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EXPERIMENTAL STUDIES

High-Efficiency Endovascular Gene Delivery Via Therapeutic Ultrasound

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OBJECTIVES We studied enhancement of local gene delivery to the arterial wall by using an endovascular catheter ultrasound (US).

BACKGROUND Ultrasound exposure is standard for enhancement of in vitro gene delivery. We postulate that in vivo endovascular applications can be safely developed.

METHODS We used a rabbit model of arterial mechanical overdilation injury. After arterial overdilation, US catheters were introduced in bilateral rabbit femoral arteries and perfused with plasmid- or adenovirus-expressing blue fluorescent protein (BFP) or phosphate buffered saline. One side received endovascular US (2 MHz, 50 W/cm^2, 16 min), and the contralateral artery did not.

RESULTS Relative to controls, US exposure enhanced BFP expression measured via fluorescence 12-fold for plasmid (1,502.1 $\pm$ 927.3 vs. 18,053.9 $\pm$ 11,612 μm^2, $p < 0.05$) and 19-fold for adenovirus (877.1 $\pm$ 577.7 vs. 17,213.15 $\pm$ 3,892 μm^2, $p < 0.05$) while increasing cell death for the adenovirus group only (26 $\pm$ 5.78% vs. 13 $\pm$ 2.55%, $p < 0.012$).

CONCLUSIONS Endovascular US enhanced vascular gene delivery and increased the efficiency of nonviral platforms to levels previously attained only by adenoviral strategies. (J Am Coll Cardiol 2001; 37:1975–80) © 2001 by the American College of Cardiology

Cardiovascular diseases are by far the leading cause of death in Western nations (1). Gene therapy to express specific therapeutic factors was expected to revolutionize management of these diseases. Unfortunately, this potential remains unfulfilled largely because of problems with safety and efficiency (2). In order to deliver genes to the cells, several viral and nonviral vectors exist, with various idiosyncratic advantages and limitations, but no current system achieves all necessary requirements for practical application. Adenovirus-mediated strategies, although highly efficient (3), are limited at therapeutic doses by cytotoxicity, inflammatory infiltrate, phenotypic perturbation and transient transgene expression (4) and, when applied clinically at these doses, have even resulted in death. In contrast, direct introduction of deoxyribonucleic acid (DNA) into the cell using a plasmid is inefficient. After charge neutralization of DNA, only 0.1% to 1% of target cells are transfected in vivo. Even with the aid of transfection agents, these rates are typically too low to achieve therapeutic effects in vivo (5,6).

Ultrasound permeabilizes cell membranes (7), and many publications report in vitro US enhancement of transfection using plasmids or liposomes with and without the addition of stabilized gas bubbles (8–12). Investigators have demonstrated that application of extracorporeal US can also alter in vivo drug (13) or gene (14) delivery within constraints of US penetration. However, these methods have yet to be successfully applied to the leading cause of morbidity and mortality.

Because restenosis represents an important clinical target for vascular gene therapy, we hypothesized that catheter-mediated endovascular ultrasound can enhance blue fluorescent protein (BFP) gene delivery in a rabbit model of arterial mechanical overdilation injury. Expression and distribution of BFP were assessed after angioplasty and plasmid- or adenoviral-mediated gene delivery with or without endovascular US. Specific histochemical studies of local macrophage infiltrate, apoptosis and cell death were also undertaken on treated artery segments to determine the toxicity of this new adjunctive method.

METHODS

Vectors for gene delivery. A replication-deficient recombinant adenovirus-expressing BFP gene driven by a cytomegalovirus (CMV) promoter was constructed (Adv/CMV-BFP) (Quantum Biotechnologies, Inc., Quebec, Canada). The plasmid used for nonviral experiments expressed BFP gene under control of a CMV promoter (p/CMV-BFP), obtained from Quantum Biotechnologies.

Ultrasound performance characteristics. The EKOS ultrasound catheter (EKOS Corp., Bothell, Washington) has an outside diameter of 2.5 French, a central infusion lumen of 180 μm and contains a cylindrical US transducer at the tip that emits US energy in a radial pattern directed into the artery wall. The US excitation signal used in the present work was a 2 MHz sine wave in burst mode. A pulse

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Abbreviations and Acronyms

Adv = adenovirus
BFP = blue fluorescent protein
CMV = cytomegalovirus
DNA = deoxyribonucleic acid
p = plasmid
PBS = phosphate buffered saline
US = ultrasound

repetition rate of 30 Hz was used with burst duration of 1.02 ms. The applied electrical power was adjusted to achieve a target pulse average acoustic intensity of 50 W/cm^2.

Surgical procedure. All animal experiments were performed within institutional and National Institutes of Health guidelines. New Zealand White male rabbits (three per treatment) weighing 2.3 to 3.2 kg underwent general anesthesia with isofluorane (3%) after induction with ketamine (35 mg/kg) and xylazine (6 mg/kg). Bilateral femoral cutdowns were performed. Common femoral arteries were exposed from inferior epigastric arteries to superficial femoral arteries and isolated with atraumatic microclamps. Superficial femoral arteries were cannulated. A 2 mm $\times$ 2 cm Cordis SAVVY angioplasty balloon (Cordis Inc., Miami, Florida) was introduced and advanced to the proximal clamp, inflated to 6 atm for two cycles of 1 min and removed. The EKOS CT 2.5F US catheter was then inserted. Through this catheter, arteries were infused with 150 μl of phosphate-buffered saline (PBS, control group), 150 μl of 1×10^9 pfu/ml Adv/CMV-BFP or 150 μl of 7.6 μg/ml p/CMV-BFP complexed with a 5:1 ratio of Superfect (Qiagen, Valencia, California) and incubated 16 min each. During this period three arteries per treatment group underwent intravascular US treatment, with contralateral vessels treated using an identical control catheter without activation of US. Saline irrigation was applied perivascularly to maintain temperature below 34°C as measured by thermocouple in the US catheter.

Tissue harvesting and evaluation of delivery efficiencies. Seven days after angioplasty and gene delivery, treated artery segments were excised and divided into four equal portions. One portion was snap frozen in mounting medium while both remaining segments were fixed in 10% neutral buffered formalin for 12 to 16 h. Formalin-fixed specimens were paraffin-embedded and sectioned (5 μm) for evaluation of BFP expression, macrophage infiltration and apoptosis as described. Frozen specimens were sectioned for viability study as described.

Evaluation of BFP expression. Efficiency of gene delivery was assessed via BFP fluorescence as recorded on a Diagnostic Instruments SPOT digital camera from a Nikon E600 epifluorescence microscope (Nikon Corporation, Tokyo, Japan) with plan apochromat lenses using an FITC-HYQ filter (EX 460-500, DM 505, BA 510–560). An observer blind to conditions used an ImagePro Plus image analysis suite to determine total BFP positive area in pixels. Mean and standard error were tabulated for each treatment with significance determined as described later.

Evaluation of local macrophage infiltrate. To visualize presence of macrophages at the 7-day timepoint, three sections per artery were incubated overnight at 37°C with a primary monoclonal antibody to rabbit macrophage (clone RAM-11; dilution 1:800; DAKO, Carpinteria, California). Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Sections were incubated with peroxidase-conjugated secondary antibody to mouse IgG (X56; dilution 1:100; Pharmingen, San Diego, California) and developed in CN/DAB substrate (Pierce, Rockford, Illinois) to visualize RAM-11 antigenic sites. An observer blind to conditions tabulated number of RAM-11 positive cells per cross section.

Evaluation of cellular viability. Frozen sections were washed with saline, incubated with 0.4% trypan blue for 2 min, then allowed to stand in saline for 5 min before evaluation. An observer blind to conditions analyzed images for percent of cross-section that failed to exclude blue dye.

Evaluation of apoptosis. Rates of apoptosis at the 7-day timepoint were assessed using the Klenow-FragEL DNA fragmentation detection kit (Oncogene, Cambridge, Massachusetts) according to the manufacturer's recommendations. Images of the processed slides (two per vessel to minimize slide-to-slide variability) were acquired at high resolution. An observer blind to conditions analyzed the percent of total area that was positive for peroxidase staining of DNA fragmentation for each slide using the ImagePro Plus analysis suite.

Statistical analysis. All values are expressed as mean and standard error for each treatment. Statistical significance for these analyses was determined using one-factor analysis of variance repeated measures, with significance determined at 95% (with Bonferroni, Tukey-A, Fisher PLSD, and Student-Newman-Keuls performed where applicable in SPSS 6.1 and Statview).

RESULTS

Catheter intensity. A US intensity field was generated from a hydrophone scanning tank. Peak intensity is located on the catheter surface at the center of the US transducer and falls off radially as seen in Figure 1.

Gene delivery efficiencies. Blue fluorescent protein expression was used to measure gene delivery efficiency, with results summarized in Figure 2. No statistically significant differences in BFP expression were detected among the plasmid, adenoviral and control groups without ultrasound ($p > 0.05$). After US exposure, plasmid-mediated BFP expression increased 12-fold over plasmid control ($p < 0.05$), and adenoviral-mediated BFP expression increased 19-fold over adenoviral control ($p < 0.05$). Ultrasound also increased BFP expression for plasmid relative to adenoviral

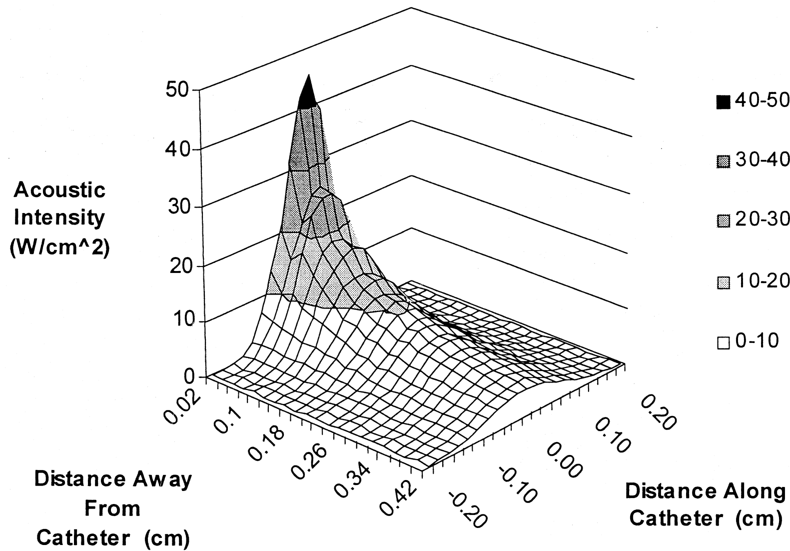

Figure 1. Ultrasound intensity field map for the EKOS catheter as measured in a hydrophone scanning tank. The peak intensity is located on the surface at the center of the ultrasound transducer and falls off away from that point. The field is axisymmetric around the catheter.

treatment alone ($p < 0.025$). Representative photographs depicting these results are presented as Figure 3.

Cell viability. Nonviable cell area was evaluated on frozen arterial cross-sections by trypan blue dye exclusion. Ultrasound did not increase cell death in the plasmid group relative to the controls ($p > 0.05$). In the adenoviral group US exposure significantly increased cell death relative to adenoviral treatment alone ($p < 0.012$). All other comparisons were not significant ($p > 0.05$, Table 1).

Apoptosis rates. Apoptosis was evaluated with the Klenow-FragEL DNA fragmentation detection kit (Oncogene, Cambridge, Massachusetts) to assess the impact of apoptosis on the total population of nonviable cells. Ultrasound did not significantly increase apoptosis among control, plasmid and adenoviral groups ($p > 0.05$, Table 1).

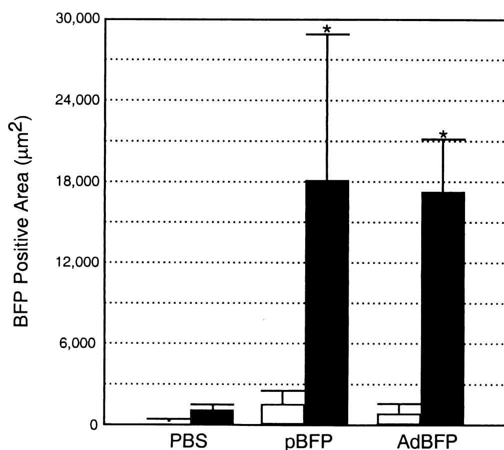

Figure 2. Blue fluorescent protein (BFP) expression. Expression of BFP 7 days after delivery of buffer (PBS), plasmid expressing BFP (p/BFP), or adenovirus expressing BFP (Adv/BFP), each either with ultrasound treatment **(black bars)** or without **(white bars)**. Values represent mean ± standard error.

Local macrophage infiltration. Cross-sectional macrophage content was evaluated via immunohistochemistry at the 7-day timepoint. No statistically significant alterations in macrophage infiltration occurred in any group ($p > 0.05$). These results did not change after US exposure ($p > 0.05$, Table 1).

DISCUSSION

Summary of results. This study shows that US exposure after balloon angioplasty significantly enhances in vivo plasmid-mediated BFP expression 12-fold over plasmid controls and adenoviral-mediated BFP expression 19-fold over adenoviral controls. Most interestingly, US increased BFP expression for plasmid relative to adenoviral treatment alone. Ultrasound enhancement of gene delivery resulted in increased toxicity by only one criterion measured in the present experiment (cell death rates for the adenoviral group), with no significant alterations in apoptosis or inflammation.

Catheter design and clinical application. For practical application of localized cardiovascular gene therapy, the US probe should be catheter-based, accessible to the human arteries (especially coronary arteries) and should offer an infusion port for gene delivery. A therapeutic ultrasound catheter was designed to meet these requirements. The settings for these experiments were determined from initial pilot experiments examining toxicity and delivery efficiency in vitro and in vivo.

Because restenosis represents a crucial clinical application for which endovascular access is available, we evaluated impact of US-mediated enhancement of gene delivery in a rabbit model of arterial mechanical overdilation injury. Because toxicity of gene delivery increases with dose for either plasmid or adenovirus, we selected safer doses that

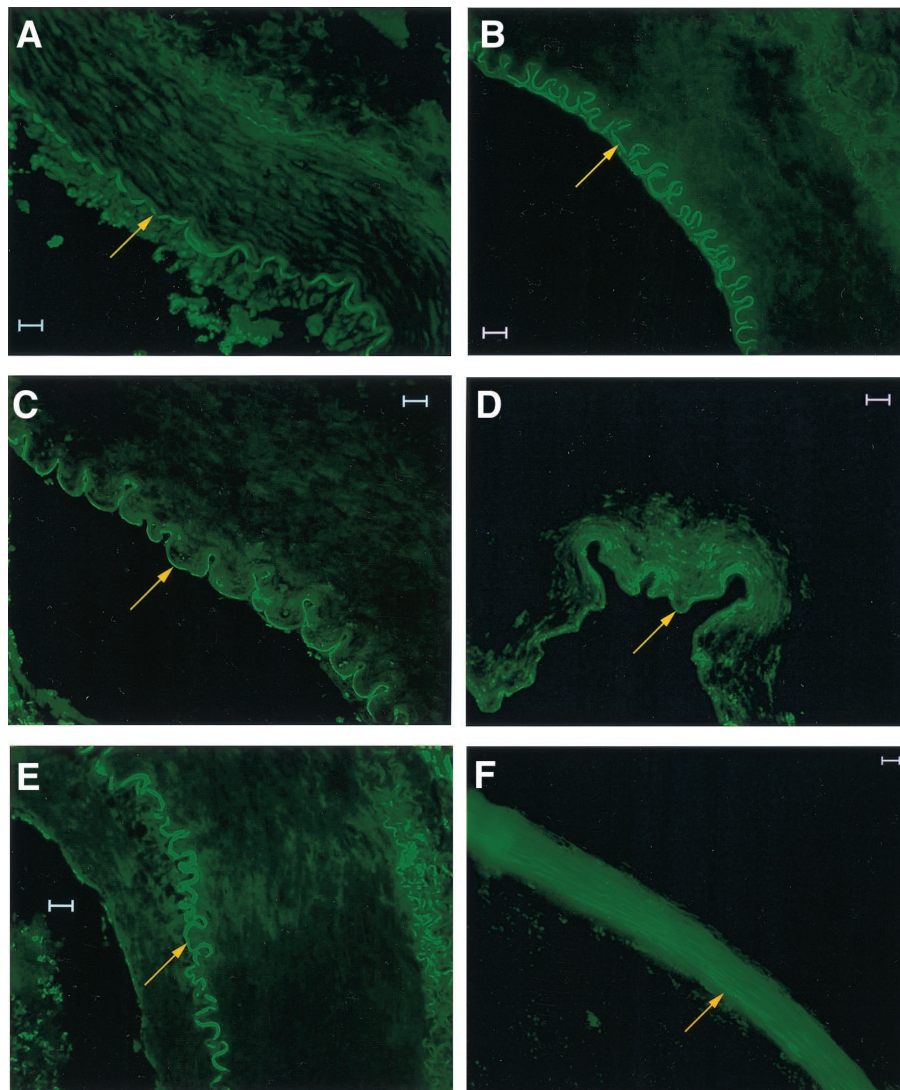

Figure 3. Representative photomicrographs. Photomicrographs (20× magnification) depicting blue fluorescent protein (BFP) expression as fluorescence through an FITC-HYQ filter with internal elastic lamina noted by yellow arrow after treatment with **(A)** saline alone, **(B)** saline with ultrasound (US), **(C)** pBFP alone, **(D)** pBFP with US, **(E)** AdBFP alone or **(F)** AdBFP with US. Scale = 20 μm.

would be subtherapeutic without US enhancement. Blue fluorescent protein expression was used to monitor gene delivery efficiency after femoral angioplasty, and plasmid- or adenoviral-mediated gene delivery with or without endovascular US and was compared to control (saline) treatment. As expected, no statistically significant differences in BFP expression occurred among plasmid, adenoviral and PBS groups at these doses without US ($p > 0.05$). Prior work has shown that 0.1 μM BFP molecules, approximately 10^5 copies per cell of 1 to 2 pl volume (15), are needed to surpass detectable endogenous autofluorescence of a mammalian cell. The delivery efficiencies of plasmid and low-dose adenovirus, while not strictly zero, do not yield statistically significant increases in signal intensity relative to these sensitivity thresholds. The impact of US on these efficiencies was tremendous. After US exposure, plasmid-mediated BFP expression increased 12-fold over plasmid control ($p < 0.05$), and adenoviral-mediated BFP expression increased 19-fold over adenoviral control ($p < 0.05$). Given low baseline plasmid and adenoviral-mediated gene delivery

Table 1. Macrophage Count, Apoptosis Evaluation and Cell Death

	Macrophages	Apoptosis	Cell Death
PBS	5.66 ± 1.06	1.43 ± 0.51	24.16 ± 3.74
PBS-US	6.11 ± 1.08	1.53 ± 0.50	9.29 ± 3.68
Adv/BFP	2.11 ± 0.56	0.53 ± 0.30	13.00 ± 2.55
Adv/BFP-US	4.44 ± 1.26	0.85 ± 0.23	26.00 ± 5.78
P/BFP	4.00 ± 1.62	0.24 ± 0.06	12.14 ± 3.75
P/BFP-US	4.44 ± 2.04	0.26 ± 0.06	16.66 ± 5.11

Macrophage infiltration, rates of apoptosis, and rates of total cell death 7 days after delivery of buffer (PBS), plasmid expressing BFP (p/BFP) or adenovirus-expressing BFP (Adv/BFP), each either with or without ultrasound (US) treatment. Macrophages represent total number of RAM-11 positive cells per cross-section. Apoptosis is expressed as the percent of cross-section positive by fragel staining. Cell death is expressed as the percent of cross-section that failed to exclude Evans Blue dye. Values represent mean ± standard error for each parameter.

efficiencies at the doses employed here, it is promising that high copy numbers per cell (at least 10^5) can be achieved with in vivo US exposure. But the most interesting result is that US increased BFP expression for plasmid relative to adenoviral treatment alone ($p < 0.025$). Given the cost, handling and safety advantages of plasmid-mediated gene delivery, these results are particularly exciting. Although the present study selected 16 min incubation total for gene transfer, transducer design alone (i.e., to the length of desired transfection) should allow reduction to a single 4-min increment to achieve the observed results. Dose-response studies may offer still further enhancements with accompanying reductions in transfection time.

Evaluation of cell toxicity. Above certain thresholds for intensity and duration, US has been found to induce breaks in DNA (16), apoptosis (17), membrane damage (7) and enhanced free radical delivery (18). We thus quantitatively evaluated cell viability, apoptosis and local infiltrate of macrophages in each group as potentially limiting complications of the strategy in vivo. Nonviable cell area was evaluated on frozen arterial cross-sections by trypan blue dye exclusion, with the results presented in Table 1. As expected from pilot experiments used to determine the US settings, US did not increase cell death in the plasmid group relative to the controls ($p > 0.05$). However, US exaggerated the toxicity of low-dose adenovirus, possibly through additive injury achieved by increased viral delivery ($p < 0.012$). All other comparisons were not significant ($p > 0.05$).

Further, we quantitatively examined apoptosis on histologic sections to assess contributions to the total population of nonviable cells, with results compiled in Table 1. No differences in rates of apoptosis occurred among control, plasmid and adenoviral groups, and US exposure did not significantly alter rates for any group in the present study ($p > 0.05$ for all comparisons), suggesting that US-facilitated changes in adenoviral-mediated cell viability may be due largely to the cellular response to physical cell injury in the presence of the viral vector. More importantly, these results suggest that transgene expression observed at a given timepoint may not be lost subsequently to already predestined cell death, although future experiments must confirm these effects.

Previous work has demonstrated that adenoviral gene delivery at doses substantially higher than those employed here induces a vigorous local inflammatory response (4) relative to low-dose strategies (19). Because macrophages play a crucial role in atherogenesis and constitute the predominant inflammatory cell type in acute inflammation within 48 h (20), we directly evaluated local macrophage infiltration as a potential limitation of the present strategy. As depicted in Table 1, no statistically significant alterations in macrophage infiltration occurred in any group in the present work ($p > 0.05$). These results did not change after US exposure ($p > 0.05$). In this experiment, US enhancement of gene delivery resulted in increased cell death only in the viral group, with no significant alterations in apoptosis or inflammation in the other groups.

Conclusions. Gene therapy is expected to alter management of vascular diseases but requires improvement of gene delivery before successful clinical applications can be realized. As concerns regarding safety of viral-mediated gene transfer have been reinforced by recent adverse events in clinical trials, the present strategy of local delivery and US enhancement may allow therapeutic levels to be achieved at safer viral doses. More interestingly, these results demonstrate that US can increase the efficiency of safer nonviral platforms for arterial delivery to levels previously attained only by adenoviral strategies. Future characterization of these promising effects must be undertaken to confirm that therapeutic doses can be achieved through this strategy without any additive long-term limitations. After appropriate dose-response and toxicity studies, evaluation of putative therapeutic transgenes may be more safely undertaken with these strategies. This technique may also be used to target gene therapy to any organ or lesion accessible to this catheter-based device.

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