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DNA uptake, intracellular trafficking and gene transfection after ultrasound exposure

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ABSTRACT

Ultrasound has been studied as a promising tool for intracellular gene delivery. In this work, we studied gene transfection of a human prostate cancer cell line exposed to megahertz pulsed ultrasound in the presence of contrast agent and assessed the efficiency of fluorescently labelled DNA delivery into cell nuclei, which is necessary for gene transfection. At the sonication conditions studied, ~30% of cells showed DNA uptake 30 min after sonication, but that fraction decreased over time to ~10% of cells after 24 h. Most cells containing DNA had DNA in their nuclei, but the amount varied significantly. Transfection efficiency peaked at ~10% at 8 h post sonication. Among those cells containing DNA, ~30% of DNA was localized in the cell nuclei, ~30% was in autophagosomes/autophagolysosomes and the remainder was "free" in the cytoplasm 30 min after sonication. At later times up to 24 h, ~30% of DNA continued to be found in the nuclei and most or all of the rest of the DNA was in autophagosomes/autophagolysosomes. These results demonstrate that ultrasound can deliver DNA into cell nuclei shortly after sonication and that the rest of the DNA can be cleared by autophagosomes/autophagolysosomes.

1. Introduction

Gene transfection of cells requires overcoming both extracellular and intracellular barriers to DNA transport into the cell nucleus. These barriers include the plasma membrane, which is the barrier of intracellular DNA uptake; the cytoskeletal meshwork in the cytoplasm, which hinders DNA trafficking in the cytoplasm; and the nuclear envelope, which limits DNA entry into the nucleus. Therefore, a gene delivery system should facilitate DNA transport across these barriers and into the nucleus to enable transcription. This process needs to be fast, because nucleases in the cytoplasm start to degrade exogenous DNA in minutes [1,2]. However, passive diffusion of plasmid DNA in the cytoplasm is generally slow, especially for large DNA molecules (e.g., >1000 base pairs), because the multiple cytoskeletal elements (e.g., microfilaments, microtubules and intermediated filaments) in the cytoplasm form a complex and crowded latticework that significantly impedes the diffusion of large molecules [3]. Therefore, DNA trafficking in the cytoplasm after delivery into cells is of interest because it is believed to be a limiting step for non-viral gene delivery methods [4].

Many studies of intracellular DNA trafficking have focused on chemical-based gene delivery systems that employ lipids, polymers and other chemical components [5]. In these methods, multiple trafficking steps are typically required, such as cell attachment, endocytosis and entrapment into endocytic vesicles, maturation of endosomes into lysosomes, escape from vesicular compartments, migration toward the nucleus periphery, dissociation between carriers and exogenous DNA, and finally entry of DNA into the nucleus. There are fewer reports of DNA trafficking studies using physical delivery systems such as electroporation [6], microinjection [7,8] and ultrasound [9], which involve significantly different pathways because they cause uptake through breaches in the plasma membrane and generally do not rely on endocytosis, although some studies have reported endocytic pathways playing a role [10,11]. This study seeks to understand the non-endocytic pathways of pDNA trafficking in cells following ultrasound exposure.

Ultrasound (US) is a promising tool for gene therapy that has been shown to facilitate DNA transfection of cells. US-mediated delivery is of interest due to its expected low toxicity, low immunogenicity, potential for repeated application, organ specificity and broad applicability to acoustically accessible organs [12–14]. Various studies have examined gene transfection in different types of cells *in vitro* [15–18] and with various organs and tissues *in vivo*, including skeletal muscle [19–22], brain [23–25], heart [26–28], liver [29] and kidney [30–33]. However, US-mediated gene transfection is still in preclinical development and has the major challenge of relatively low transfection efficiency compared to viral vectors and optimal chemical formulations [34].

Previous studies have shown that gene delivery using viral vectors can be very efficient and has been evaluated in numerous clinical trials, but may be limited by safety concerns and adverse immune responses [35–37]. Lipid and polymer-based formulations have also been used

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for intracellular gene delivery. While some systems appear to be best suited to in vitro studies, a number of such chemical formulations have shown success for delivery *in vivo* [38–40]. Often, these formulations are optimized to increase gene delivery while minimizing cytotoxicity.

US-mediated delivery of DNA into cells offers advantages and limitations compared to other intracellular delivery methods. An advantage is that only acoustic energy is introduced into the cellular environment, which avoids possible safety concerns associated with chemical, viral or other materials introduced and left behind by other methods. Another advantage is that US-mediated delivery has been seen in many different cells types and therefore may be broadly applicable, in contrast to other methods that often require reformulation for specific cell types. A limitation of the US-based approach is that transfection efficiency is often lower than by other leading methods. This study seeks to address this limitation by examining the pathways of DNA trafficking in cells, which may provide insight into strategies to improve transfection.

Unlike chemical delivery systems, US-mediated DNA uptake is often reported to be non-endocytotic. Acoustic cavitation is believed to play a major role in the cell membrane permeabilization that facilitates DNA uptake [41,42]. US can possibly deliver plasmid DNA (pDNA) to the periphery of the cell nucleus and facilitate rapid transfection by altering the cytoskeletal network [43,44]. There is, however, a need to better understand the DNA trafficking pathways in cells after sonication in order to identify the rate-limiting steps that should be addressed to improve transfection efficiency.

In this study, we use confocal microscopy and flow cytometry to analyze the location of pDNA during its intracellular trafficking after US exposure both qualitatively and quantitatively. We consider pDNA localization in the nucleus, in endosomes/lysosomes, in autophagosomes/autophagolysosomes and "free" in the cytoplasm. This study seeks to test the hypothesis that pDNA introduced into the cytoplasm during sonication is either transported rapidly into the nucleus (i.e., within 30 min) or removed by autophagosomes/autophagolysosomes. DNA that reaches and remains in the nucleus can be transcribed and translated to produce its expression product.

2. Materials and methods

2.1. Cell sample preparation

DU145 human prostate cancer cells (American Type Culture Collection, Manassas, VA, item no. HTB-81) in RPMI-1640 medium (Cellgro, Mediatech, Herndon, VA) were cultured as monolayers in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Cellgro).

DU145 cells were harvested by trypsin/EDTA (Cellgro) digestion and re-suspended in RPMI-1640 medium at a final concentration of 1×10^7 cells/mL before sonication. Cell concentration was determined by a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). The pDNA gWiz-GFP (Aldevron, Fargo, ND) encoding green fluorescent protein (GFP) and the gWiz control vector, gWiz Blank (Aldevron) were used for gene delivery and transfection studies. The pDNA was added to the cell suspension at a concentration of 20 μg/mL before sonication. Definity US contrast agent (Bristol-Myers Squibb Medical Imaging, North Billerica, MA) was added to cell samples at a concentration of 1 vol% to serve as cavitation nucleation sites.

2.2. Ultrasound apparatus

Sonication was carried out using an immersible, focused, piezoceramic US transducer (Sonic Concepts, Woodinville, WA, USA, model no. H-101) supplied with matching resistance networks allowing production of sound at 1 MHz. The transducer had a diameter of 70 mm, a 52 mm focal length and a 1.5 mm focal width at half-amplitude (−6 dB). A sinewave was provided by two programmable waveform generators (Stanford Research Instruments, Sunnyvale, CA, model no. DS345 and Agilent, Austin, TX, model no. 33120A) and amplified by an RF broadband power amplifier (Electronic Navigation Industries, Rochester, NY, model no. 3100LA).

The transducer was submerged in deionized and partially degassed water at 37 °C placed in a polycarbonate tank (30.5 × 29 × 37 cm) to sonicate a 375 μL sample held within a disposable micropipette (Samco, San Fernando, CA). A 5 cm thick acoustic absorber (SC-501 Acoustic Rubber, Sonic Concepts) was placed opposite the transducer in the tank to minimize standing-wave formation. A three-axis positioning system (10 μm resolution, Velmex, Bloomfield, NY) was mounted on top of the tank to position samples and a hydrophone at desired locations in the tank.

The US transducer was calibrated versus the peak-to-peak voltage of the signal by a PVDF membrane hydrophone (NTR Systems, Seattle, WA, model no. HMA-0200) at a distance of 1 cm from the transducer. Sonication was carried out at a peak positive amplitude pressure of 0.78 MPa and total treatment time of 1 min with a burst length of 0.25 ms and a duty cycle of 25%. The corresponding acoustic energy fluence was therefore 306 J/cm^2. Additional characterization of the US apparatus has been reported previously [45].

2.3. Fluorescent probes

To measure pDNA uptake efficiency and track the localization of pDNA in the cytoplasm, MirusLabelIT Tracker Cy3 kit and Cy5 kit (Mirus, Madison, WI) were used to label pDNA before sonication. The labeling reaction was conducted according to the procedure recommended by the manufacturer.

Hoechst 33,342 (trihydrochloride, Invitrogen, Carlsbad, CA) was added to cell samples at a final concentration of 10 μg/mL for 10 min at room temperature to stain cell nuclei.

LysoTracker Green DND-26 (Molecular Probes, Invitrogen) was used to label acidic organelles in live cells. The reagent was added to cells in growth medium at a final concentration of 50 nM and incubated for 5 min at growth conditions. Cells were then washed three times before analysis.

EEA-1 (final dilution ratio 1:250, BD Bioscience, San Jose, CA), CD63 (final dilution ratio 1:10, Developmental Studies Hybridoma Bank, Iowa City, IA) and LAMP-1 (final dilution ratio 1:5, Developmental Studies Hybridoma Bank) were used as primary antibodies to label early endosomes, late endosomes and lysosomes, respectively. Alexa fluor 488 Donkey anti-Mouse A-21,202 (Invitrogen) was used as the secondary antibody at a final concentration of 2 μg/mL. Cells were plated on a coverslip (VWR International, West Chester, PA), fixed in 2% formaldehyde for 10 min at room temperature and washed twice with phosphate-buffered saline (PBS). Cells were treated with 10% FBS and 0.02% (w/v) sodium azide in PBS for 5 min at room temperature and washed twice with PBS. Cells were then incubated with the primary antibody diluted in PBS with 10% FBS and 0.2% saponin for 1 h in darkness at room temperature, and washed twice in PBS with 10% FBS. After that, cells were incubated with the secondary antibody diluted in PBS with 10% FBS and 0.2% saponin for 1 h in darkness at room temperature, and washed twice in PBS with 10% FBS.

2.4. Flow cytometry

In this study, we used flow cytometry as a means to acquire data from large populations of cells, but without spatial resolution. We coupled that with data from fluorescence microscopy to obtain data with spatial resolution, but only from small populations of cells. Uptake of pDNA, transfection efficiency and cell viability were determined by a BD LSR benchtop flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed by FCS Express V3 (De Novo Software, Los Angeles, CA). Typical analyses sampled approximately 10,000 cells. Samples