Ultrasound Enhances Reporter Gene Expression After Transfection of Vascular Cells In Vitro

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Background—Restenosis after percutaneous coronary intervention remains a serious clinical problem. Progress in local gene therapy to prevent restenosis has been hindered by concerns over the safety and efficacy of viral vectors and the limited efficiency of nonviral techniques. This study investigates the use of adjunctive ultrasound to enhance nonviral gene delivery.

Methods and Results—Cultured porcine vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) were transfected with naked or liposome-complexed luciferase reporter plasmid for 3 hours. Ultrasound exposure (USE) for 60 seconds at 1 MHz, 0.4 W/cm^2, 30 minutes into this transfection period enhanced luciferase activity 48 hours later by 7.5-fold and 2.4-fold, respectively. Luciferase activity after lipofection of ECs was similarly enhanced 3.3-fold by adjunctive USE. USE had no effect on cell viability, although it inhibited VSMC but not EC proliferation.

Conclusions—Adjunctive USE was associated with enhanced transgene expression in VSMCs and ECs and reduced VSMC but not EC proliferation in vitro, which suggests that ultrasound-assisted local gene therapy has potential as an antirestenotic therapy. **(*Circulation*. 1999;99:2617-2620.)**

Key Words: restenosis ■ genes ■ ultrasonics ■ transfection

Restenosis remains a major clinical problem and results from negative remodeling and an obstructive neointima comprised of VSMCs and extracellular matrix. Adjunctive systemic therapies have largely failed to prevent restenosis. An alternative strategy is single-dose local administration of agents that can modify the vascular response to injury, including local gene therapy.[1] Viral vectors achieve the highest efficiency, but substantial concerns remain over their clinical safety and long-term efficacy.[1] Although relatively safe, nonviral gene delivery, including lipofection, is currently at least 10-fold less efficient.[1]

Ultrasound exposure (USE) has been shown to permeabilize plasma membranes and reduce the thickness of the unstirred layer at the cell surface,[2,3] which should encourage DNA entry into cells. Furthermore, many lipofection reagents contain dioleoylphosphatidylethanolamine (DOPE), which encourages DNA "breakout" from endosomes through a physicochemical transition that is known to be accelerated by USE.[4,5] On the basis of these observations, we investigated the hypothesis that USE may enhance transgene expression after naked DNA and/or liposome-mediated transfection of primary vascular cells.

Methods

Cell Culture and Transfection Conditions

Porcine medial vascular smooth muscle cells (VSMCs) and lumenal endothelial cells (ECs) from the thoracic aorta of Yorkshire White cross pigs aged <6 months were cultured in DMEM containing 10% porcine serum; EC cultures were supplemented with EC growth factor (20 μg/mL; Sigma) and heparin (90 μg/mL; Sigma). All transfections were performed for 3 hours at 37°C in 24-well plates with cells at 60% to 70% confluence and were stopped by dilution with 1 mL of fresh culture medium. Naked DNA transfections were performed in 200 μL of DMEM containing 10% porcine serum and 7.5 μg/mL luciferase plasmid DNA (pGL3; Promega) per well. Lipofections used Promega Tfx-50 (which contains DOPE), according to conditions optimized for VSMCs (200 μL of DMEM containing 10% porcine serum; DNA:lipid charge ratio of 4:1; 7.5 μg/mL final DNA concentration) and ECs (200 μL of serum-free DMEM; DNA:lipid charge ratio 3:1; 5 μg/mL final DNA concentration).

Thirty minutes after the transfection was begun, USE was performed for 60 seconds with a custom-built, 10-mm-diameter, 1-MHz piezoelectric ceramic transducer within the transfection medium 2 mm above the cell monolayer and the 24-well plates suspended in a polystyrene water bath at 37°C to minimize acoustic reflections (<5%) and consequent standing wave formation. The transducer was calibrated to produce continuous-wave 1-MHz ultrasound at a spatial

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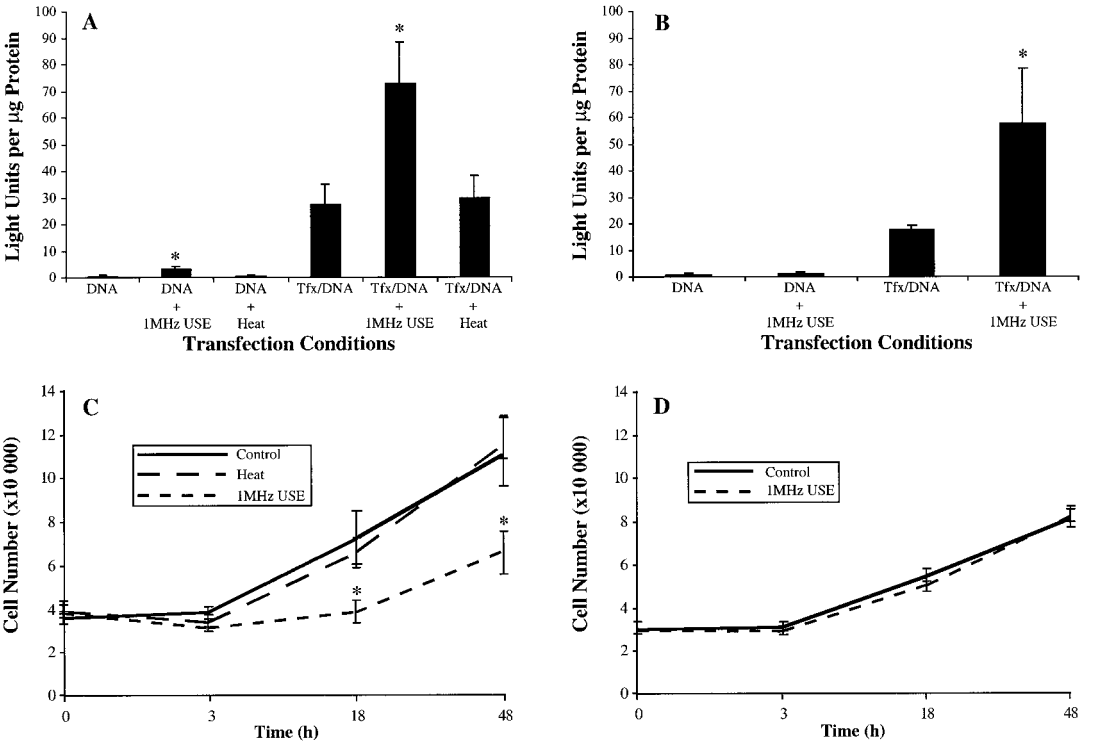

Figure 1. Porcine VSMCs (A) and ECs (B) were transfected for 3 hours with naked or liposome (Promega Tfx-50) complexed luciferase DNA (n=12), and luciferase activity in cell lysates was assayed after 48 hours at 37°C (A and B). Parallel adherent VSMC (C) and EC (D) cell counts were performed at baseline (time 0) and 3, 18, and 48 hours after transfection. Where applicable, 30 minutes into the 3-hour transfection period, USE (1 MHz, continuous wave, 0.4 W/cm^2) was performed for 60 seconds. Asterisks indicate significant differences between control and ultrasound-exposed cells ($P<0.05$).

average temporal average intensity of 0.4 W/cm^2. USE caused only minor acute damage to the cell monolayer and had no effect on naked or Tfx-50–complexed plasmid integrity as assessed by agarose gel electrophoresis. Temperature was recorded continuously by a thermistor placed adjacent to the ultrasound transducer. A 10-mm-diameter heating probe was constructed to mimic the rate of rise and final temperature achieved during USE.

Assays for Luciferase Activity, Adherent Cell Number, and Viability

Luciferase activity in cell lysates 48 hours after transfection was measured with the GenGlow kit (Labtech International) and 1253 Luminometer (BioOrbit) and expressed as light units (LU) per microgram of protein. Background luminescence was zero in untransfected cell lysates. Parallel wells were trypsinized at 0, 3, 18, and 48 hours after treatment. Cell counts and viability were assayed by Coulter counter and fluorescent activated cell sorter (FACS) analysis of propidium-iodide and fluorescein diacetate exclusion.[6]

Time-Lapse Video Microscopy

Identically seeded subconfluent VSMCs in 24-well plates were observed by time-lapse video microscopy (TLVM) with the use of an inverted microscope (Leica UK Ltd) within a 37°C chamber. One frame of a high-power field was recorded every 2.4 minutes for 48 hours beginning 3 hours after USE (where applicable) with a monochrome video camera (Sony), Super-VHS video recorder (Panasonic), and a BAC900 animation controller (EOS electronics AV Ltd). A mitotic event was recorded when 2 daughter cells appeared from a single dividing cell. An apoptotic event was recorded when an individual cell underwent the typical morphological changes and dislodgement.

Statistical Analysis

All data are presented as mean±SEM. Treatments were compared by Friedman ANOVA and the Wilcoxon signed rank test for post hoc comparisons. Significance was defined as $P<0.05$, with the Bonferroni correction for multiple comparisons applied where appropriate. The n numbers quoted refer to the number of separate experiments; on each occasion, each treatment was performed in triplicate wells.

Results

Luciferase activity was barely detectable in VSMC lysates after naked plasmid transfection (0.4±0.2 LU/μg) but was enhanced 7.5-fold by USE (3.0±2.0 LU/μg; n=12; $P<0.02$ compared with naked DNA alone), representing 11% of that achieved after optimal lipofection alone (27.6±6.9 LU/μg) (Figure 1A). USE during lipofection enhanced luciferase activity nearly 3-fold (72.8±17 LU/μg; n=12; $P<0.002$ compared with lipofection alone) (Figure 1A). Luciferase activity in EC lysates after naked DNA transfection (0.7±0.1 LU/μg) was not enhanced by adjunctive USE (1.2±0.2 LU/μg; n=4; P=NS compared with naked DNA alone) (Figure 1B), whereas USE during lipofection enhanced luciferase activity >3-fold (17.7±1.1 versus 57.8±20.2 LU/μg; n=4; $P<0.04$).

Culture-medium temperature increased progressively during USE, reaching 51±1°C at 60 seconds but returning to the 37°C baseline within 45 seconds. Exposure of VSMCs to an identical rate and final temperature rise over 60 seconds in the absence of USE had no effect on adherent cell number or luciferase activity

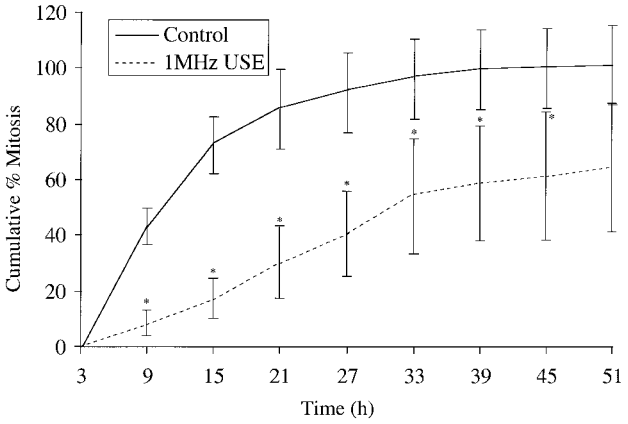

Figure 2. Two wells of subconfluent porcine VSMCs were observed concurrently by TLVM for 48 hours beginning 3 hours after USE (where applicable), and the cumulative rate of mitosis was analyzed (n=3). One of the 2 wells was exposed to ultrasound (1 MHz, continuous wave, 0.4 W/cm^2, 60 seconds) before filming. Asterisks indicate significant differences between control and ultrasound-exposed cells ($P<0.05$).

(Figure 1, A and C), excluding the possibility that heating was responsible for the observed effects of USE.

USE had no significant effect on adherent VSMC number at 3 hours but was associated with much smaller subsequent increases compared with control or heat-exposed wells (Figure 1C). TLVM analysis confirmed this to be due to a reduced rate of VSMC mitosis (Figure 2) and not increased apoptosis and VSMC detachment in ultrasound-exposed wells (cumulative apoptosis in control wells, 9.9±4.2% at 24 hours and 11.6±5.0% at 48 hours; in ultrasound-exposed wells, 4.8±4.3% at 24 hours and 7.6±5.2% at 48 hours; n=3; P=NS for all comparisons). Adherent VSMC viability and EC viability were identical under all conditions. USE had no effect on adherent EC number at any time point (Figure 1D).

Discussion

This study demonstrates that adjunctive USE enhances reporter gene expression after optimized naked DNA transfection and/or lipofection of primary vascular cells in vitro. Recent studies in nonvascular, primarily immortalized cells confirm this effect of USE, and transfection rates of up to 15% using naked DNA and 2- to 1000-fold enhancements in reporter gene expression after lipofection have been reported.[7–11] The lower enhancements recorded in the present study may arise for several reasons. First, we studied nontransformed cells, which are known to be relatively resistant to transfection. Second, USE was performed from above to minimize standing-wave formation resulting from reflection at fluid/air and plastic/air interfaces. This constrained transducer design such that only one third of each cell monolayer was covered by the transducer. Finally, we elected to use 1-MHz continuous-wave ultrasound at <1 W/cm^2 because this mirrors the mean output of diagnostic transducers[12] and had no effect on DNA integrity or vascular cell viability in vitro. Nevertheless, it is likely that further optimization of USE conditions (eg, intensity, frequency, exposure time, and duty cycle) will be possible.

Measurements of reporter gene activity cannot define the site(s) of action of ultrasound, which may include not only potential effects on plasmid DNA entry (ie, transfection per se) but also on intracellular trafficking, lysosomal degradation, nuclear translocation, RNA transcription, or protein translation. Although USE is known to increase permeability to large macromolecules, including plasmid DNA[7] and high-molecular-weight dextrans,[2] further studies are required to elucidate whether USE-enhanced transgene expression is entirely explicable in terms of increased DNA entry and/or lysosomal breakout as originally proposed.

Many transfection techniques are associated with significant cell death. In our experiments, low-intensity USE was associated with substantial enhancements in reporter gene expression without significant acute cell death or damage, as has also been shown in nonvascular cells.[10] Intriguingly, adherent VSMC but not EC counts rose more slowly in ultrasound-treated wells than in control or heat-exposed wells during the 48 hours after USE. Analysis by TLVM indicated that this was not due to increased death and detachment of VSMCs but to a reduced rate of proliferation. This effect of USE was not seen in ECs. The mechanisms and kinetics of this effect remain to be determined, although other studies have shown cell-type and frequency-dependent effects of USE on cell proliferation in vitro, including stimulation of rat fibroblast proliferation (1 MHz, 0.4 W/cm^2),[13] and inhibition of human[14] but not bovine[15] VSMC proliferation with low-frequency (20 kHz) ultrasound.

The results presented herein provide encouragement for ultrasound-assisted gene therapy to prevent restenosis. Furthermore, USE at the site of angioplasty may also arrest VSMC proliferation in response to vascular injury without hindering endothelial regeneration. The proven safety and practicality[16] of intravascular therapeutic ultrasound make this possibility particularly attractive in contrast to lingering concerns over alternative adjunctive technologies such as intracoronary brachytherapy[17] and adenoviral or retroviral gene therapy.[1]

Acknowledgments

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