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The effects of albumin-coated microbubbles in DNA delivery mediated by therapeutic ultrasound

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Abstract

The application of therapeutic ultrasound (TUS) in combination with contrast agents (USCA) to mediate gene delivery relies on the understanding of the bioeffects involved. The objective of this study was to evaluate the various bioeffects generated by albumin-coated microbubbles: Optison, an USCA, when applied with TUS operated for 10–30 min, on cells and on DNA transfection. This study reveals that Optison microbubbles were still acoustically active after long-term TUS application of 30 min. Optison enhances TUS-gene transfection by increasing the number of plasmids in the cells and also by distributing the plasmids to more cells, without significant decrease in cell viability. Optison also interacts with the DNA to further enhance transfection in a mechanism not necessarily involving cavitation. However, Optison affects mainly the cell cytoplasmatic membrane, without interfering with DNA intracellular trafficking. Using high-resolution scanning electron microscopy (HRSEM), the bioeffects on cell membrane induced by TUS–Optison were observed, demonstrating that Optison lead to a rougher surface, characterized by depressions that are reversible within 24-h post TUS. These effects are different from those observed when only TUS was applied. The findings from this study suggest that albumin-coated microbubbles enhances transfection when using TUS for 10–30 min, and that microbubbles play a major role in elevating cell transfection level and efficiency.

Keywords: Therapeutic ultrasound; Optison; Gene delivery; Cavitation; Bioeffects

1. Introduction

Ultrasound (US) has gained wide interest in gene therapy due to its potential to deliver genes into cells and tissues [1–4]. Most studies using US for gene delivery have applied low frequencies (<1 MHz) [5,6] which are known to induce cavitation [7]. In the process of cavitation the ultrasonic field interacts with gas bubbles in the liquid leading to their growth and eventual implosion [8–10]. This collapse initiates shear stress, shock waves, and microjets that affect the cells in the nearby vicinity [4,7,10,11]. It is believed that these effects lead to the permeabilization of the cell membrane [6,8,10–13]. On the other hand these effects, if not properly controlled, may lead to cell death [6,14].

In the past years several studies have demonstrated that therapeutic ultrasound (TUS) can deliver genes to cells and tissue [1,2,15–17]. TUS operates at frequencies of 1–3 MHz, intensities of 0.5–2 W/cm^2 and at pulse mode [1,2,15–17]. TUS is known to be safer than low-frequency US in terms of tissue damage, and it is also approved for clinical applications, making it a promising tool for the delivery of genes in the clinical settings [1,2,15–19]. However, the frequencies and intensities associated with TUS are known to be sub-cavitational, i.e. below the energy threshold for cavitation [3,7]. This has been suggested to be responsible for the low transfection level achieved, as TUS is applied when compared to low frequency US.

Therefore, recent studies using TUS for gene delivery have used ultrasound contrast agents (USCA, gas filled microbubbles), which are known to lower the threshold for cavitation by acting as cavitation nuclei, hence inducing cavitation under therapeutic ultrasound conditions [7–9,11,20,21]. The addition of USCA provides a control over the cavitation process,

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allowing to alter cell membrane permeability without significant damage to cell viability [8,9,11].

Air-filled microbubbles (e.g. Albunex) [3,8] or liposomes, were the first USCA used for gene delivery [12,16]. Today, a second generation of contrast agents is preferred, due to their coating materials, which stabilize the bubbles and the insoluble gases used to fill the bubbles (such as perfluorocarbon). These second generation USCA are more stable and provide better performance [22,23]. Among this group, Optison, is frequently used in TUS transfection studies in vitro [4,17,19,24] and in vivo [25–29]. In these studies, Optison was administrated in different ways; in vitro—DNA and Optison were mixed before TUS application [30], or each added alone [24]. In vivo, some studies injected the DNA and the Optison separately (DNA to the site of interest and Optison i.v.) [31,32], while other studies mixed the DNA and Optison together before injection [4,20,28,29]. Thus, the effects of the interaction between DNA and Optison due to their mixing were not addressed.

Another issue that needs to be addressed is the effect of US exposure time on USCA. Previous studies performed on perfluorocarbon exposed sonicated dextrose albumin (PESDA) microbubbles applied diagnostic US for exposure times of less than 1 min [21,33]. Other studies that evaluated the effect of US on Optison, or the effect of Optison on cells, have used low-frequency US (<500 kHz) for short terms of less than 3 min [6,13,34]. Studies using Optison in TUS mediated gene delivery have also applied short-term (less than 5 min) TUS [3,15,35], probably due to the notion that USCA are destroyed in the first seconds or minutes of TUS application [3,8,36]. Recently, we have shown that long-term application of TUS (>20 min) results in high transfection levels and DNA localization in the nucleus, even without the use of USCA [17]. However, addition of Optison to this process resulted in an additional increase in cell transfection and bioeffects, even when TUS was applied for 20 min and more [37]. Still, the mechanism by which Optison promotes transfection when applied with TUS is not clearly understood. In particular, the effect of TUS on Optison and the effect of Optison on cells when applied with long-term TUS need to be clarified. This includes the stability of Optison during long-term TUS application and the likelihood of other mechanisms involved in TUS mediated gene delivery when long-term TUS exposure is applied. This study addresses these important issues, while attempting to understand the role of Optison in this process.

2. Materials and methods

2.1. Plasmids

Two reporter plasmids were used for the various studies: pGL3-Luc (Promega) containing the firefly luciferase gene and pIRES-EGFP-N1 (Clontech) containing the EGFP gene. Plasmid DNAs (pDNA) were amplified and purified using JET-Star (Genomed, Germany) according to the manufacturer's protocol and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA). The purified plasmids were quantified using absorbance at wavelength of 260/280 nm. A pGeneGrip plasmid (pGG, Gene Therapy Systems, USA) [38] labeled with rhodamine and containing the GFP gene was used for the pDNA uptake studies.

2.2. Cell culture

Baby hamster kidney cells (BHK-21, ATCC) were grown in DMEM (Biological Industries, Israel) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin/streptomycin solution (Biological Industries, Israel) and fungizone (Gibco). Cells were cultured as monolayers in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.3. Therapeutic ultrasound apparatus

A therapeutic ultrasound with a 1 MHz applicator and a 2 cm^2 surface area probe (Ultra-Max, XLTEK, Canada) was used for all experiments. The coupling quality and total energy delivered were monitored at all times. The TUS set-up and measurements of acoustic pressure, mechanical index (MI) and temperature at all TUS parameters were as previously described [17].

2.4. In vitro gene transfection

BHK cells were counted and seeded in 6-well plates at a density of 1×10^5 cells/well. Four milliliters of medium containing plasmid DNA (7.5 μg/ml) were added to the cells. TUS transducer was immersed in the well, on top of the cells, and the cells were then exposed to 1 MHz pulsatile ultrasound (20%, 30% duty cycle (DC)) at intensities of 1–2 W/cm^2 (correspond to 0.114–0.159 MPa, or mechanical index (MI) of the same values [17]) for total exposure times of 10, 20, 30 or 40 min (correspond to 60–1440 J/cm^2 [17]). Control cells received only DNA, without the application of TUS, or TUS alone. The effect of ultrasound contrast agent on cell transfection was performed using Optison™, human albumin microspheres (Amersham Health). Optison (10% v/v) was added to the plasmid solution, mixed and kept for 10 s. Cells were then transfected using the same parameters as without Optison. For all studies, cell viability was detected using MTT assay (5 mg/ml, Sigma Aldrich) 2 h post TUS application. Cell viability is presented as the percentage of viable cells post TUS application relative to control (indicated as 100% viability).

2.5. Measurements of luciferase activity and GFP expression post TUS

Measurements of luciferase activity were performed 3 days post TUS application, with or without the addition of Optison, according to the company's protocol (Promega, USA) and as described [17]. For each sample, total protein was calculated using BCA protein assay reagents (Pierce Biotechnology, USA). Luciferase activity is reported as relative light units (RLU) obtained from the sample divided by total protein weight measured, for each sample.

Transfection efficiency was evaluated using pIRES-EGFP. Cells expressing GFP were followed under inverted fluorescent microscope (TE2000-S, Nikon). Transfection efficiency was