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Involvement of cytoskeleton in sonoporation and drug delivery

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Abstract— The mechanisms by which sonoporation increases the native plasma membrane permeability are still unknown but various hypotheses have been suggested including pore formation and endocytosis. We have shown recently that caveolae-mediated endocytosis plays a major role during sonoporation. In addition, some studies have reported the clear participation of actin in mammalian cell endocytosis. Thus, this study aims to investigate the effect of sonoporation on actin microfilaments and microtubules and to identify the role of both cytoskeletons on sonoporation-mediated membrane permeabilization. Adherent U-87 MG cells were insonated at 1MHz, 1 W/cm², 20% duty cycle for 60 s, in the presence of BR14[®] microbubbles. SYTOX[®] Green was used to assess the membrane permeabilization, by flow cytometry. The cells were incubated with phalloidin-TRITC to stain actin microfilaments and tubulin antibody Alexa Fluor $^{\circ}$ 555 to stain tubulin. The ultrastructural changes of plasma membrane were monitored by scanning electron microscopy. To inhibit the polymerization of actin and tubulin cytoskeleton, the cells were treated with cytochalasin D (cytoD) and nocodazole (Noco), respectively. Immunofluorescence results show alteration of actin and tubulin cytoskeleton, immediately after sonoporation while control cells present a filamentous cytoskeleton with polygonal shape. However, the disorganization of the cytoskeleton network is reversible since 60 min postsonoporation, only few cells show a tubulin (8%) and actin (25%) cytoskeleton disruption. Moreover based on SEM study reveals that the treatment of the cells with both cytoD and Noco induced a strong decrease in the number of TPS (transient and permeant structures): $98.5 \pm 0.2\%$ and $96 \pm 0.6\%$, respectively. Moreover, flow cytometry results showed that cytoD and Noco lead to a decrease in the membrane permeabilization rate: 58% and 87%, respectively. In conclusion, this study demonstrates the transient alteration of actin and tubulin cytoskeleton following sonoporation. It suggests that cytoskeleton plays a role during sonoporation, as cytoskeleton inhibitors provoke a decrease in the cell permeabilization rate. Its implication could occur during both the entry and transport of endocytosed molecules.

Keywords— Sonoporation, Ultrasound, Microbubbles, Cytoskeleton, Immunofluorescence, Scacnning electron microscopy

I. INTRODUCTION

It is well known that sonoporation increases the plasma membrane permeability. This physical method is based on the combination of ultrasound and microbubbles [1-3]. The enhancement of the native permeability of cells, during the sonoporation process, requires perforations on the plasma membrane, that allow the intracellular uptake of poorly permeant therapeutic molecules (*i.e.*, bleomycin, irinotecan, nucleic acids [4-6]. However, the exact mechanism of sonoporation is poorly understood. One of the agreed mechanisms is (i) formation of non-selective poration on the cell membrane [7-9]. The agreed mechanism consists in the stimulation of endocytosis either in the absence [10] or presence of microbubbles [9, 11-13]. Otherwise, the plasma membrane movements are ensured by firstly actin microfilaments. During sonoporation, the cytoskeleton could be altering, since it is closely linked to the plasma membrane and represents a network of fibers throughout the cytoplasm.

The present study aims to determine the sonoporation influence on actin microfilaments and microtubules in human glioblastoma cells (U-87 MG), and their potential role in sonoporation-mediated membrane permeabilization.

II. MATERIAL AND METHODS

A. Cell Culture

Human glioblastoma cells (U-87 MG) were seeded on 18 mm diameter glass cover slips, placed in 24 well-plates and containing Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA) supplemented with 10% v/v fetal calf serum (Life Technologies, Carlsbad, CA) and incubated at 37° C under an humidified atmosphere in 5% CO₂ incubator. Cells were cultured until 80% confluence before ultrasound exposure.

B. Ultrasound Exposure

Ultrasound waves were transmitted at 1MHz frequency using 20% duty cycle for 60 s at 1 W/cm², (SoniGeneTM systems, VisualSonics). Ultrasound contrast agent, BR14[®] (Bracco Research, Switzerland) was added into the well at a microbubble/cell ratio of 5.

C. Cell Permeabilization

SYTOX[®] Green, a small and non-permeant molecule was used, at a final concentration of 1 μ M, to monitor the membrane permeabilization, by flow cytometry [14]. The cell mortality was assessed by flow cytometry after propidium iodide (PI) staining. Fluorescence histograms were recorded with a flow cytometer (Beckman-Coulter, Fullerton, CA) and

analyzed using the Kaluza software supplied by the manufacturer. A minimum of 10 000 events was analyzed to generate each histogram.

D. Scanning Electron Microscopy

The ultrastructural modifications of the cells, after sonoporation, were monitored by scanning electron microscopy (SEM). For SEM experiments, the cells were insonified and fixed immediately after sonoporation (0 min) by immersion in paraformaldehyde (PFA) and glutaraldehyde fixative solution. The transient and permeation structures were counted manually using the same set of images for each condition. Ten cells per condition were included in the analysis.

E. Direct immunofluorescence assay

The cells were fixed using 4% PFA 4% in PBS at room temperature, immediately (0 min), 30 and 60 min postsonoporation. The cells were incubated with TRITC (tetramethylrhodamine-5, 6 – isothiocyanate)- labeled phalloidin (Sigma-Aldrich[®], St. Louis, MO), used to stain the ACTIN cytoskeleton. Alexa Fluor[®] 555-conjugated anti-TUBULIN antibody (Sigma-Aldrich[®]) was used to label the microtubules. ProLong[®] Antifade Mountant purchased from Invitrogen, is a liquid mountant used to cover the slides. It contains DAPI (4',6-diamidino-2-phenylindole) that stain the nucleus in blue fluorescence. Confocal images were captured using Zeiss LSM510 confocal microscope.

F. Inhbitors Treatment

To inhibit the polymerization of actin and tubulin cytoskeleton, the cells were seeded on glass cover slips and treated with 50 μ M of cytochalasin D and 10 μ M of nocodazole (Sigma-Aldrich[®]), respectively. The cells were incubated with inhibitors before sonoporation during one hour at 37°C.

III. RESULTS AND DISCUSSION

Initially, this study aimed to investigate the effects of sonoporation on the U-87 MG cytoskeleton. The insonified U-87 MG cells were immunostained with TRITC-conjugated phalloidin and Alexa Fluor® 555-labelled anti-TUBULIN antibody. As expected, control cells exhibit filamentous network of actin and tubulin cytoskeleton, while insonified cells are distinguished by punctiform diffuse staining. Indeed, immunofluorescence images show a rearrangement of actin and tubulin cytoskeleton immediately after sonoporation (Figure 1). As phalloidin does not bind nonpolymerized actin, the diffuse staining attests the rearrangement of actin filaments in monomeric actin. Furthermore, Juffermans et al., have shown that combining ultrasound with microbubbles caused a significant increase in the number of F-actin stress fibers, suggesting an instant effect of ultrasound on the cytoskeleton, which may be related to the initiation of membrane permeability.



Figure 1: Effect of sonoporation on ACTIN and TUBULIN cytoskeleton in U-87 MG. White arrows show the actin and tubulin network in control cells, while arrowhead designate the disorganization of cytoskeleton immediately after sonoporation.

In order to complete these microscopic observations, we evaluate the recovery of cytoskeleton, by fixing the cells 30 and 60 min post-sonoporation. This second step consists of counting cells with disrupted cytoskeleton. The quantitative study shows that 30 min post-sonoporation, the percentage of cells with cytoskeleton disruptions decreases and approximately 60% and 70% of sonoporated cells recover the integrity of actin and tubulin cytoskeleton, respectively.



Figure 2: Recovery of native cytoskeletons (N = 500 cells).

This decrease is emphasized over time since only 8% of cells present a tubulin cytoskeleton disruption 60 min postsonoporation. For actin cytoskeleton, this decrease is half compared to the cells 30 min post-sonoporation, suggesting that actin cytoskeleton takes longer to get back up compared to tubulin one, following sonoporation. Together these results give evidence that alterations caused by sonoporation are transient, and reversible since the cells regain their cytoskeleton integrity within the 60 min following sonoporation.

To further investigate the involvement of cytoskeleton during sonoporation, U-87 MG cells were treated with microfilament inhibitors of actin and microtubule polymerization before sonoporation. SEM images show that the inhibitors treated cells present less transient and permeant structures (TPS) on their plasma membrane compare to the insonified cells in the absence of inhibitors treatment (Figure 3). Indeed, the quantitative study shows that cytochalasin D treatment induces a strong decrease in the mean number of TPS $(98 \pm 0.2\%)$. In a similar manner, nocodazole also decreases the TPS on the plasma membrane $(96 \pm 0.6\%)$.



Figure 3: SEM micrographs of the U-87 MG membrane morphology (N = 10 cells).

The results obtained by flow cytometry revealed a decrease in the incorporation of SYTOX[®] Green rate in the insonified and inhibitors treated cells. We note a decrease of 87% after cytochalasin D treatment, while the treatment with nocodazole induces 58% decrease in SYTOX[®] Green uptake (**Figure 4**). These findings suggest the involvement of both cytoskeletons (ACTIN and TUBULIN) in membrane permeabilization during sonoporation process. In fact, several studies demonstrate the involvement of filamentous actin and microtubules cytoskeletons in membrane invaginations, vesicle formation and trafficking during endocytosis [15-18]. Together these data suggest that intact cytoskeleton is required for membrane permeabilization and endocytosis during sonoporation process.



Figure 4: Effects of inhibitors of actin microfilament and microtubule polymerization on sonoporation-mediated membrane permeabilization.

IV. CONCLUSIONS

In conclusion, our results showed that sonoporation induces the rearrangement of actin and tubulin cytoskeleton. This reorganization is transient. Since, 60 min post-sonoporation majority of cells recover the integrity of cytoskeleton. Our observations give evidence of the implication of cytoskeleton during sonoporation process. The cytoskeleton involvement could occur during the early stages of endocytosis (entry and transport of molecules), thus explaining a strong increase of endocytic pathways (caveolae and clathrin-mediated endocytosis) previously demonstrated. In addition, the presence of cytoskeleton fibrous structure could be one of the criteria for an effective transfer of molecules.

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