TRANSMEMBRANE EXTRACTION OF FLUORESCENT PROTEINS WITH ULTRASOUND AND MICROBUBBLES

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Abstract - Recent studies have shown that microbubbles under ultrasound (US) activation permeabilize plasma membrane. But the mechanism of permeabilization is not completely understood. The recurrent hypothesis assumes the formation of pores in the cell membrane allowing molecule delivery into cells. The aim of our study is to comfort this assumption by investigating whether US and microbubbles facilitate outward transport of molecules across the plasma membrane. Stably transfected Hela-GFP cells (GFP + cells) and propidium iodide (PI) were used to follow the US-microbubbles mediated permeabilization. To correlate these observations with molecular incorporation, US waves were generated from a 1 MHz unfocused transducer with pressures from 200 kPa to 600 kPa and duty cycles of 40 % and 75 %. Exposure time was 2 min with or without BR14 microbubbles (Bracco Research, Geneva). Immediately after insonation, the cells were immersed in a 4°C bath to interrupt cell activity. The percentage of GFP positive cells and the mean cell fluorescence intensity (FI) were measured by flow cytometry to evaluate the GFP release. We observed a significant decrease of FI and the percentage of GFP+ cells using US in presence of BR14 microbubbles. These effects were maximal at 400 kPa, 75 % of duty cycle and 2 minutes insonation compared to control conditions. In parallel, the number of GFP + cells that incorporated PI was higher than 50 % for the same acoustic parameters. US alone had a slight impact on cell membrane permeability. These observations were also correlated to an increase of FITC-dextran incorporation that varied also *vs* acoustic parameters in presence of BR14 microbubbles. This result demonstrates the possibility to incorporate extracellular molecules into Hela cells throw probably pore formation. All observations would be explained by pore formation occurring under activation of microbubbles with ultrasound. This phenomenon leads to probably gradient dependant transport through pore opening during insonation.

I. INTRODUCTION

Recent studies have shown that microbubbles activation under specific US beam induce transient cell membrane permeability [1, 2]. This sonoporation process is actually under investigation to incorporate extra cellular molecules into cells. But the principal interrogation concerns the mechanism of cell membrane permeabilization and molecular internalization into cells. The main hypothesis assumes the formation of pores in the plasma membrane allowing molecules incorporation into cells through a passive transport [2]. Using a high speed camera, it has been observed that US waves at 200 kHz induce deformation and reparable rupture of lipidic membrane vesicle as well as the leakage of the fluorescent vesicle content in the external medium [3]. Moreover, Taniyama and colleagues have shown the appearance of holes with a maximum size of 100 nm at the cell surface after treatment with US and microbubbles on human aortic endothelial cells and vascular smooth muscle cells [1]. These observations have been carried out with scanning electron microscopy and thus the cells were not necessarily in a viable situation. Morphological changes have also been reported after cell treatment with US and microbubbles. Using electron microscopy observations, it has been demonstrated a modification of cell size and cell membrane roughness associated with removal of cell surface receptor [4, 5]. A reduction of cell size was also reported. This effect is likely attributed to leakage of cytosolic fluid leading to an escape of cytoplasmic content trough probably reversible membrane disruption or pore formation.

In this context, the aim of our study was to comfort the hypothesis of pore formation by investigating whether US and microbubbles facilitate outward transport of fluorescent molecules across the cell membrane. For this reason Hela cells constitutively expressing GFP protein (27 kDa) were used in our study [6]. If pore opening occurs during sonoporation, a leakage of GFP content across the destabilized plasma membrane would occur. This process leads to a simultaneous decrease of the number of cells expressing GFP and the associated cell fluorescence intensity (FI). The effects of variable acoustic parameters were also investigated.

II. MATERIALS AND METHODS

Cell culture

Hela and Hela-GFP cells expressing constitutively the enhanced green fluorescent protein (GFP) [6] were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10 % heat-inactivated foetal calf serum (FCS), 2mM-L glutamine and 100 units/ml streptomycin and 100 µg/ml penicillin at 37°C, in humidified atmosphere in the presence of 5 % CO_2.

Microbubbles and ultrasound set-up

BR14, perfluorocarbon gas microbubbles were generously provided by Bracco (Bracco Research, Geneva, Switzerland). A concentration corresponding to 20 bubbles per cell was used.

Ultrasound waves were generated from a transducer with centre frequency of 1 MHz. The transducer was driven with an electrical signal generated by an arbitrary waveform generator and amplified with a power amplifier (ADECE, Artannes, France).

Cell insonation protocol

After washing with opti-MEM (Invitrogen, Carlsbad, USA) supplemented with 1% FCS, cells were introduced in a holder consisting of a thin plastic tube. The tube was introduced in a water tank maintained at room temperature. During insonation with or without BR14 microbubbles, the medium was gently stirred. After treatment cells were immediately immersed in 4°C bath.

In parallel, the incorporation of FITC-dextran (70 kDa) into Hela cells was also investigated. FITC-dextran (1 mg/ml) was introduced with microbubbles (20 bubbles per cell). After ultrasound exposure, cells containing tubes were immediately immersed in 4°C bath and then cell associated-fluorescence intensity was measured by flow cytometry.

Quantification of GFP leakage and dextran-FITC incorporation

After washing, cells were harvested and suspended in 500 µl of PBS. FITC-dextran incorporation and GFP extraction were assessed by flow cytometry with LSR flow cytometer (Becton Dickinson, Sunnyvale, CA) to measure the percentage of cells still expressing GFP, fluorescent dextran uptake and the mean cell FI. Percentage of GFP + cells and cells that incorporated FITC- dextran was determined for the whole cell population. Simultaneously propidium iodide incorporation was evaluated in order to assess cell membrane destabilization on GFP + cells immediately after US insonation.

III. RESULTS

Effects of BR14 microbubbles on GFP-Hela cell membrane permeabilization

Compared to control condition, US alone at 400 kPa and 40 % duty cycle during 2 minutes insonation induced a slight decrease in the number of GFP + cells and FI (Fig. 1). Adding microbubbles enhanced significantly this phenomenon. A reduction about 40 % of the percentage of GFP + cells and 50 % of the FI was observed (Fig.1). Simultaneously, the number of cells incorporating PI has doubled when microbubbles were present compared to US alone. This result was negatively correlated to the percentage of GFP + cells as well as the FI.

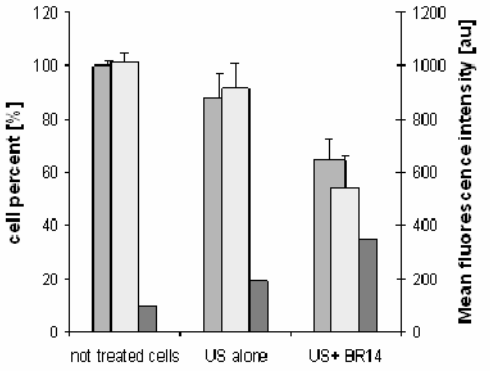

Figure 1. Percentage of GFP+ cells (grey bar), FI (white bar) and PI incorporation (dark bar) at 400 kPa, 40 % duty cycle, 2 min insonation, 20 bubbles per cell.

Effects of acoustic pressure on GFP-Hela cell membrane permeabilization

Figure 2 shows the effect of acoustic pressure on cell membrane permeabilization and outward of GFP protein. At 200 kPa a minimal effect on GFP extraction is induced as shown by the slight change in number of cells expressing GFP molecule. Increasing the acoustic pressure to 400 kPa decreases the total number of GFP+ cells and FI while this is associated with an increase in the number of cells incorporating PI. GFP extraction phenomenon was not enhanced at 600 kPa as shown in the total number of GFP+ cells and in PI incorporation.

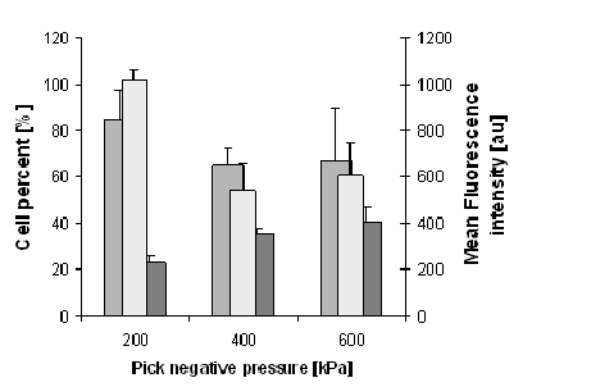

Figure 2. Percentage of GFP+ cells (grey bar), FI (white bar) and PI incorporation (dark grey) *vs* acoustic pressure using US at 40 % duty cycle, 2 min insonation, 20 bubbles per cell.

Effects of duty cycle on GFP-Hela cell membrane permeabilization

Varying duty cycle seems to have a significant incidence on both GFP + cell percent, FI, and PI incorporation as shown in Fig. 3. US at 75 % duty cycle reduced further the percent of GFP+ cells compared to US at 40 % duty cycle. The FI was also reduced by approximately a factor 2 and was also negatively correlated to PI cell percent incorporation.

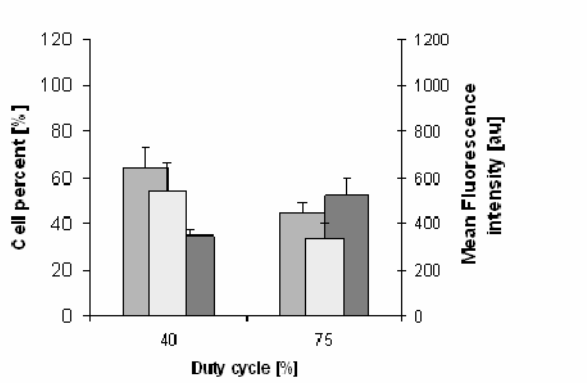

Figure 3. Effect of duty cycle on GFP extraction, at 400 kPa, 2 min insonation, 20 bubbles per cell. grey: GFP+ cells %, White: Mean fluorescence intensity (FI), Dark grey: % of PI cell incorporation

Cell viability and GFP recovery:

GFP recovery has been verified at different times after sonoporation (data not shown). For that purpose, insonified cells have been incubated at 37ºC then GFP expression and IP incorporation have been measured with the flow cytometer at different times. 4 and 48 hours after sonoporation at 400 kPa, 75% of duty cycle excitation with 20 microbubbles per cell, the percentage of GFP + cells and FI have increased while simultaneously the percentage of IP incorporation has decayed. This initial result indicates that cells recover their GFP content since the number of GFP+ cells has increased as well as FI compared to immediately after insonation. PI cell incorporation reduction shows that cell membrane integrity has returned to its initial state 48 h after sonoporation.

Correlation between Hela-GFP cell membrane permeabilzation and FITC-dextran incorporation

Hela cells were used to assess FITC-dextran incorporation at different acoustic pressures as shown in Fig. 4. Using US at 40 % of duty cycle and 2 minutes of insonation and BR14 microbubbles, the results show a weak incorporation rate at 200 kPa. Internalization rate was significantly increased 3 times at 400 kPa and exceeded 60% at 600 kPa. This improvement of FITC-dextran incorporation as function of acoustic pressure was also associated with a concomitant increase of FI and PI inward into cells.

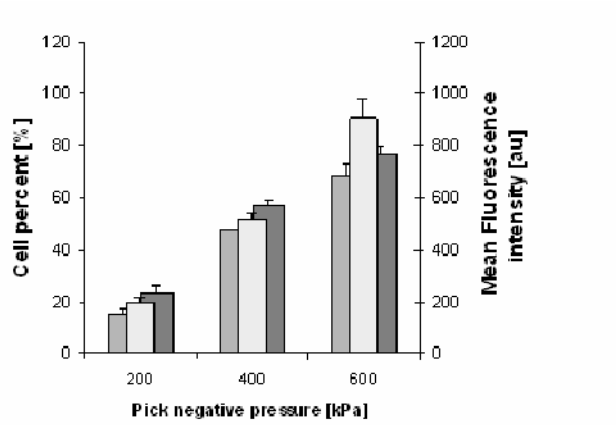

Figure 4. Effect of acoustic pressure on FITC-dextran incorporation using US at 40 % duty cycle, 2 min insonation and BR14 (20 bubbles per cell). Grey bar: dextran uptake; white bar: FI; dark grey: % of PI cell incorporation.

IV. DISCUSSIONS – CONCLUSIONS

Ultrasound in combination with contrast microbubbles have shown enhanced molecule incorporation into cells. *In vitro* and *in vivo* optimizations as well as the understanding of the permebilization mechanism are crucial before using this method for treating human diseases. The main hypothesis assumes pore formation induced by bubbles acoustic responses depending on ultrasound wave characteristics. Our results have demonstrated a decrease of the percentage of GFP+ cells and FI, probably attributed to an outward transmembrane transport of GFP after membrane destabilization. This depended on acoustic pressure as well as duty cycle.

V. REFERENCES

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