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Enhanced gene transfection using calcium phosphate co-precipitates and low-intensity pulsed ultrasound

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ABSTRACT

The capability to controllably disrupt the cell membrane by ultrasound (US), thus facilitating entry of exogenous species, has now reached a state of some maturity. However, a compelling question asks whether there is a residual role for US in enhancing transfection: that is, once the genetic material has been delivered to the cytosol, can US assist in its transport into the nucleus? The present experiment was designed with a view to addressing this question. As such, our experimental setup discriminates between: (i) the precursor cell membrane permealization step, and (ii) any subsequent intracellular trafficking into the nucleus. In this study, calcium phosphate co-precipitates (CaP) were used to internalize plasmid DNA encoding for luciferase (pDNA-Luc) (>90%) in HeLa cells. After 2 h incubation with the CaP-pDNA-Luc, cells were washed and insonated for varying durations. The results showed that US can indeed enhance the intracellular trafficking of previously internalized genes when longer insonation periods are implemented, culminating with an increased probability for successful nuclear localization, as inferred from an enhanced luciferase expression. Moreover, the results suggest that the intracellular role of US might be mediated through a pathway that appears not to be limited to destabilizing the endosomal vesicles. The study thus provides new information regarding the intracellular effects of US, and in effect represents a new modality combining US and CaP carriers for improved efficiency in gene delivery.

1. Introduction

The cell membrane is recognized as a primary target for ultrasound (US) based disruption, especially in the context of molecular delivery. However, recent and emerging literature indicates that US may also directly influence biological processes at the sub-cellular level (Or and Kimmel, 2009). For example, in US-induced cell death, it appears possible that US can directly affect the mitochondria (Zhong et al., 2011) and endoplasmic reticulum, as well as the genomic DNA as intracellular targets for initiating cell death programs (Furusawa et al., 2012). Also, from a delivery perspective, the role of US in macromolecular trafficking and nuclear localization continues to form a significant discussion point within the community. During non-viral transfection, exogenous DNA faces three dynamic constraints en route to the nucleus; (i) the cell membrane barrier, (ii) [volume diffusion/or active transport] through the cytoplasm, and (iii) traversal through the nuclear membrane. Each of these constraints represents a critical transport challenge that acts to inhibit the transfection process. In US-mediated transfection (sonotransfection), US has been accepted to permealize the cell membrane through the induction of physical pores (Mehier-Humbert et al., 2005; Zhao et al., 2008). A remaining issue here involves the determination of those acoustic parameters that optimize the transience of such pores, thus, minimizing irreversible damage and achieving the highest percentage of *viable* transfected cells (Hutcheson et al., 2010; Miller and Dou, 2009). Regarding the other constraints, Duvshani-Eshet and Machluf have reported that US (at 1 MHz, 2 W/cm^2, 30% duty cycle) was able to localize exogenous plasmid DNA (pDNA) in the nucleus (Duvshani-Eshet et al., 2006). These studies were characterized by relatively long insonation periods [up to 30 min] after manoeuvring the exposure setup to minimize cell detachment and death (Duvshani-Eshet and Machluf, 2005), and indeed, the report highlighted that nuclear localization depended on the sonication time, with the suggestion that such prolonged insonation simply led to more permeabilized cells (demonstrated as the number of fluorescent cells) and greater macromolecular loading to each respective cell (demonstrated as an increase in the relative fluorescence). This report further stated that at sonication periods lesser than 10 min, DNA was "hardly" seen in the nucleus.

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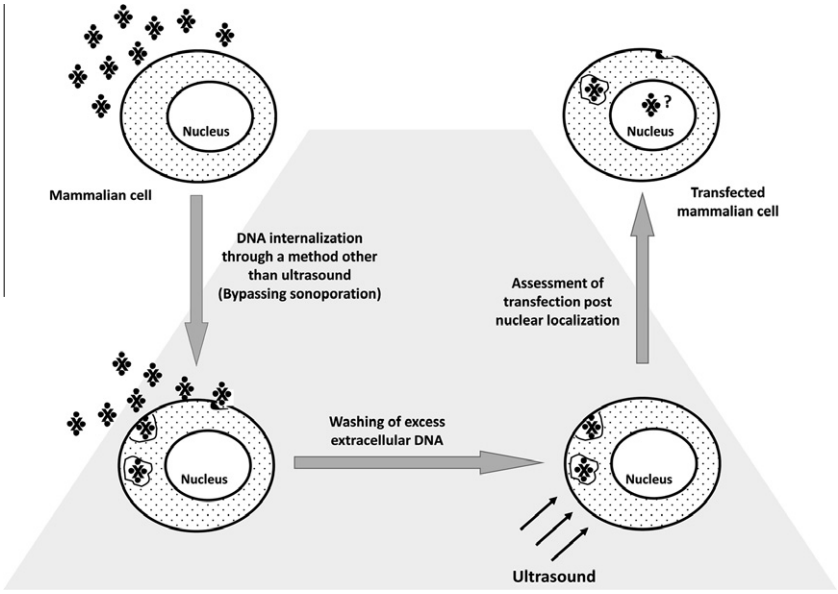

Fig. 1. Illustrative diagram describing the dissection of ultrasound roles. A drug carrier (in this study calcium phosphate co-precipitates; CaP) internalizes the exogenous plasmid DNA (pDNA) through endocytosis. Excess CaP-pDNA is then removed from the extracellular room prior to ultrasound irradiation. In such case, increased nuclear uptake compared to ultrasound treatment or the drug carrier alone should be attributed to ultrasound-facilitated trafficking.

In light of that study, it can be deduced that prolonged sonication might sustain the overall permeabilization state of cells, increasing the extent of gene-loading per cell, which in turn would enhance nuclear localization due to saturation of the degrading enzymes in the cytoplasm and/or due to enhancing the diffusivity of the pDNA through the cytoplasm by establishing steeper concentration gradients. In other words, the role of prolonged US could be in part due to an effect on the cell membrane (Hrazdira et al., 1998). An alternative scenario might involve a purely intracellular effect, such as changing the cytoplasmic viscosity, inhibiting the degrading enzymes or permealizing the nuclear membrane. This intracellular role of US cannot be proven as long as excess exogenous DNA remains in the extracellular medium during sonication. Therefore, any experiment that aspires to deliver decisive evidence in this respect must discriminate the effects of US on the cell membrane, from those presumed to be exerted intracellularly, and thus remove any possible interference from extracellular DNA. An illustration of this premise is given in Fig. 1. In this study, we have used calcium phosphate co-precipitates (CaP) to deliver a pDNA with high efficiency showing clearly that US, generated over a longer timescale, can exert an intracellular role in nuclear localization. We also found there to be a distinct advantage in using CaP in combination with US for efficient transfection, which reduces any cytotoxic response that may be exerted by trying to effect similar transfection levels with either US (Miller and Dou, 2009) or CaP (Jordan et al., 1998) by themselves.

2. Materials and methods

2.1. Cell culture

Human cervical adenocarcinoma (HeLa) cells (Health Science Research Resources Bank, Japan Health Sciences Foundation, Tokyo, Japan) were cultured in RPMI 1640 medium (WAKO Pure Chemical Industries, Ltd., Osaka, Japan, code # 189-02025) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic mixture under humidified air and 5% CO_2. The cells were harvested 24 h before the experiments by trypsinization and seeded in polystyrene culture dishes (diameter = 35 mm, base thickness = 0.5 mm, Corning Inc., Corning, NY) at a cell density of 1×10^6 cells.

2.2. Preparation of plasmid DNA

The plasmid vector pBKCMV-luc (pDNA-Luc) containing the luciferase gene under the control of cytomegalovirus early promoter was used in this study. The vector was amplified in DH5α bacteria, purified using FlexiPrep kit (GE Healthcare, Bucks, UK) according to the manufacturer's protocol and quantified spectrophotometrically. The average A_{260}/A_{280} ratio was 1.9 ± 0.0, indicating the absence of contaminants (Lee and Peng, 2007).

2.3. Preparation and loading of calcium phosphate co-precipitates

Calcium phosphate co-precipitates (CaP) loaded with pDNA-Luc (CaP-pDNA-Luc) were prepared according to the method described by (Lindell et al., 2004) with some modifications. In brief, sufficient volumes of stock solutions of pDNA-Luc (100 μg/ml) and $CaCl_2$ (1 M) were mixed with the high phosphate-RPMI medium to attain a final concentration of 5 μg/1.5 ml and 14 mM of calcium ions (Ca^{2+}), respectively. The suspension was lightly vortexed and left for 1 min before use. The transfection suspension was stable over 24 h.

2.4. Transfection with plasmid DNA-loaded calcium phosphate co-precipitates in vitro

Pre-cultured HeLa cells were incubated with 1.5 ml of the CaP-pDNA-Luc suspension medium for 24 h. The cells were then collected quantitatively for luciferase assay and total protein measurements, as described below. Parallel experiments were conducted using a mixture of 4KD-FITC-dextran and $CaCl_2$ in RPMI medium to attain a final concentration of 100 μg/ml of the fluorogenic marker and 14 mM of Ca^{2+}, respectively. The suspension was prepared and handled in the same manner as the CaP-pDNA-Luc suspension, before being dispensed into culture dishes in aliquots of 1.5 ml/dish. The dishes were then incubated for 2 or 4 h. Cells were then harvested quantitatively, washed twice with cold phosphate buffered saline (PBS) and injected into a flow cytometer