama, 2002).

One such protein, BMP2 has been shown to improve the efficacy of the spinal fusion procedure in primates (Boden et al., 1998). In this approach, a collagen sponge soaked with recombinant human BMP2 was inserted into specimen rhesus monkeys and fixed with a titanium threaded cage. It is especially appealing that BMP2 expression adjacent to the spine does not lead to nerve root compression. Unfortunately, practical delivery of recombinant BMP2 to the damaged area suffers from limitations; the protein has a relatively short half-life when administered directly in vivo (Boden, 2000). This limitation warrants the use of a gene delivery vehicle to induce prolonged expression of BMP2 at the site of injury.

Adequate bone formation using gene therapy via liposomes, gene gun ballistics, polymer-DNA complexes, or injection of naked DNA has been elusive (Alden et al, 2002). The most effective transfer of the BMP2 gene to the injured area in mice, rabbits, and primates has been accomplished using viral vectors, particularly vectors derived from adenovirus (Boden, 2000; Lieberman et al., 2002).

**Adenovirus.** Ad is an icosahedral capsid dsDNA virus. The characteristic Ad capsid morphology includes three major proteins: hexon, penton base, and knobbed fiber (figure 1) (Russel, 2000). A fiber protrudes from a penton base at each of the 12 capsid vertices. Most Ad vectors used for gene therapy are derived from

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![Figure 1. Diagram of basic Ad morphology (adapted from Barnett et al., 2002).](image)
human serotypes 2 and 5, which are also most common in nature (Barnett et al., 2002).

Native Ad infection commences with the primary Ad receptor, Coxsakie and Adenovirus Receptor (CAR). In this mechanism, the viral fiber knob first binds to cell-surface CAR and then the viral penton base interacts with cellular integrins to prompt clathrin-mediated internalization (Russel, 2000). It has been shown that the penton base contains an RGD motif (Arginine-Glycine-Aspartic Acid) that directly interacts with the integrin subunit (Stewart et al., 1997).

After cell entry, the endosome is lysed and the viral genome is released into the cytoplasm where it is taken up into the nucleus but not integrated into the host chromosome. The early region 1 (E1) encodes over 25 individual gene products, mostly transcription factors and apoptosis inhibitors, which are immediately turned on and begin the process of viral replication (Musgrave et al., 1999). Other genes are activated later to transcribe the viral genome, produce viral peptides, and package new viruses for reinfection. Up to 10,000 viral progeny can be produced in a single cell before lysis (Kay et al., 2001).

**Adenoviral Vectors.** Ad vectors have been used to treat a variety of ailments as extensive as autoimmune diabetes, Tay-Sachs disease, and a wide spectrum of cancers (Russell, 2000). The utility of Ad is described by its safety and efficiency: Ad is non-oncogenic, can be rendered replication-deficient by simple molecular techniques, and can achieve a relatively high level of transduction in a variety of cell types – including dividing and quiescent cells (Krasnykh et al., 2000). Ad is also a practical clinical option because it can be produced inexpensively in high titer stocks (up to $10^{13}$ virions mL$^{-1}$).
First generation Ad vectors were rendered replication-defective by deletion of E1 viral early genes. Researchers later discovered that low level expression of undesirable viral genes persisted with E1-deleted vectors. It was suspected that E2 and E4 early genes, including Ad DNA Polymerase and DNA binding protein, were involved in the persistent production of viral proteins (Musgrave et al., 1999). Subsequent generations of Ad vectors contained deletions of E2 and E4 regions, which further diminished the viral toxicity (Lusky et al., 1998) and increased the duration of gene expression (Chen et al., 1997). Deletion of these genes allowed additional space in the viral genome for the insertion of a therapeutic gene. These vectors with large deleted regions have been shown to have an insertional DNA capacity of up to 36 kb (Krasnykh et al., 2000).

An important criterion for evaluating a vector is the ability of the virus to target only desired cells. In the case of Ad, CAR is widely expressed, but not always in tissues of desired clinical application. It is therefore necessary to design Ad vectors targeted to alternate cellular receptors, thus circumventing the natural CAR-mediated infection pathway and delivering high viral dosage to CAR-negative cells. Although many methods exist for designing targeted Ad vectors (Barnett et al., 2002), we have designed a novel vector with the ability to bypass CAR and take up virus directly through integrins. This altered tropism is achieved by incorporating an RGD motif, similar to that of the penton base, in the HI loop of the knob domain of the viral fiber protein thus allowing internalization directly via integrins (Krasnykh et al., 1996).

**Gene Therapy.** Two main types of Ad therapeutic approaches exist to promote ossification of the injured area. Viral vectors may be used to deliver
therapeutic genes directly to the site of repair (Alden et al., HGT 1999; Baltzer et al., 2000), or they may be used to transfect MSCs which are then physically planted in the injured area (Wang et al., 2002). While both systems have proven operative to varying degrees, cell-mediated delivery is safer and more effective (Hollinger et al., 2000). By implanting transfected cells rather than direct virus, viral toxicity and associated hazards are reduced. Also, ex vivo transfection allows for more efficient gene transfer and accommodates the improvement of vector targeting by providing a clear set of target cells. Although both models are susceptible to immune-related difficulties, researchers have been more successful in overcoming those problems in the ex vivo model (Kim et al., Spine 2003).

MSC-mediated gene therapy is the most physiologic of all current spinal fusion therapy systems (Lieberman et al., 2002), but suffers from inefficient gene transfer. Although some Ad gene delivery can be attained using native tropism vectors, optimal therapy requires an Ad vector that infects via a CAR-independent mechanism. Ad vectors must therefore be retargeted to alternative receptors expressed at high levels on MSC target cells. In this paper, we will demonstrate the potential for improved bone formation by enhancing gene transfer to CAR-negative MSCs by enlisting a tropism-modified adenovirus targeted to \( \alpha_v \beta_5 \) integrins.

**Materials and Methods**

**Cell Culture.** CAR-positive human embryonic kidney 293 cells (Graham, 1977) were purchased from Microbix (Toronto, Canada), CAR-negative human
glioma-derived U118 cells were obtained from the American Type Culture Collection (Manassas, VA), and mesenchymal stem cells (MSCs) were kindly provided by Dr. Xu Cao at the University of Alabama at Birmingham (UAB, Birmingham, AL). All cells were propagated at 37°C in 5% CO₂. 293 and U118 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) fetal calf serum (FCS), penicillin (100 U mL⁻¹), L-glutamine (2mM), and streptomycin (100 μg mL⁻¹). MSCs were grown in Poietics MSCGM SingleQuot Media (Cambrex, Walkersville, MD).

**Adenoviral Vectors.** Vectors used in this experiment are first generation, E1-, E3-deleted recombinant adenovirus of serotype 5 (Ad5). Ad5Luc1 expresses firefly luciferase and Ad5BMP2 expresses human bone morphogenic protein 2, both under the control of the cytomegalovirus immediate-early promoter (Krasnykh, 2001). Ad5LucRGD and Ad5BMP2-RGD are tropism-modified vectors which contain an RGD (Arginine-Glycine-Aspartic Acid) motif in the HI loop of the knob domain of the viral fiber protein (Krasnykh et al., 1996). Vectors expressing BMP2 were made from linearized pAdenoVator-CMV-BMP2 shuttle vector which was co-transformed into recombination-competent E. coli strain BJ5183 with AdenoVator viral backbone plasmid (Q Biogene, Carlsbad CA). Recombinant vectors were propagated in 293 cells, purified by two rounds of CsCl₂ density centrifugation and plaque titered on 293 cells as previously described (Anon, 1998).

**Luciferase Assay.** Monolayers of confluent cells were washed with Dulbecco’s phosphate buffered saline (DPBS; UAB Media Core Facility, Birmingham, AL) and detached with trypsin (UAB Media Core Facility, Birmingham, AL). Detached cells were centrifuged for 5 minutes at 1800 rpm
and resuspended in appropriate growth media. Mixtures containing a 10 μL aliquot of suspended cells and 90 μL of trypan blue (UAB Media Core Facility, Birmingham, AL) were prepared and placed in a bright-line hemacytometer for counting. Concentrations of suspended cells were adjusted to 1 x 10⁶ cells mL⁻¹ by adding appropriate volumes of growth media. 50 μL aliquots of cells (5 x 10⁴ cells) were placed in 24-well plate and incubated for 24 hours at 37°C.

Adenoviruses containing luciferase reporter gene were diluted in DMEM supplemented with 2% (v v⁻¹) fetal calf serum (FCS) to multiplicity of infection (m.o.i.) of 10 (5 x 10⁶ particles mL⁻¹), 100 (5 x 10⁷ particles mL⁻¹), and 1000 (5 x 10⁸ particles mL⁻¹). Cell medium was aspirated and 200 μL of diluted virus or 2% FCS control was added. Cells were incubated 1 hour at 37°C, virus was aspirated, and 500 μL normal growth medium was added to each well. Cells were incubated 24 hours and washed with DPBS. 100 μL lysis buffer was added to wells and allowed to incubate 10 minutes. 20 μL lysis mixture was added to 100 μL luciferase assay buffer (10 μg mL luciferin in DPBS) in sterile microfuge tube and placed in luminometer (Promega, Madison, WI). Measurements were recorded in relative light units. 5 μL of lysis mixture or IgG protein standard was added to 25 μL Reagent A and 200 μL Reagent B from Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA) in 96-well plate and analyzed by spectrophotometry (λ=650 nm). IgG protein standard dilutions were used to generate a standard curve and concentrations of proteins for individual wells was calculated. Relative light unit measurements were normalized for protein according to calculated protein concentrations. Statistical analysis was performed by one-way ANOVA using Microsoft Excel (Version 10.2, Microsoft, Redmond, WA).
Flow Cytometry. Confluent cells were rinsed with DPBS (UAB Media Core Facility, Birmingham, AL) and released with Versene (UAB Media Core Facility, Birmingham, AL). Detached cells were centrifuged for 5 minutes at 1800 rpm and resuspended in DPBS containing 1% bovine serum albumin (BSA; Boehringer, Indianapolis, IN). Mixtures containing a 10 μL aliquot of suspended cells and 90 μL of trypan blue (UAB Media Core Facility, Birmingham, AL) were prepared and placed in a bright-line hemacytometer for counting. Concentrations of suspended cells were adjusted to 1 x 10^6 cells mL⁻¹ by adding appropriate volumes of DPBS-BSA. 200 μL aliquots of cells (2 x 10^5 cells) were incubated with 25 μL of diluted primary antibody (10 μg mL⁻¹) or DPBS-BSA control for 1 hour at 4°C with constant shaking. Primary antibodies employed were Ad5knob (anti-human CAR; Douglas, Birmingham, AL) and P1F6 (anti-human αvβ5 integrin; Covance, Berkeley, CA). Cells were then washed with 4°C DPBS, centrifuged at impulse speed for 1 minute, then washed and centrifuged again. Supernatant was aspirated to leave 270 μL of liquid and cells were resuspended. Cells were incubated with 30 μL of diluted secondary mouse anti-His-IgG antibody (10 μg mL⁻¹; Jackson Immunoresearch, West Grove, PA) for 1 hour at 4°C with constant shaking. Cells were washed as described above and incubated with 30 μL of diluted tertiary goat anti-mouse-IgG FITC-conjugated antibody (10 μg mL⁻¹; Jackson Immunoresearch, West Grove, PA) in a dark area for 1 hour at 4°C with constant shaking. Samples were washed as described above and analyzed by flow cytometry in the University of Alabama at Birmingham FACS Core Facility on a FACSComp machine using Cellquest analysis software (Becton-Dickinson, Franklin Lakes, NJ).
**Western Blot.** Confluent cells infected with Ad5BMP2 or Ad5BMP2-RGD were incubated at 37°C and 5% CO₂ for 24 hours. 1.5 mL aliquots of cell medium were then obtained and stored for 24-48 hours at -20°C. Polyacrylamide gels were constructed using a 15% polyacrylamide resolving gel (5.375 mL 40% acrylamide-bis, 3.75 mL 1.5 M Tris-HCl [pH=8.8], 5.75 mL H₂O, 20 µL tetramethylethelylenediamine [TEMED], and 100 µL 10% ammonium persulfate [APS]) leveled with isobutanol and a 4% polyacrylamide stacking gel (0.4 mL 40% Acrylamide-Bis, 1.25 mL 0.5 M Tris-HCL [pH=6.8], 2.35 mL H₂O, 10 µL TEMED, and 50 µL 10% APS). 20 µL aliquots of thawed protein samples or Benchmark pre-stained protein ladder (Invitrogen, Carlsbad, CA) were loaded into acrylamide gel in electrode buffer (3.03 g L⁻¹ Tris base, 14.4 g L⁻¹ Glycine, 0.10 g L⁻¹ SDS) in Mini-PROTEAN 3 apparatus (Bio-Rad, Hercules, CA). Gels were then subjected to 150 V current for 30 minutes. Gels were placed on methanol-equilibrated Immun-Blot PVDF protein blotting membrane (Bio-Rad, Hercules, CA) and inserted into MiniPROTEAN gel tank with transfer buffer (electrode buffer, 20% methanol). Gels were subjected to 0.3 A current at 4°C for 1 hour. Membrane was blocked by incubating 1 hour on rocking surface with 5% milk (1 g Carnation nonfat dry milk, 20 mL TBS). Membrane was washed 4 times with TBS and incubated with dilute biotinylated anti-BMP-2/4 antibody (0.2 µg/mL in TTBS; R&D Systems, Minneapolis, MN) on rocking surface at 4°C for 18 hours. Membrane was washed 4 times with TBS and incubated with Vectastain Elite ABC kit (avidin-horseradish peroxidase; Vector Laboratories, Burlingame, CA) for 30 minutes on rocking surface. Membrane was washed 4 times with TBS and incubated with Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA) for 1 minute. Membrane was then photographed on Kodak X-
OMAT Autoradiography Film by a Kodak X-OMAT processor (Eastman Kodak, NewHaven, CT).

Results

A preliminary experiment confirmed the potential for native Ad vectors to infect MSCs. In this experiment, 293, U118, and MSC cell monolayers were infected with a native Ad vector at 10, 100, and 1000 multiplicity of infection (m.o.i.). The vector, Ad5Luc1, carried a luciferase reporter gene and a luciferase assay system was used to evaluate the efficacy of gene delivery to target cells (figure 2). Gene transfer was reduced between 2 and 4 orders of magnitude in U118 and MSC cells compared to CAR-positive 293 cells.
Expression levels of CAR and $\alpha_v\beta_5$ integrins on target cells was evaluated by fluorescence-activated cell sorting (FACS) using Ad5knob anti-human CAR integrin antibodies (figure 3). CAR and $\alpha_v\beta_5$ expression on MSCs were compared with CAR-positive 293 cells and CAR-negative U118 cells. It was found that MSCs express low levels of CAR and elevated levels of $\alpha_v\beta_5$ integrins. These data suggest a need for an adenoviral vector that bypasses the normal CAR-mediated infection pathway.

To circumvent problems related to low-level CAR expression, a tropism-modified adenoviral vector was used to bypass the normal CAR-mediated infection pathway for direct uptake via $\alpha_v\beta_5$ integrins. The vector, Ad5LucRGD, expressed a luciferase reporter gene and included an RGD motif in the HI loop of
the viral fiber knob protein. 293, U118, and MSC cell monolayers were infected with Ad5Luc1 and Ad5LucRGD at 10, 100, and 1000 m.o.i. and a luciferase assay

Figure 4. Adenovirus-mediated gene transfer to CAR-negative cells is improved with tropism-modified vector. Monolayers of CAR-positive 293 cells, CAR-negative U118 cells, and MSCs were infected with Ad5Luc1 (black columns) and Ad5LucRGD (gray columns) at 10 (a), 100 (b), and 1000 (c) m.o.i. After 1h incubation at 37°C, virus was aspirated and cells were incubated at 37°C for 24h. Cells were lysed, assayed for luciferase activity, and normalized for protein. Charts represent mean ± standard deviation of triplicate experiments. * p < 0.01, ** p < 0.005, and *** p < 0.001 for Ad5LucRGD versus Ad5Luc1 for each cell type.

Table 1. Data supplement to figure 4. Cells were treated and analyzed as described in figure 4. Unshaded rows represent mean values of triplicate experiments in relative light units [g⁻¹ protein]. Shaded rows represent the expression ratio of tropism-modified virus (Ad5LucRGD) divided by native virus (Ad5Luc1). The ratio is expressed as a decimal number and as the log_{10} of that number.
system was used to evaluate the efficacy of gene delivery to target cells (figure 4). Gene transfer was increased over 30 times (1.5-fold) in MSC and over 50 times (1.8-fold) in U118 when compared to native tropism vector (table 1).

Once tropism-modified vectors were shown to improve the efficacy of gene transfer to MSCs, vectors expressing BMP2 were used to induce ossification. Two vectors, Ad5BMP2 and Ad5BMP2-RGD, at 10, 100, and 1000 m.o.i. were used to infect 293, U118, and MSC cell monolayers. Cell media was obtained and a western blot was performed using biotinylated anti-BMP2/4 antibody (figure 5). BMP2, a 16 kDa protein, was not detected in two separate infection and western blot experiments using stock adenovirus. Although no loading controls were used in this experiment, background proteins of high molecular weight were detected erratically.

Other members of our laboratory had recently reported similar difficulty detecting BMP2 when stock Ad5BMP2 and Ad5BMP2-RGD viruses were used to...
deliver the gene. To resolve any potential for stock contamination or degradation, new Ad5BMP2 and Ad5BMP2-RGD viruses were generated as described above. Follow-up experiments by other laboratory members using new vectors will confirm expression of BMP2 in MSCs (J.T. Douglas, personal communication).

Discussion

In this report, we found that native adenoviral gene transfer to MSCs suffers from limitations. Native-tropism vectors do not infect MSCs as well as CAR-positive cells (figure 2). We have demonstrated that MSCs express low levels of cell surface CAR, thus accounting for the low levels of gene transfer (figure 3). Elevated levels of $\alpha_v\beta_5$ integrins were detected on MSCs, thus suggesting the utility of enlisting a tropism-modified vector (figure 3). In subsequent experiments, the efficiency of gene transfer to MSCs was increased when a vector targeted to $\alpha_v\beta_5$ integrins was employed (figure 4). The log$_{10}$ of the calculated expression ratio indicated that gene transfer was increased by approximately 1.5 orders of magnitude in CAR-negative MSCs when tropism-modified vector was used (table 1).

We then enlisted native tropism and tropism-modified vectors expressing BMP2 to induce bone formation. In preliminary experiments, BMP2 was not detected by western blot (figure 5). Stock BMP2 adenoviruses had been stored at -20°C for several years in a freezer common to all laboratories on the floor. Other members of our research group had recently encountered difficulty detecting BMP2 expression with that virus, even when using different cell lines and
antibodies. To eliminate the potential for stock contamination or decay, both Ad5BMP2 and Ad5BMP2-RGD vectors were reconstructed. Further experiments have indicated that BMP2 is detectable in cells infected with new vectors (data not shown). In a subsequent experiment, MSCs and controls will be infected with new BMP2 vectors and silver nitrate will be used to detect calcification in vitro.

In future experiments, human MSCs will be infected with Ad5BMP2 and Ad5BMP2-RGD and will be allowed to incubate. 24 hours post-infection, cells will be washed and injected into leg muscles of separate immunodeficient mice. Non-infected cells will be injected into another mouse as a control. Radiologic analysis of mice will be performed in weekly increments. Approximately 12 weeks post-injection the mice will be sacrificed and evaluated histologically for ectopic bone formation using Von Kossa stain (Baltzer et al., 2000). Increased bone formation is expected in muscles that receive cells infected with tropism-modified Ad vector in comparison to non-modified vector and non-infected cell controls.

These studies should provide a basis for future collaboration with an orthopedic surgeon using rabbit model of spinal fusion (Lieberman et al., 2002). In this model, an osteoperiosteal defect is surgically created in skeletally mature rabbits. The defect is stabilized by plating and wires similarly to the method used in humans. The gene therapy study will involve the addition of untreated MSCs and MSCs treated with native and modified Ad vectors. Radiographic and histiologic analysis of defects will analyze the progression of bone healing. Rabbits will be sacrificed and the bone will be removed for stress testing to evaluate functional effectiveness of this treatment (Baltzer et al., 2000). Successful
completion of this research in spinal fusion will have potential for direct applications human patients.

Gene therapy is an avenue of research with applications in several clinical fields, including orthopedics, genetics, and oncology (Kim et al., BJC 2003). Conceptually, gene therapy is the simple process of delivering genetic material to cells for therapeutic purposes; unfortunately, this simple process is often obstructed by technical difficulties. Only with the advent of better genetic delivery systems will the treatment gain the efficiency necessary to be both safe and effective. Research in targeted gene therapy vehicles, such as the RGD tropism-modified Ad vector, is essential to the success of gene therapy as a clinical option.
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