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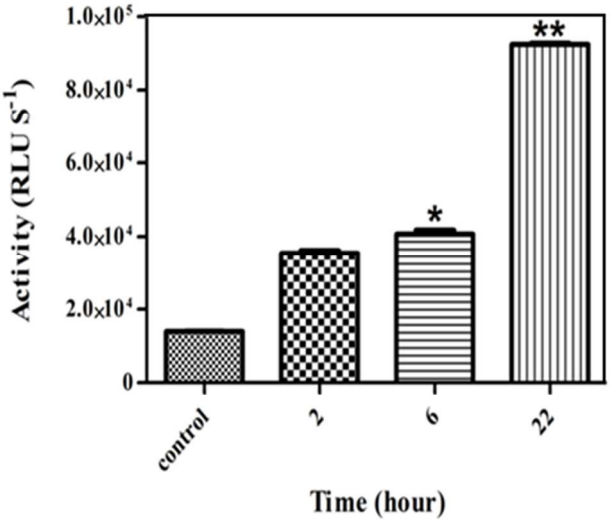
The luminometric assay based on RLuc/Annexin V, which is called “adherent state apoptosis assay (ASA)”, is a new, rapid and reliable method to functional assay apoptosis in adherent cells.



Adherent State Apoptosis Assay (ASA): a Fast and Reliable Method to Detect Apoptosis in Adherent Cells

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Abstract

Apoptosis is a tightly controlled biochemical process for cell death. Although the induction of apoptosis is an important mechanism for screening of many valuable products such as drugs, due to the false signals that usually cause during isolation of cells from the surface of the culture plate, techniques have limitation to measure apoptosis in adherent cells. In this study, we investigated the use of RLuc/Annexin V, a probe which obtained by fusion Renilla luciferase (RLuc) with annexin V and bound to phosphatidylserine (PS) on the surface of suspended apoptotic cells, as a potentially luminescent probe to assay apoptosis in adherent cells such as Chinese Hamster Ovary (CHO) cells. The probe was overexpressed in *Escherichia coli* BL21 (DE3) and purified by immobilized metal ion chromatography. The probe assayed for detection of apoptosis in CHO cells. The results showed that Rluc/Annexin V binds to the CHO cells with no additional treatment for cell suspension, and the signal of RLuc can be detected by luminometer. The new assay based on the Rluc/Annexin V is named adherent state apoptosis assay (ASA). It may be a new avenue for studying apoptosis in adherent cells in a rapid, reliable, non-invasive method.

Keywords: Apoptosis, Adherent cells, Rluc/Annexin V, CHO cells.

INTRODUCTION

Apoptosis, or programmed cell death, is a well-documented phenomenon in many cellular systems [1]. It plays a key role in tissue and organ development during embryogenesis as well as in regulating cell turnover in adult tissues. Besides the biomedical importance, the apoptosis assay is important in evaluation of the toxicity of some valuable bio-products such as drugs. Over the past decade, several methods have been reported to detect the apoptosis [2, 3]. Although apoptosis was originally defined based on morphology, the conventional methods used to identify apoptotic cells are based on the measurement of a single parameter associated with biochemical or molecular changes that are characteristic of apoptosis. Among current techniques and protocols, the sensitive and quantitative methods such as the flow cytometric and luminometric analysis are fascinating methods and usually work based on the binding ability of annexin V.

Annexin V (Mr 36-kDa), a member of the annexin family of calcium-dependent phospholipid binding proteins, has an affinity to phosphatidylserine (PS)-containing lipid bilayers [4, 5]. When PS is exposed on the extracellular face of a cell membrane, annexin V binds with a high affinity to the phosphatidylserine with the aim of Ca^{2+} [4]. Both, fluorochrome conjugates of annexin V and recombinant annexin V fused with photoproteins were used to monitor changes in cell membrane phospholipid asymmetry [6, 7], and thereby providing a useful tool for detection of apoptotic cells.

Although the detection of apoptosis by fluorochrome conjugates of annexin V in flow cytometry is a sensitive, rapid and reproducible method, the use of this method for adherent cells is associated with some difficulties. Studies show that when the annexin V-based probes are to be used for the study of apoptosis in adherent cells, then a problem is encountered, in that specific membrane damage occurs during harvesting [8]. Therefore, assays using many of the commercial kits and studies are based on suspension cells.

Recently, the luciferase-based methods have been reported to detect apoptosis [9]. The split luciferase was used for the assay of apoptosis by Torkzadeh *et al*, and interestingly the apoptotic signal increased up to 150 times after apoptosis induction [9]. Although this is an interesting and real-time technique to detect apoptosis using luciferase, the time-consuming and complicated processes involved in making cell reporters are the limiting factors for using split luciferases in apoptosis assay. Recently, moreover, a new reporter based on the luciferase to detect apoptosis has been reported by fusion annexin V and renilla luciferase (RLuc) [10]. The new reporter, Rluc/Annexin V, can detect “eat me” signal on the surface of

apoptotic cells. Although the ability of new reporter to detect apoptotic signal in both neutrophils and suspended cells was shown, the accuracy of Rluc/Annexin V to detect apoptosis in adherent cells has been questioned.

In this study, we have introduced a new assay based on Rluc/Annexin V probe that overcomes the technical difficulties that occurred with the assay of apoptosis in CHO cells as an adherent cell model. Unlike many protocols and methods such as that was reported previously for RLuc/Annexin V [10], the new assay is specified for detection of apoptosis in adherent cells using the bioluminescent probe and a plate-reader luminometer. Furthermore, unlike the flow cytometric assays that use resuspended cells, the assay does not require the isolation of cells from plate and the challenging step for cell resuspension is omitted. Finally, the new method, which is called the "adherent state apoptosis assay (ASA)", is a rapid and non-invasive method for assaying apoptosis in adherent cells and may be a new avenue for studying the effects of drugs on apoptosis in adherent cells in a high throughput manner.

MATERIAL AND METHODS

Preparation of RLuc/Annexin V

Preparation of RLuc/Annexin V was carried out on a Ni-Sepharose column and quantitated by the Bradford assay as described previously [10, 11]. Briefly, *E. coli* BL21 (DE3) cells, incubated until reaching an OD 600 of 0.9, were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated an additional 12 h at 22□°C. The cell extract was achieved by sonication, clarified by centrifuge (12000 rpm for 20 min), and was purified on the Ni-Sepharose column according to manufacturer's protocol (Qiagen).

RLuc/Annexin V Western Blot

Samples were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) 10% in the presence of β-mercaptoethanol and electro-blotted to nitrocellulose paper according to standard procedures [12, 13]. Blots were blocked with skim milk 5% overnight and incubated with a mouse monoclonal anti-His_6-peroxidase antibody (Roche). Bands containing renilla luciferase, Annexin and RLuc/Annexin V probe were visualized by chemiluminescence (Amersham ECL kit) and exposure of x-ray film for 60 s.

Cell Culture and Induction of Apoptosis

Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 mg/L penicillin, and 100 mg/L streptomycin [14]. Cultures were incubated at 37 °C in 5% CO2. For induction of apoptosis, the cells were trypsinized with 0.05% trypsin-EDTA solution for 5 min at 25 °C and used for cell counting. Then, cells were seeded in 12-well plates at concentration of 1×10^5 cells per well. Apoptosis was induced in confluent cells by various concentrations of actinomycin D (0.5, 1, 10 and 50 μM dissolved in H2O) for 2, 6 and 22 h.

Cell Viability Determinations, Dye Exclusion, and XTT Assays

The viability of actinomycin D- treated CHO cells was determined by trypan blue stain exclusion [15]. Cells were trypsinized, harvested, suspended in DMEM medium, and transferred to centrifuge tubes. The cells were washed and resuspended in PBS. The trypan blue stain was added to the cell suspension, and the cells were counted in a hemocytometer. The number of blue cells and white cells were counted until a minimum of 200. The cell viability, moreover, was determined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay [16]. Briefly, CHO cells were grown to confluence in 96-well plates. Cells were treated with actinomycin D (0.5, 1, 10 and 50 μM) and grown at 37 °C, 5% CO2 for 2, 6 and 22 h. To determine the percentage of surviving, metabolically active cells in each well, 50 μl of XTT and N-methyl dibenzopyrazine methyl sulphate (PMS) were added to each well to a final concentration of 1 mg ml^{-1} and 0.32 mg ml^{-1} respectively and incubated at 37 °C, 5% CO2 for an additional 3 h. The number of cells actively converting the dye to the formazan metabolite was determined in triplicate using an ELISA plate reader (Thermo, USA) measuring absorbance at 492 nm. The IC50 was determined using GraphPad Prism 5.1 software.

Semi-Quantitative Analysis of Apoptosis by DAPI Staining

Semi-quantitative analysis of apoptosis was carried out using 4',6-diamidino-2-phenylindole (DAPI) staining. CHOs treated with 10 μM of actinomycin D for the various indicated time intervals (2, 6 and 22 h) were stained with DAPI for 5 min at room temperature and then examined under a fluorescence microscope (Olympus IX71 Microscope and DP71 Camera Instructions). Apoptosis was defined as nuclear condensation, and the results were expressed as the percent of apoptotic cell number to total number of nuclei/field.

Quantitative Analysis of Apoptosis by Flow Cytometry

CHO cells were cultured in DMEM culture medium supplemented with 10% at 37 °C and 5% CO2 until cell adhesion. Actinomycin D (10 µM) was added to wells and 22 h after treatment, adherent cells were harvested to make single cell suspension fixed with 70% ethanol, overnight at 4 °C. The suspension was washed with PBS and incubated for 30 min after addition of RNaseA (100 µg). The cells were stained with propidium iodide (PI) and were analyzed by flow cytometer PAS (Particle Analysing System, Münster, Germany) and FlowJo 7.6.1 software. The percentage of apoptotic cells was evaluated by determining the sub G0/G1 DNA content. The procedure was repeated three times.

Assay for caspase-3 activity. CHO cells were grown in DMEM and treated with the actinomycin D (10 µM). Both treated and untreated Cells were collected after 12 h by scraping and spun down at 500 × g for 5 min. then, cells were lysed and the activity of caspase-3 were assayed using Caspase Colorimetric Protease assay Kit (Invitrogen) according to the manufacturer's instructions. Briefly, fifty microliters of the reaction buffer (containing DTT) was added to the cytosol extracts (2 mg/mL) of both induced and uninduced cells. Five microliters of the caspase-3 substrate conjugate was then added to each sample followed by incubation of the reaction mixtures at 37°C for 2h and the absorbance was measured at 405 nm.

Adherent State Apoptosis Assay (ASA)

CHO cells were cultured in 96-well plates (white plates for luminescence with maximum reflection and minimal auto-luminescence) and the induction of apoptosis was carried out by adding 10 µM of actinomycin D. After 0, 2, 6 and 22 hours of induction, the medium of cells were collected and the adherent cells washed three times with annexin V binding buffer (10 mM HEPES NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) without any additional trypsinization treatment to suspend the cells. Then adherent cells, which were bound to the 96-well plates, incubated at 25 °C for 30 min with 50 µl annexin V-binding buffer containing 20 µg ml^{-1} of RLuc/Annexin V. Subsequently, the incubation medium was collected and the adherent cells were washed with binding buffer. The cell-associated luminescence was measured by adding the coelenterazine (1 mM) in a luminometer (Perkin Elmer/Wallac Victor 2 multi-label counter). The data were analyzed by non-linear regression using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Statistical Analysis and Data Mining

The data were presented as mean-SD. Analysis of variance was carried out with GraphPad Prism software (version 5.1). A P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

In this paper, we have evaluated the ability of RLuc/Annexin V for assay of apoptosis in CHO cells and we have optimized the procedure that allows a rapid assay of apoptosis. The assay, moreover, is called adherent state apoptosis assay (ASA).

Production of RLuc/Annexin V

A vector contenting the RLuc/Annexin V was expressed under the control of T7 promoter in *E. coli* BL21 (DE3). The signal of RLuc/Annexin V was detectable within 2 h after induction of protein expression in *E. coli*. Moreover, the signal intensity reached a plateau after 12 h of induction. The purification of recombinant RLuc/Annexin V was performed by affinity (Ni-NTA Sepharose) chromatography. The purified probe had purities more than 95 % on the basis of the analysis by SDS-PAGE in which RLuc/Annexin V was present as a band of 72 kDa (Fig 1A). Moreover, the result from SDS-PAGE in support of the result from the western blot that confirms the Rluc and annexin V construct produce probe of the correct size (Fig. 1B). The purified probe RLuc/Annexin was used for further assay development.

Microscopic and Biochemical Analysis of Apoptotic Cells

The ability of actinomycin D to induce apoptosis in different cell lines has been reported [17, 18]. To determine whether actinomycin D can induce the apoptosis in CHO cell line, cells were treated with four different concentrations of actinomycin D (0.5, 1, 10 and 50 μM) for 2, 6, and 22 h and their proliferation was assessed by XTT assay (Fig 1B). Furthermore, cells were investigated under a microscope for morphological variations of apoptosis such as cells shrinkage, nuclear segmentation and chromatin condensation (Fig 1 C and D).

The actinomycin D 50% inhibitory concentration (IC50) value for CHO cell line was observed to be 10 μM for a 22 h treatment. The results, moreover, showed that after treatment with actinomycin D at the 10 μM for 22 h, the majority of cells sustained apoptosis and showed typical apoptotic features such as densely compacted nuclei as shown in Fig. 1D.

Semi-Quantitative Analysis of Apoptosis

A semi-quantitative analysis of apoptosis was done using DAPI. Cells were treated with the actinomycin D (10 µM), and then processed for DAPI staining up to 22 h post-induction. Apoptosis was measured in the induced and uninduced cells by determining the percentage of fluorescent-positive cells undergoing nuclear blebbing, DNA fragmentation, and condensation [19-21]. As seen in Fig 2, actinomycin D induces an obvious apoptosis in CHO cells, compared with the untreated control. The DAPI staining assay is consistent with the changes in the shape and morphology of the CHO cells after induction of apoptosis. Moreover, the results from DAPI staining are consistent with the microscopic and biochemical analysis that show actinomycin D at concentration of 10 µM for 22 h can trigger a significant and detectable apoptosis in CHO cells.

Quantitative Analysis of Apoptosis by Flow Cytometry

The preliminary studies using XTT and DAPI indicated that actinomycin D induce apoptosis in CHO cell. The further evaluation was carried out using flow cytometry. The redistribution of cell cycle phases were studied after the treatment with actinomycin D (10 µM) for 22 h. The proportion of the cells in the sub-G0/G1 increased significantly in CHO cells after the treatment with actinomycin D when compared with control. Therefore, the cells with a sub-G0/G1 DNA content, a characteristic feature of apoptosis, were seen at 22 h in CHO cells following exposure to actinomycin D (Fig 3).

Measurement of Caspase-3 Activity

As a hallmark of apoptosis, we performed caspase-3 assay in actinomycin D -induced CHO cells. As shown in Fig 4 B, a statistically significant increment at the caspase-3 activity in induced cells was recorded when compared with control (Fig 4A). When the cells were treated with the actinomycin D, the absorbance at 405 nm reached to 0.4 which is about forty times more than the uninduced CHO cells. The result from caspase activity indicates that caspase-3 activation plays a role in actinomycin D -induced apoptosis at CHO cells. These data, moreover, are consistent with the results obtained with microscopic, DAPI staining and sub-G0/G1 studies.

Adherent State Apoptosis Assay (ASA) in CHO Cells

The biochemical and cell biology assays including DAPI staining, caspase-3 assay and sub-G0/G1 studies showed that apoptosis occurred in CHO cell lines using actinomycin D. CHO cells were inoculated into 96-well plates (white plates for luminescence with maximum reflection and minimal autoluminescence). Apoptosis was initiated by incubating the confluent cells with actinomycin D (10 µM) for 2, 6 and 22 h and the apoptotic adherent cells

were labelled for PS externalization using Renilla/Annexin V. The adherent cells washed with Ca^{2+} containing buffer to remove the excess probe, and the functionality of RLuc/Annexin V, which was bound to the apoptotic cells, was assayed by adding coelenterazine hcp in a luminometer (Perkin Elmer/Wallac Victor 2 multi-label counter). The results from the statistical analysis showed that there was a statistically significant difference between the experimental data (P-value <0.05). Furthermore, as shown in Fig. 5, the signal of RLuc/Annexin V increased with treatment time and increased up to 5 times after 22 h in CHO cells when compared with control. The results show that RLuc/Annexin V can bind to the apoptotic adherent CHO cells with no additional treatment to suspend the cells.

The assay of apoptosis has been an interesting component of many biological studies. Therefore lots of methods and protocols have been presented so far for developing the assay of apoptosis. Among them, methods based on flow cytometry are quantitative and the caspase activity and 96-well-based ethidium bromide and acridine orange (EB/AO) staining are reported to be one step procedures. Due to the complexity for preparation of suspended cells, the measurement of apoptosis in adherent cells using flow cytometry is combined with the difficulties [8]. The assay might damage the cell membranes and change the cell population distribution of live, apoptotic and/or necrotic cells [22]. Moreover, the staining and the assaying processes require multi-step and time-consuming procedures such as overnight incubation at 4 °C or the treatment with RNaseA. The apoptosis assay based on the activity of caspase is a powerful method. However, this procedure is end-point assay which lead to loss of the sample. Ribble *et al* described a modified method based on EB/AO staining to assay the apoptosis in both suspension and adherent cells. The assay is high-throughput and optimized for the 96-well plates [22]. Although it is a fascinating method and can overcome some of the difficulties associated with the assay of apoptosis in adherent cells, the visual microscopic analysis might limit its application as a rapid method for high-throughput screening (HTS) and increase the rate of errors. Nevertheless, the adherent state apoptosis assay (ASA) and modified EB/AO staining assay may use as complementary techniques for apoptosis assay in adherent cells. Finally, the luminometric assay based on RLuc/Annexin V, which takes less than an hour to carry out, is a new, rapid and reliable method to functional assay apoptosis in adherent cells.

Conclusion

Apoptosis is an important form of cell death and is the mechanism of action of many toxins such as some drugs used in treating cancers. Several commercial probes can detect apoptosis based on the externalization of a plasma membrane phospholipid, phosphatidylserine (PS), during the apoptosis and the specific affinity of annexin V for binding to PS [6,7]. Although these probes are useful for suspended cells, it has found that isolation of cells from the surface of the plate often cause false signals especially when they assayed by flow cytometry. Just recently a new probe, RLuc/Annexin V, has been reported for detection of apoptosis and its potential to assay suspended cells was shown [10]. In this paper, we evaluate the ability of RLuc/Annexin V to assay apoptosis in adherent cells. Therefore, along with the other commercial probes and protocols, RLuc/Annexin V is a probe for detection of apoptosis not only for suspended cells but also for adherent cells. The new assay is named adherent state apoptosis assay (ASA). The assay may have potential for further application in high throughput screening of toxicity of new drugs, and it may be a new avenue for studying the effects of drugs and bioproducts on apoptosis in adherent cells.

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FIGURE LEGENDS

Figure 1A. The purified RLuc/Annexin V on the SDS-PAGE. The purification was done using immobilized metal ion chromatography. Lane M is the molecular marker. **1B)** Immune-blotting of purified renilla luciferase, annexin V and RLuc/Annexin V probe. **1C**, the effect of actinomycin D on the viability of CHO cells. CHO cell line was treated with actinomycin D at different concentrations and time intervals and their proliferation was assessed by XTT assay. Data are the average results of three independent experiments. The analysis demonstrate that the inhibition concentration (IC50) of actinomycin D after 22 hour treatment is about 10 μM. **1D and E**, Apoptosis induction by actinomycin D in CHO cells. Microscope analysis of control cells (**1D**) and cells treated with actinomycin D (10 μM) for 22 h (**1 E**).

Figure 2. Actinomycin D-induced apoptosis in CHOs. CHOs were exposed to actinomycin D (10 μM) for the indicated time periods (0, 2, 6 and 22h). Apoptosis was evaluated by DAPI staining. The morphological changes of nuclear chromatin in apoptotic cells were monitored by fluorescent microscopy (A). Results are presented as a semiquantitative estimate based on the percent of apoptotic cell number to total number of nuclei/field (B).

Figure 3. Flow-cytometric analysis of sub-G0/G1 DNA content in control (A) and actinomycin D -treated CHO cells (B). The cells were treated with actinomycin D (10 mM) for 22 h, stained with propidium iodide and assayed by flow cytometry. The distribution of sub-G0/G1 phase is expressed as % of total cells.

Figure 4. Caspase-3 activity in control and apoptotic CHO cells. A, uninduced CHO cells. B, The assay indicates CHO cells that were treated with the actinomycin D for 12 h. After a 12-h induction of apoptosis in presence of actinomycin D, the absorbance at 405 m increased significantly.

Figure 5. Activity of RLuc/Annexin V after induction of apoptosis in CHOs cell line, values represent the mean of three independent experiments. In all cases, measurements were normalized using renilla luciferase activity. Mean and standard deviation (SD) are shown for one representative out of three independent replica experiments. All strains were tested a minimum of three times in triplicate samples. For further details, see Methods.