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ORIGINAL PAPER

Cellular imaging assay for early evaluation of an apoptosis inducer

Sang Youn Hwang · Seung Hun Cho · Byung Heon Lee · Yoon-Jae Song · E. K. Lee

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Abstract The objective of this study was to propose a feasibility of a cellular imaging assay as an alternative to the conventional cytotoxicity assay, such as MTS assay, for apoptosis monitoring. As an apoptosis monitoring parameter, affinity interaction between phosphatidylserine (PS) and annexin V was chosen. First, the specific binding affinity between annexin V and PS in phospholipid bilayers consisting of various molar (0–15%) composition of PS was measured using a surface plasmon resonance biosensor. As PS composition increased, the binding level of annexin V increased proportionally. Second, various concentrations (0.1–10 μM) of staurosporine were used as to induce apoptosis and introduced to MCF-7 breast carcinoma cells. The cellular fluorescence images from annexin V-FITC conjugate were obtained by confocal microscopy, and their fluorescence intensities were quantified by image scanning. Dose–apoptosis (or cell death) relationships were very similar to those from MTS and FACS assays. In summary, our cellular imaging method could serve as a quicker and simpler alternative to MTS (end point assay) and FACS (flow cytometry) to screen potential apoptosis inducers.

Keywords Apoptosis · Cell death · Phosphatidylserine · Annexin V · Staurosporine · Confocal microscopy · MTS assay · Early screening

S. Y. Hwang
Department of Chemical Engineering, Hanyang University, Ansan, Korea

Present Address:
S. Y. Hwang
Hanmi Pharmaceutics Research Center, Giheung, Korea

S. H. Cho
Department of Bionanotechnology, Hanyang University, Ansan, Korea

Present Address:
S. H. Cho
Korean Research Institute of Biosciences and Biotechnology, Daejeon, Korea

B. H. Lee
Department of Biochemistry and Cell Biology School of Medicine, Kyungpook National University, Daegu, Korea

Y.-J. Song
Department of Life Science, Kyungwon University, Seongnam, Korea

E. K. Lee (✉)
College of Bionanotechnology, Kyungwon University, Seongnam, Korea
e-mail: eklee@kyungwon.ac.kr

Introduction

The conventional assays for apoptosis including enzyme-linked immunosorbent assay (ELISA), caspase activity assay, and flow cytometry have some disadvantages, which include time-consuming wash steps for ELISA, an additional cell lysis step required for caspase activity assay, and a delicate sample preparation step for flow cytometry. Another conventional cytotoxicity assay, MTS(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay is a surrogate, end-point assay requiring a long incubation time (48–60 h). A general problem with conventional biochemical assays is that they provide only limited information on either biochemical or morphological parameters [1]. Therefore, developing a cell-based imaging method would be useful for extracting both morphological and biochemical data.

As an early apoptosis marker, annexin V is widely used in flow cytometry and microscopy because of its strong binding affinity for PS [2]. PS is an important phospholipid component of the plasma membrane and a well-known 'biosignature' in the apoptotic pathways. PS becomes exposed to the outer surface of cell membranes very early in the apoptotic process, regardless of cell type or apoptosis inducer [3–5]. Binding of a PS receptor to the exposed PS evokes a macropinocytic response in the phagocyte [6, 7]. Annexin V is a protein, consisting of 320 amino acids and a molecular weight of 36 kD [8], which binds to anionic phospholipid surfaces in a Ca^{2+}-dependent manner [9]. Several studies have used PS translocation to detect apoptosis [10]. In addition, imaging of annexin V has been shown to serve as a suitable biomarker system in evaluating cell death, modifying compounds, and plaque stabilizing strategies [11]. Several conjugates of fluorophore and annexin V have been successfully used for apoptosis monitoring [12–14].

The aim of our study was to quantitatively monitor apoptosis in breast cancer cells (MCF-7) in an in vitro manner by imaging PS translocation to the outer membrane surface. Cellular imaging techniques have been used recently as acceptable tools for the evaluation of cellular cytotoxicity [15]. In this study, a commercial conjugate of fluorescein isothiocyanate (FITC) and annexin V was used to bind to the translocated PS for visualization under confocal microscopy. Staurosporine (SSP), a nonspecific protein kinase inhibitor, was used as a model apoptosis inducer [3, 16]. After exposing the cells to SSP, the FITC fluorescence intensity was analyzed to quantify the fraction of apoptotic cells. To assess the validity of the cellular imaging assay, the assay results were directly compared with the results from MTS and FACS assays.

Materials and methods

Cells and reagents

MCF-7, a human breast cancer cell line, was purchased from ATCC (American Type Culture Collection) (Rockville, MD, USA). Dulbecco's modified Eagles medium (DMEM) was purchased from Invitrogen (Gaithersburg, MD, USA). MCF-7 cells were cultured in 60 cm^2 dishes in the DMEM media containing 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were split every 3–4 days and seeded in a 96-well imaging plate (Greiner Bio-one GmbH, Germany) at 5×10^3 cells/well. FBS, trypsin–EDTA, penicillin and streptomycin were purchased from Gibco (Carlsbad, CA, USA). The kit for apoptosis detection containing both FITC-annexin V and PI was purchased from BD Biosciences (Palo Alto, CA, USA). SSP was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of PS/PC complex lipid membrane

PS (Avanti Polar Lipids, USA) and PC (phosphatidylcholine, Sigma, USA) was each dissolved in chloroform to a 10 mM concentration. The molar ratios of PS/PC were: 0% PS/100% PC, 5% PS/95% PC, 10% PS/90% PC, and 15% PS/85% PC. The dissolved PC and PS were each dispensed into a round-bottom flask to a predetermined molar ratio and further mixed with chloroform. After chloroform was removed by evaporating for 10 min, the film coating inside the rotating flask was hydrated with 1 ml of PBS buffer and sonicated using pulses (10 s ultrasound pulse followed by 20 s resting) for 10 min to detach the coated lipid layer from the flask wall. The hydrated multilamellar vesicular (MLV) liposome solution was passed through a nitrocellulose filter with 0.2 μM pores (Advantec, MFS, CA, USA) and the filtered liposome solution was stored at 4°C before use.

SPR monitoring of annexin V binding to PS on lipid membrane surface

To evaluate the interaction between PS and annexin V, we used a SPR system (BIACORE® 3000, GE Healthcare, USA) equipped with an L1 chip, of which the surface was coated with lipophilic linkers. After cleaning the chip surface three times with 20 mm CHAPS (Sigma-Aldrich, USA) for 1 min each at 10 μl/min, the PS/PC complex liposome vesicles were captured on the chip surface. The volume of injected liposomes was adjusted so that the immobilization level for each molar ratio of PS and PC was nearly the same. To remove loosely bound liposomes from the L1 chip, the surface was washed with 50 mM NaOH for 1 min at 10 μl/min. To block non-specific binding, the chip surface was treated with 0.1% bovine serum albumin (BSA) for 1 min at 10 μl/min. Annexin V (500 nM) diluted in the running buffer (10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4) was injected for 1 min at 5 μl/min. A CHAPS buffer with NaOH was used to clean the L1 chip surface. All analyses were performed at 25°C.

Cellular imaging assay by annexin V-FITC staining and confocal microscopy

PS translocation was previously measured by the specific binding of FITC-conjugated annexin V to externalized PS [17]. Logarithmically growing, ca. 5×10^3 MCF-7 cells were seeded and cultured in a 96-well imaging plate for 24 h at 37°C in humidified 5% CO_2 atmosphere. Various concentrations of SSP (0.01, 0.05, 0.1. 1, 5, 10 μM) were

added to the cells. Low concentrations (0.01, 0.05 μM) were intentionally included to determine whether their effects could be monitored by the imaging assay. Following a 4 h incubation, the cells were washed twice with the binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) and incubated in the binding buffer containing annexin V-FITC for 15 min. To remove unstained annexin V-FITC, the cells were washed with the binding buffer. Fluorescent intensity of annexin V-FITC was measured with a confocal laser scanning microscope (TCS SL, Leica, Germany) equipped with an argon ion laser using a 40× oil lens. The excitation wavelength was 488 nm and the emission wavelength was 500–540 nm for annexin V-FITC. The green FITC intensity associated with the externalized PS was quantified by using TSL SL software supplied by Leica; the ROI (region of interest) map of cell colony was set on the transmitted images to calculate cell dimensions, and then the map was applied to the fluorescent images to determine their fluorescence intensity. Ten ROIs, each containing 10–15 cells, were randomly taken for each well and their signal densities were averaged for reporting. The fluorescence intensity was determined as follows: after a cell edge was determined, the cell interior was scanned by Leica software to obtain 'total fluorescence' and total number of pixels. 'Mean fluorescence intensity' was calculated by dividing the total fluorescence by the total number of pixels. This procedure was done manually for this work, but software to identify each cell boundary, obtain its cross-sectional area and perimeter, and calculate the mean intensity is being developed with our collaborator (CBS Biosciences, Inc., Daejeon, Korea). It is expected the software package for apoptosis monitoring (by PS-annexin V binding) become commercially available by early 2012.

MTS assay

The cytotoxic effect of SSP was measured by MTS assay and its result was compared with that of cell imaging assay, based on the hypothesis that once a cell became apoptotic it would eventually result in cell death and be captured by the MTS assay. SSP was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 1 mM and stored at −20°C. Logarithmically growing MCF-7 cells were seeded at ca. 5×10^3 cells/well into a 96-well plate and allowed to adhere for 24 h at 37°C. Then, the supernatant was replaced by 100 μl of the culture medium, and 25 μl of the SSP solution at various concentrations (0.1, 0.2, 0.5, 1, 2, 5,10 μM) was added to induce apoptosis. Parallel cultures of MCF-7 cells in a medium containing only DMSO were used as negative controls. After 60 h incubation at 37°C in humidified 5% CO_2 atmosphere, 20 μl of cell titer $96^{®}AQ_{ueous}$ one solution reagent (MTS and phenazinemethosulfate (PMS); the electron coupling reagent) (Promega, USA) was added into each well, and the plate was incubated for 2 h. The absorbance at 490 nm was read with a 96-well plate reader (Power Wave XS, BioTek, USA).

Flow cytometry assay

Flow cytometry was performed using the FACS Calibur system (BD Biosciences, USA). Approximately 1×10^6 MCF-7 cells/well were cultured in a 6-well plate for 24 h, and treated with the various concentrations of SSP (0.1, 1, 10 μM). After 4 h the cells were washed twice by PBS. In order to dissociate the adhered cells from the plate surface, 0.05% trypsin–EDTA was used. After removing trypsin–EDTA by centrifugation, the binding buffer was mixed with the cell pellet. 5 μl of both annexin V-FITC and PI were added into the cell solution (approximately 1×10^5 cells/well of 100 μl). PI was used to differentiate necrotic cells against apoptotic cells, since this dye is a nuclear marker of dead or dying cells, unable to penetrate healthy plasma membranes [18]. After 15 min of incubation, stained cell solution was diluted with 400 μl of the binding buffer to the final concentration of approximately 2×10^4 cells/well and fed to the FACS system.

Results and discussion

SPR analysis of specific binding of annexin V to PS

Phospholipids in the cell membrane include phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin, and phosphatidylserine (PS). PS is one of the key regulation factors involved in binding mechanisms to membrane surface. In normal cells, PS exists mainly in the inner leaflet of the membrane bilayer, but as apoptosis starts, it is translocated across the membrane to the outer leaflet. Because of its high affinity to PS, annexin V binds specifically to the cells with exposed PS in the presence of Ca^{2+}. Figure 1 shows a schematic diagram of annexin V binding to the translocated PS.

Liposomes with different compositions of PS were prepared and immobilized on the L1 chip surface, and annexin V binding was monitored by SPR. The mass increase from the liposome immobilization was in the range of 6,000–6,800 RU (Fig. 2a). Treatment with 50 mM NaOH after the immobilization did not cause any significant change in the immobilization level. The unoccupied area of the L1 chip was blocked with 0.1% BSA for 1 min to prevent non-specific binding. The blocking resulted in a mass increase of 9–33 RU.

The binding levels of annexin V increased proportionally with an increase in PS composition (0, 5, 10, 15%)

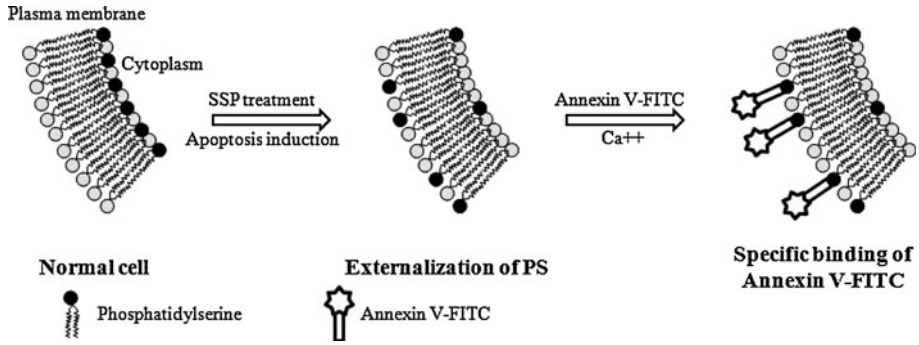

Fig. 1 Schematic illustration of translocation and externalization of phosphatidylserine in apoptosis and its binding to annexin V-FITC conjugate

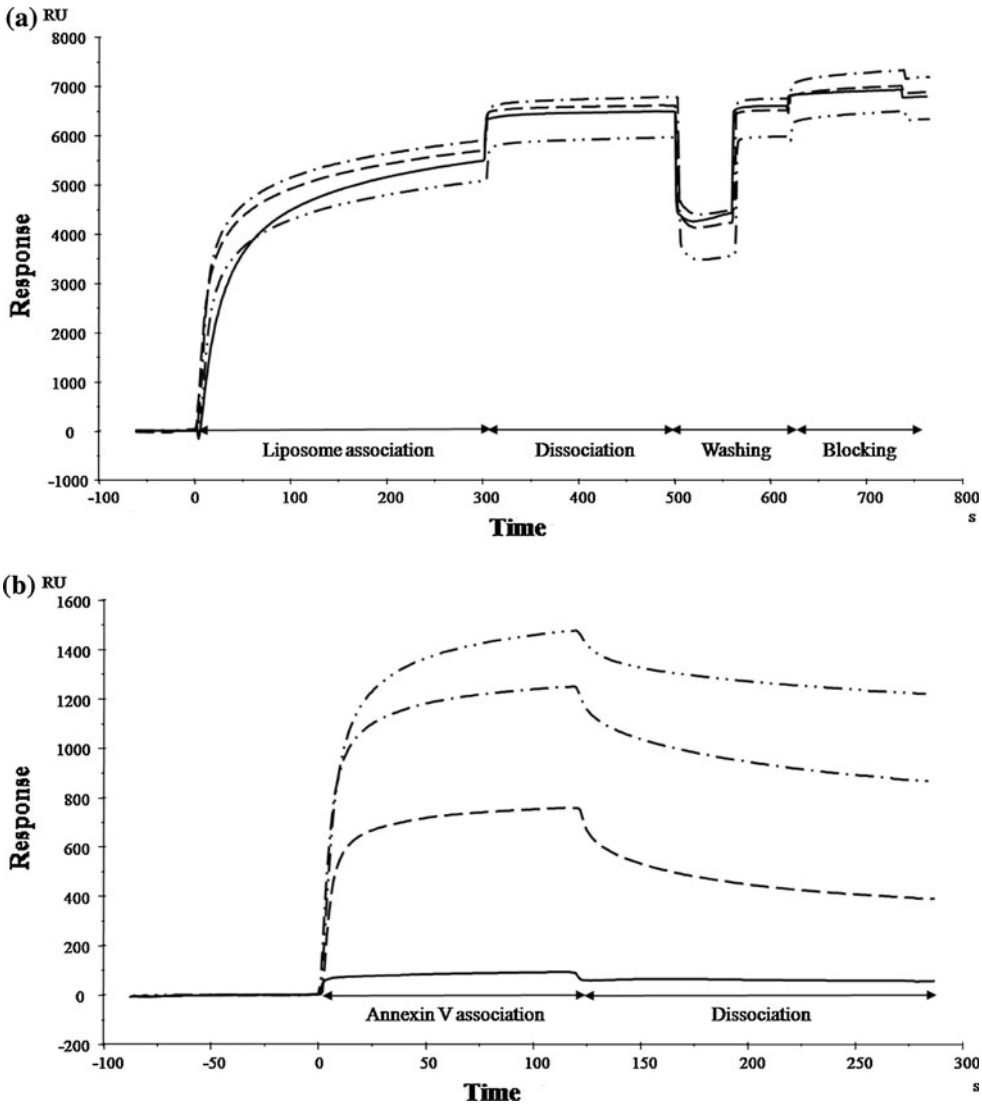

Fig. 2 Sensorgrams of annexin V binding to PS as measured by SPR. **a** Immobilization of several molar ratios of the PS/PC liposome, **b** Annexin V binding on the PS/PC liposome surface, *solid line* 0% PS/100% PC, *long dashed line* 5% PS/95% PC, *dash-dot-dash line* 10% PS/90% PC, and *dash-dot-dot-dash* 15% PS/85% PC

(Table 1; Fig. 2b). The 15% PS composition was determined as the maximal, considering its composition of erythrocyte plasma membrane. This result confirmed that an increase in PS concentration at the membrane surface would indeed increase annexin V binding levels [19]. In addition, it was important to note that the bound annexin V hardly dissociated, which suggested that the binding was strong enough to determine PS flipping by annexin V imaging as previously reported [20].

Cell imaging assay

PS translocation is not an absolutely specific biomarker for apoptosis, since it was reported that in cell senescence and

Table 1 SPR data on binding levels of annexin V to phospholipids complexes containing various PS compositions

Composition of lipids complex	PC (mg/ml)	PS (mg/ml)	Immobilized on chip (RU)	Annexin V binding (RU)	Mass ratio (AV/LIP%)
0% PS/100% PC	7.28	0.000	6597	61	0.92
5% PS/95% PC	6.92	0.039	6475	575	8.87
10% PS/90% PC	6.55	0.079	6737	1092	16.2
15% PS/85% PC	6.19	0.118	5979	1369	22.9

antigen-activated immune cells the PS was exposed for non-apoptotic events and the exposed PS could flip back inside if there was no persistent antigen stimulus. However, PS translocation/exposure is still widely accepted as an early apoptosis biomarker. Figure 3 shows one example of raw data on cell images. Figure 3a–d and e–h show the transmission and fluorescence images, respectively. Note that the transmission images were overlapped with the fluorescence images and the ROIs were marked by colored lines. As a negative control, MCF-7 cells were incubated in medium containing only 1% DMSO for 4 h. Very little FITC fluorescence was observed from the negative control cells by confocal microscopy (Fig. 3e). After 4 h of SSP treatment (0.1, 1, 10 μM), green fluorescence from the annexin V-FITC conjugate could be observed under confocal microscopy (Fig. 3f–h). The fluorescence image files for each SSP concentration were scanned to obtain the mean fluorescence intensity. Figure 4 showed a change in the average signal intensity of 5 repetitions with respect to SSP concentration. Between 0.01 and 0.05 μM SSP concentration, the fluorescence intensity was little changed. However, for 1, 5, and 10 μM, its increases were distinct and significant, indicating that the apoptosis was more clearly observed for 1 μM and above. This observation was in agreement with a previous report [21], in which significant levels of activated caspase-3 were observed in apoptotic MCF-7 cells treated with SSP higher than 1 μM.

Since this imaging assay required only 4 h incubation time, it could predict the cytotoxicity (leading to cell death) much earlier without having to wait for 60 h for complete death in the MTS assay. By this, early detection/screening of the efficacy of an apoptosis inducer could be possible. Further, this assay can be easily automated by using a 96 (or 384) well plate format on an X–Y staging platform, as found in a high contents screening (HCS) instrument. If needed, the confocal microscope can be replaced with a more economical non-laser microscope (e.g., light emitting diode). Although not pursued in this work, once the transmission images were converted into digital files, we could obtain the cross-sectional area and perimeter of each cell to observe morphological changes for cell shrinkage and membrane blebbing quantitatively. The quantitative data on morphology changes may help reinforce the analysis from the fluorescence imaging.

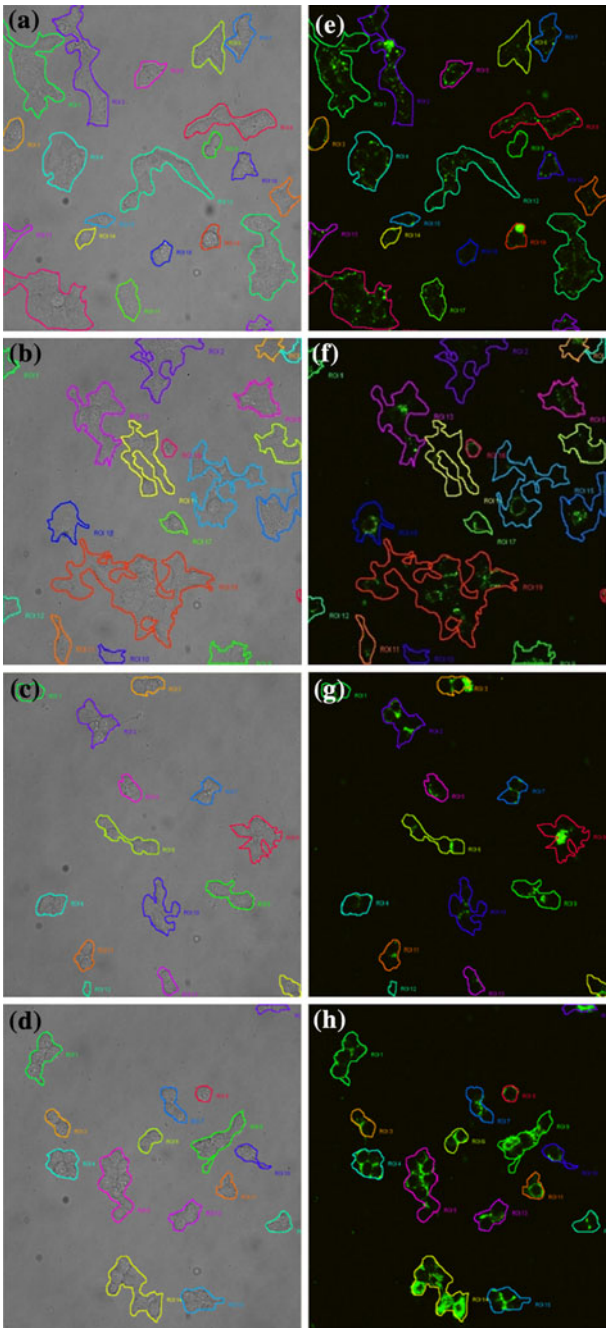

Fig. 3 Transmission (**a**–**d**) and fluorescent images (**e**–**h**) for the effect of SSP concentration **a**, **e** 0 μM; **b**, **f** 0.1 μM; **c**, **g** 1 μM; **d**, **h** 10 μM

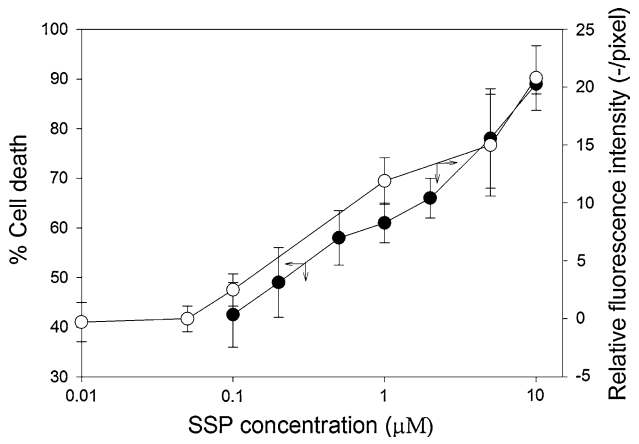

Fig. 4 Comparison of MTS assay and cellular imaging analysis for dose dependency on cell death. MTS assay *dark circle*: after 60 h of SSP incubation ($n = 6$); Imaging assay *white circle*: after 4 h of SSP incubation ($n = 5$)

Comparison of cell imaging assay with MTS assay

To assess the validity of the cellular imaging assay, MTS assay was performed for direct comparison with the cell imaging assay. Recently, MTS assay is being replaced with a simpler and more HTS-compatible CellTiterGlo assay; however, the more traditional MTS assay is still widely used in many toxicology labs. Although MTS assay would capture not only the apoptotic mechanism but also any other forms of cell death (including necrosis) or inhibition of mitochondrial metabolism, the side-by-side comparison was meaningful for two reasons: (1) SSP is known to specifically induce apoptosis, and (2) once a cell became apoptotic after the first 4 h exposure to SSP, it would result in cell death and be eventually captured by the MTS assay. For the first 4 h of incubation, the absorbance in the MTS assay did not change regardless of the SSP concentration (data not shown), which suggested that the MTS assay was not suitable as an early detector. The absorbance data after 60 h incubation were converted into '% cell death', and Fig. 4 shows that the % cell death (mean values of 6 repetitions) increased with SSP concentration.

The major potential advantage of the cell imaging assay over MTS assay would be the former's ability for early detection. From an operational viewpoint, the cell imaging assay could be advantageous in the following aspects: time- and cost-saving (MTS reagent usage could be avoided); high throughput and multiplexing capability (by using a well plate format and an X–Y staging platform), and user friendliness (if image analysis software is used). To fully evaluate the assay accuracy and reliability, comprehensive assay validation data as well as direct performance comparison data need to be generated later.

Comparison of cell imaging assay with FACS result

As another comparison, we performed a flow cytometry experiment of dual staining with both annexin V-FITC and PI. Cells that were not exposed to SSP were also stained with annexin V-FITC as a negative control, because it could bind non-specifically to the indigenous PS located on the outer membrane surface. Figure 5a shows the FACS data, in which the cells were sorted into 4 groups: (1) 'healthy' cells [denoted as FITC(−), PI(−)], (2) apoptotic cells [denoted as FITC(+), PI(−)], (3) necrotic cells (denoted as FITC(−), PI(+)), and (4) completely dead cells (denoted as FITC(+), PI(+)). As SSP concentration increased, the count of annexin V-FITC stained cells, i.e., apoptotic cells (denoted as FITC(+), PI(−) in Fig. 5), also increased. The cells stained with PI, which were the necrotic cells (denoted as FITC(−), PI(+) in Fig. 5), occupied much smaller proportions (about 3–4% of the total) than the apoptotic cells at all SSP concentrations. This was possibly because SSP is known to specifically induce apoptosis. It was interesting to note that at even

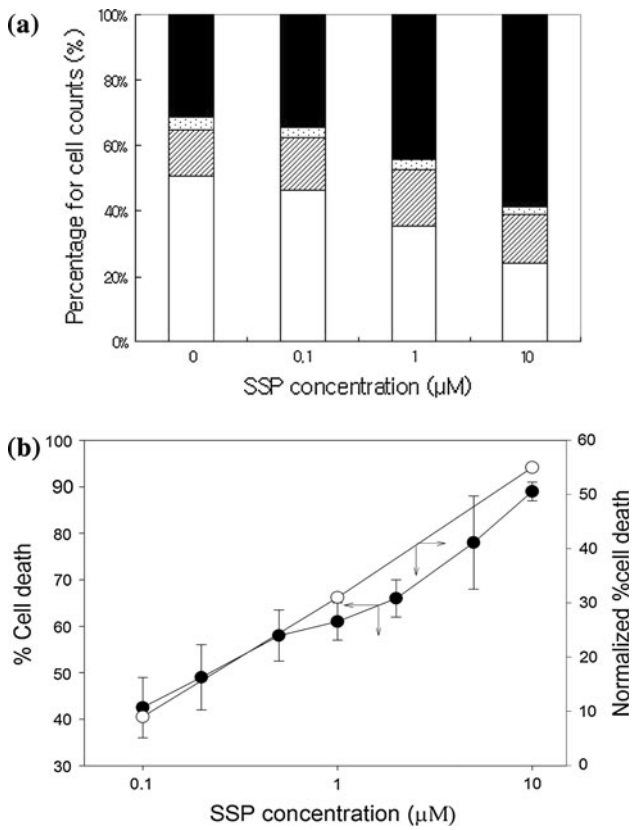

Fig. 5 **a** Flow cytometry assay using annexin V-FITC and PI. *White square*: annexin V-FITC(−), PI(−), *square with upper right to lower left fill*: annexin V-FITC (+), PI(−), *doted square*: annexin V-FITC(−), PI(+), *black square*: annexin V-FITC(+), PI(+). **b** FACS anaylsis of the effect of SSP concentration on cell death

10 μM SSP, nearly 20% of the cell population remained 'healthy' (denoted as FITC(−), PI(−) in Fig. 5). The dose-cell death relationship, which is the effect of SSP concentration on cell death, was determined from the FACS data, and was very similar to that of the cell imaging assay (see Figs. 4, 5b). This comparison result strongly supported the validity of the cell imaging assay. From an operational perspective, the cell imaging assay was considered more convenient and simpler, because in the FACS experiment, not only were the sample preparation steps tedious but the cells adhered to a well plate needed to be dissociated from the plate surface and suspended in solution for flow sorting. This step would not be required in the cell imaging assay.

Conclusions

We propose a cellular imaging assay monitoring PS translocation as an alternative to MTS and FACS assays for early screening of potential apoptosis inducers. From the SPR experiment in which PC-based lipid bilayers containing different PS compositions were immobilized, we first confirmed that annexin V binding was specific and proportional to PS concentration. When human breast cancer cells (MCF-7) were exposed to 0–10 μM of SSP, the cellular imaging assay showed an proportional increase in fluorescence intensity of annexin-FITC with respect to SSP concentration. The imaging assay result on the SSP dose dependency on cellular apoptosis was very comparable to that of the MTS assay on cell death. It was also nearly identical with the FACS data using dual staining. The potential advantage of the cell imaging assay would include time- and cost-saving, high throughput and multiplexing capability, and user friendliness. The assay accuracy and reliability need to be validated later. In conclusion, the imaging method can serve as a simpler alternative to MTS and FACS for early screening of potential apoptosis inducers.

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Competing interest The authors declare no competing interests.

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