공초점 현미경을 이용한 RAW 264.7 세포 괴사의 단세포, 실시간 형광이미지 관찰

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Single-cell based, real-time detection of RAW 264.7 apoptosis using confocal fluorescence microscopy

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Introduction

Currently, cell-based drug screening assays have been implemented for various molecular interactions including induction of apoptosis. Apoptosis is the process of cell death highly regulated by activation and deactivation of proteases, of which the main orchestrators are caspases. Caspases can be activated by signals transduced by the apoptosis inducers such as staurosporine. Most of the apoptosis monitoring involves the imaging of the fluorescence localization by using confocal laser induced microscopy. To understand the multiplexing and drug-drug interactions with a cell requires the continuous monitoring of these events in a single cell in real time. For a preliminary study for the real-time, single-cell analysis, we performed a time-lapse monitoring of the nucleation of the green fluorescence protein (GFP) to correlate it with cell membrane blebbing. And, we determined the nucleation kinetics based on the fluorescence intensity profile obtained from the real-time imaging data.

Experimental

Cell Culturing & GFP Transfection

RAW 264.7 mouse macrophage cell line (ATCC, Rockville, MD, USA) was grown in Dulbecco's modified Eagles medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 IU/I penicillin, and 10 μg/mL streptomycin in a humidified 5% CO_2 incubator at 37 °C. Plasmid used was pCaspase3-Sensor Vector, a Clontech Living Colors plasmid (Clontech, Paolo Alto, CA, USA) [1-3]. The pCaspase3-Sensor vector expressed an Enhanced Fluorescent Yellow Protein (EYFP) fused with a Nuclear Export Signal (NES) and a Nuclear Localization Signal (NLS). The NES is dominant and separated from the EYFP by a caspase3 cleavage site. In the absence of caspase3 activity, the EYFP localizes in the cytosol. In the presence of caspase3 activity, however, the NES is cleaved off and the NLS promotes the EYFP's transport into the nucleus. Cells were transfected using CLONfectin^{TM} (BD Biosciences, Paolo Alto, CA, USA) [4]. Cells were plated in a 12 well plate one day before and incubated with 4 ug of plasmid DNA and CLONfectin (4 μg in HEPES-buffered saline) for 4 hr in the CO2 incubator. Then remove CLONfectin/DNA containing medium and wash cells with medium. Apply 2 ml of fresh complete growth medium and after 24 hr sorted for maximum responsiveness with minimal background fluorescence to obtain the transient reporter cell.

Apoptosis Induction

Exponentially growing cells were centrifuged and resuspended in a fresh pre-warmed medium. For apoptosis studies, staurosporine (Sigma) was added to the culture media. Stock solution of 1 mM staurosporine (STS) was prepared in dimethylsulfoxide. Aliquots were added to the culture medium to give the final concentration of 1 μM. Staurosporine is a protein kinase inhibitor and a strong inducer of apoptosis [5-6]. The mechanism of apoptosis in response to STS is mediated by the translocation of Bax from the cytosol to mitochondria, mitochondrial dysfunction, and the release of cytochrome c [7]. A similar but slower appearance of apoptosis has been reported in STS-treated MCF-7 cells lacking caspases-3 [8]. After treating with 1 μM sturosporine, the GFP signal was translocated from the cytosol to the nucleus by pCaspase 3-Sensor Vector.

Fluorescence imaging

Cells were examined under an inverted confocal laser scanning microscope (TCS SL, Leica, Germany). Translocated green fluorescence protein (GFP) images were observed using an argon ion laser. In-vivo detection of apoptosis process on microscope stage was performed in a single perfusion chamber (Live Cell Instrument, Seoul, Korea). The laser excitation of GFP occurred at λ = 488 nm, and the emitted fluorescent light was detected between λ = 510 – 600 nm [9]. Images were taken by time-lapse analysis every 10 min with a 100× (N.A = 1.3) oil immersion lens. The fluorescence intensity profiles of the single cell were also measured to monitor the apoptosis process. The images were processed by Leica confocal software package supplied with the CLSM.

Time-lapse microscopic measurements

For confocal time-lapse analysis, cells were placed in the single perfusion chamber. Cover slips were laid on the cell-culture dish and cells were injected with medium. A cover slip brings out in cell-culture dish after a day, then the cover slip adapt into the single perfusion chamber. It was filled with the DMEM medium containing 10% FBS, penicillin, and streptomycin in the chamber. The single perfusion chamber was laid on the invert microscope stage and kept at 37℃ and 5% CO_2 concentration during the measurement using temperature and CO_2 controller (Live Cell Instrument, Seoul, Korea). Figure 1 shows the single perfusion chamber system. Image acquisition started 1 hr after the temperature stabilized at 37 ℃. Images were taken by time-lapse detection mode every 10 min for 10 hr.

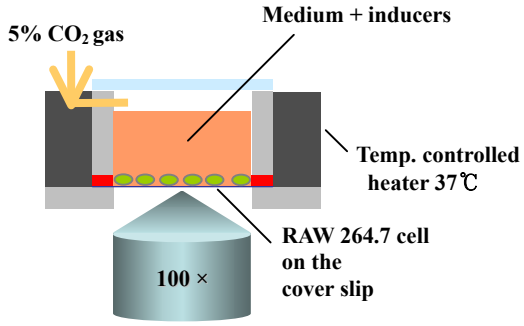

Fig. 1. Schematic of single perfusion chamber.

Results & Discussion

Medium/long term culturing using cell detection chamber

Many studies on adherent cell culture used the surface coated with proteins such as FBS (fetal bovine serum) and human fibronectin solution. We loaded RAW-264.7 cells to the chamber (the surface is glass) without coating and incubated at 37℃, 5% CO_2 concentration. Under this condition

over 90% of the cells appeared viability in 10 h (data not shown).

Staurosporine-induced death

We treated the cells with staurosporine (apoptosis inducer) and monitored the death of the cells. Fig. 2 shows the time-lapse monitoring of cell apoptosis after treating 10 μM staurosporine. It was clear that the GFP signal was translocated from the cytosol to nucleus. We found the common features of apoptosis during monitoring RAW-264.7 cells. First, at t = 3 and 4 h in Fig. 2, the blebbing in the membrane was observed. Second, a few hours later (t = 5 h, in Fig. 2), we observed the cell membrane became weak and the membrane appeared wrinkled. After 5 h, no more membrane could be observed until 10 h.

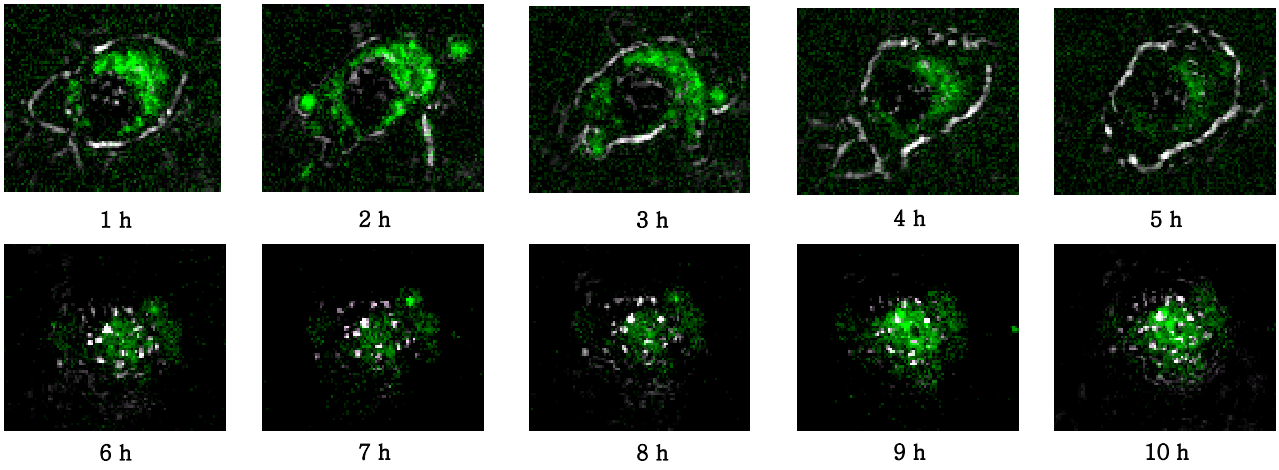

Fig. 2. Time course cell image after 1 μM staurosporine treatment.

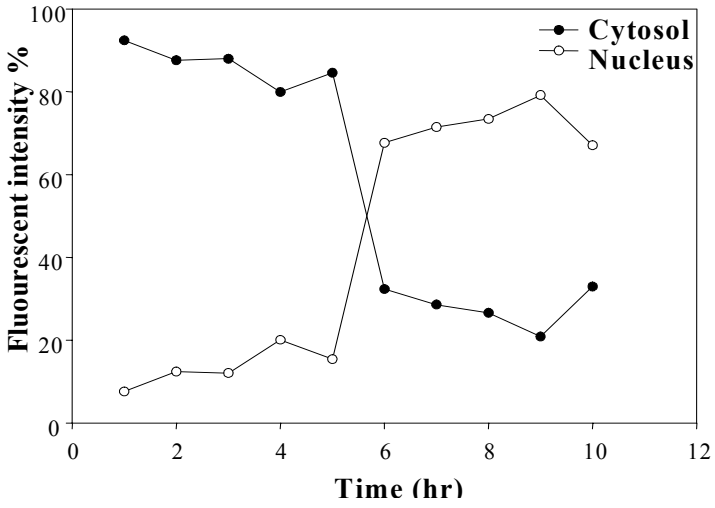

Fig. 3. Time course GPF signal transportation and the apoptosis time.

GFP signal translocation and the apoptosis time

From the GFP image monitoring, we obtained the GFP signal intensity profile. In the software, we crossed the line on the cell to collect the fluorescent intensity inside the cell. In Fig. 3, 1 h after staurosporine treatment, the GFP signal was detected in the cytosol, and few GFP signal was detected

in the nucleus. In the middle of apoptosis (t = 5 h), GFP signal in cytosol decreased significantly, whereas it increased gradually in the nucleus. In the later stage of the apoptosis (t = 10 h), most (ca. 80%) of the GFP intensity was detected in the nucleus, which meant the apoptosis was completed. With this GFP signal profile, we could determine the kinetics of the fluorescence nucleation. In Fig. 3 (d), about 50% GFP signal moved to the nucleus between 5 and 6 h. This was consistent with the apoptosis image in Fig. 2 for the starting of apoptosis. After 6 h, over 65% of the GFP intensity was detected in the nucleus. In this study we monitored the single GFP signal, but three-fluorescence signals will be monitored in the next experiments for multiplexing analysis.

Conclusion

We observed the staurosporin-induced apoptosis of GFP transfected RAW 264.7 cells by CLSM. We performed a time-lapse monitoring of the nucleation of the green fluorescence protein (GFP) with higher resolution compared with fluorescent microscope. In addition, we determined the nucleation kinetics based on the fluorescence intensity profile obtained from the real-time imaging data. With high intensity signal, this approach could be used in micro-fluidic cell culture chip.

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