THE DEVELOPMENT OF THE EFFICIENT CELLULAR AND BIOCHEMICAL ASSAY SYSTEM OF APOPTOSIS REGULATING CHEMICALS

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Abstract

Programmed cell death is genetic and biochemical process caused by specific chemicals and stress stimulation like UV-irradiation. Using Hela cell line, we have developed efficient assay system for determining apoptotic (and anti-apoptotic) properties of a number of chemical substances. Cell death inducing activities are assessed by measuring cell viability (WST-8) and cytotoxicity (LDH) using 96-well plates. Further characterization of cell death inducing chemicals was performed by one dish-based Annexin-V and DNA ladder formation assay. Additionally, activation of the cell death executing caspase-3 was detected by western blot analysis using the protein extracted from the same cell population as Annexin-V and DNA ladder formation assay. As for the initial test chemicals, we used staurosporine, TRAIL+IFNγ, tunicamycin, and brefeldin-A. External apoptotic stimuli (staurosporine) and death receptor-mediated inducer (TRAIL+IFNγ) showed rapid apoptosis induction 4-8 hr after treatment in Annexin-V, DNA ladder formation, and caspase-3 assay. In contrast, ER stress inducers (tunicamycin, brefeldin-A) showed rather slow and modest apoptosis induction activities in all the three assays. Cell nuclear and mitochondrial morphological investigation further supports the distinct properties of these acute and modest apoptosis inducers. Taken together, the apoptosis assay system established here would be strong tools to evaluate a number of chemicals including dioxins.

Introduction

Apoptosis is active programmed process leading the living cell to its own death[1]. A number of chemicals and stress induce apoptosis. Novel chemicals having pro-apoptotic or anti-apoptotic activities will be useful chemical probe to analyze mechanism of apoptosis. Out regulation of apoptosis is causing cancer, neuronal disorders as well as other diseases, and creating pharmaceutical drugs using these new chemicals as lead compounds would be also expected. Three pathways, mitochondrial pathway (intrinsic), death receptor pathway (extrinsic), and endoplasmic reticulum (ER) stress pathway of apoptosis are known[2]. We have developed efficient assay system to detect and determine apoptotic activities of chemical at both cellular and biochemical levels.

Materials and Methods

Reagents

Staurosporine, thapsigargin, tunicamycin, brefeldin-A and 7-Aminoactinomycin (7-AAD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human TRAIL and IFN-γ were from PeproTech Inc. (New Jersey, USA). All the other chemicals used were of analytical grade.

Cell Culture

Human epithelial cervical carcinoma Hela cells were cultured in E-MEM supplemented with 1% Non Essential Amino Acid, 10% heat-inactivated fetal bovine serum (Equitech-Bio Inc., Texas, USA), and penicillin (100units/ml) and streptomycin (100mg/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability and Cytotoxicity Assay

Hela cells were seeded onto 96-well plates at a density of 1 x 10^4 cells/ cm^2. After 24 hr-incubation, the cells were exposed to the indicated concentrations of the apoptosis-inducing reagents for 48 hr. The half volume of the supernatants was used to measure the cytotoxicity by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, WI, USA). WST-8 assay was performed in the residual medium containing the cells using Cell Count Reagent SF (Nacalai tesque Inc., Kyoto, Japan). The experimental results were calculated as described at the manufacture's instrument and expressed as means of triplicates ± standard deviation (SD)

FACS Analysis

Apoptosis was determined by externalization of phosphatidylserine on the outer membrane and 7-AAD exclusion. After treatment with the reagents for the indicated time, 5 x 10^5 cells were collected and washed with PBS (-). The cells were suspended in Incubation Buffer [10 mM Hepes/ NaOH (pH7.4), 140 mM NaCl and 5 mM $CaCl_2$] and stained with Annexin-V-Fluos (Roche Diagnostics GmbH. Penzberg, Germany) and 7-AAD. Then, the cells were subjected to FACS analysis using FACSCalibur (BD Biosciences, San Diego, CA, USA). The data were analyzed using the CellQuest program.

Immunofluorescence microscopy

Hela cells were transfected with pDsRed2-Mito or pDsRed2-ER plasmid by lipofection using Fugene™6 Transfection Reagent according to the manufacture's protocol and incubated for 24 hr. Then, the cells were treated with the indicated reagents. After fixation with 4% PFA, the cells were labeled with Hoechst 33258. Nuclear condensation and the morphological changes were observed under a confocal laser scanning fluorescence microscope LSM 510 (Carl Zeiss, Jena, Germany).

DNA Ladder Detection

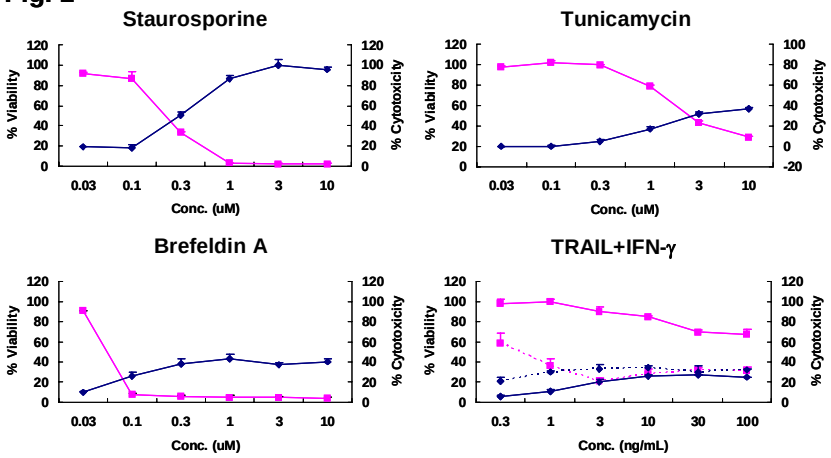
1 x 10^6 apoptotic cells were lysed in lysis buffer containing 10 mM Tris-HCl (pH7.4), 10 mM EGTA and 0.5% triton-X 100. After digestion with RNase A and Proteinase K, DNA fragments were extracted with isopropanol precipitation. The DNA obtained was electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Immunoblot Analysis

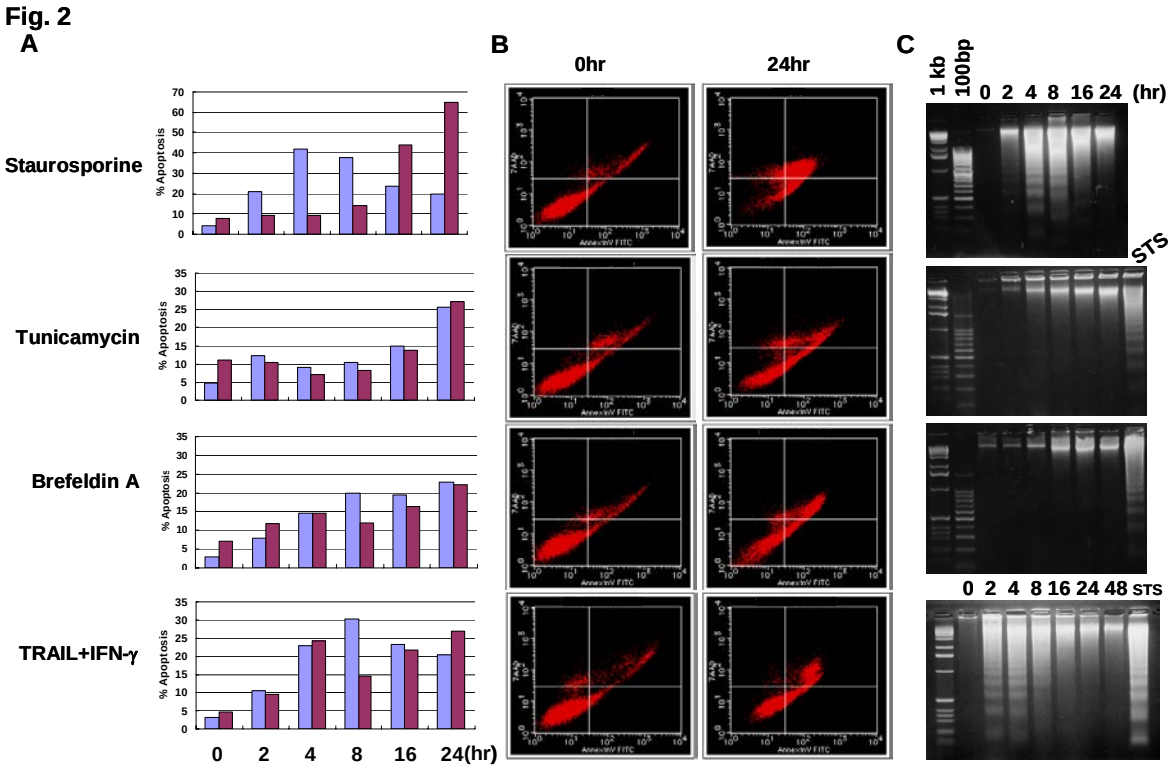
Cells were lysed in homogenate buffer [50 mM Tris-HCl (pH7.4), 250 mM Sucrose, 10 mM EGTA, 2 mM EDTA, 20 μg/ml leupeptin, 1 mM PMSF, 0.1% triton-X 100, 1 x Complete Mini (Roche)] by sonication. Protein content was measured by Protein Assay Lowry Kit (Nacalai Tesque). 40 μg of total proteins were subjected to SDS-polyacrylamide gel electrophoresis (15%) and then immunobloted using antibodies against active caspase-3 (CHEMICON, Temecula, CA, USA) and actin (SANTA CRUZ, Santa Cruz, CA, USA).

Results and Discussion

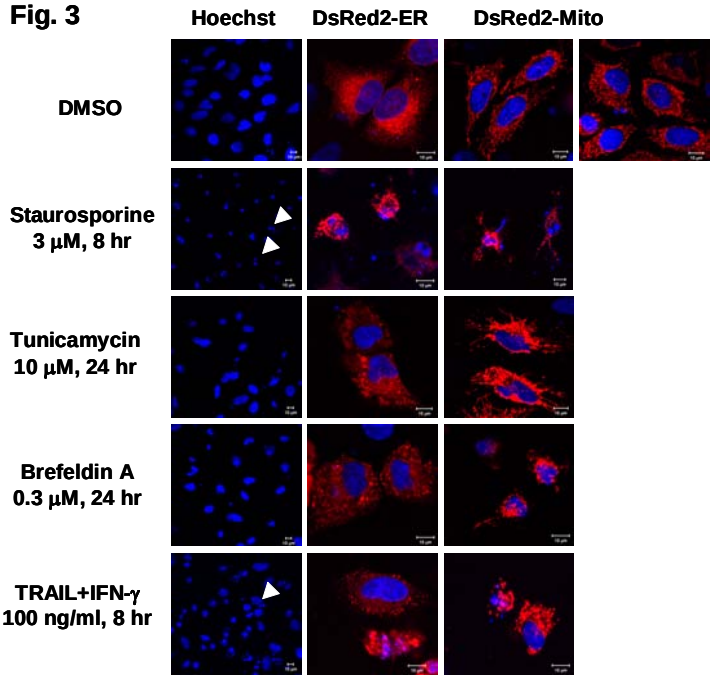
Fig. 1



To characterize in detail responses of Hela cells to various apoptosis-inducing drugs, we utilized inducers via three independent apoptotic pathways; mitochondrial apoptotic pathway triggered by external stimuli (staurosporine), death receptor-mediated pathway (TRAIL+IFNγ) and ER stress-induced pathway (tunicamycin, brefeldin-A). First, we analyzed dose-responses of Hela cells to the various drugs (Fig. 1). Cell viability (Pink line) was determined by measuring the reduction of 2-(2-methoxy-4-nitrophenyl) -3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8). Cytotoxicity (Blue line) was determined by measuring the activity of lactate dehydrogenase (LDH) released upon cell lysis into the medium. Co-incubation with 200 U/ml of IFNγ (dot line) enhanced cell death induced by TRAIL itself (solid line).



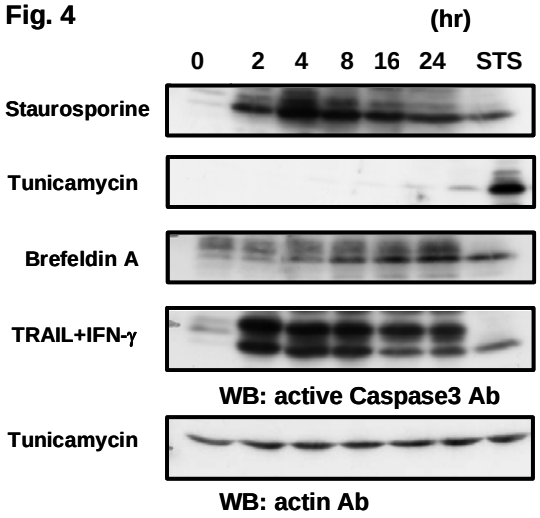
To distinguish between necrosis- and apoptosis-mediated cell deaths, we performed FACS analysis using Annexin-V conjugated with FITC and 7-AAD (Fig. 2, A and B). The drugs were used at concentration which showed maximum cytotoxicity in Fig.1. Hela cells treated for the indicated times were collected and counted. Then, 5 x 10^5 cells were subjected to FACS analysis, and 1 x 10^6 cells were used for DNA ladder detection (Fig. 2C). The same amounts of protein from the residual cells were applied for immunoblot analysis using anti-active caspase-3 antibody (Fig. 4). Thus, these data was generated from the same dish. Moreover, we used 3 μM staurosporine as a positive control in all of the experiments for reliable and comparable data. Staurosporine and TRAIL triggered rapid apoptosis, demonstrated by Annexin-V positive and 7-AAD negative population (blue bar in Fig. 2A). Early apoptosis population was reached to the maximum 4-8 hr after treatment, and then Annexin-V and 7-AAD double positive-cell death population (red bar in Fig. 2A) was more increased. On the other hand, ER stress induced slow apoptosis. Interestingly, staurosporine and TRAIL, which induced rapid apoptosis, showed DNA ladder formation, but not ER stress inducers.



Next, we observed morphological changes of

nucleus, ER and mitochondria in the apoptotic Hela cells. In DMSO-treated control cells, nuclear stained with Hoechst dye was round, and both fused and fragmented mitochondria were observed (Fig. 3). Staurosporine or TRAIL treatment induced nuclear condensation and fragmentation as the arrow heads show, while ER stress made nuclear shrink slightly. This indicated that the nucleosomal DNA ladder could be isolated only from the morphologically fragmented nucleus in apoptotic cells. Structure of ER and mitochondria in staurosporine or TRAIL-treated cells were severely destructed and fragmented, which were more damaged than ER stress inducers. Because exposure time of ER stress inducers was upto 24 hr in Fig.2 and 3, we then examined if the nucleosomal DNA ladder is not really induced in ER stress even after 48 hr-treatment. As a result, 48 hr-exposure fragmented the DNAs into units of single or multiple nucleosomes (Data not shown). This suggested that ER stress induced slowly-proceeded apoptosis.

Fig. 4



To examine caspase-3 activity in the apoptotic cells, we next performed immunoblot analysis using anti-active caspase-3 specific antibody (Fig. 4). Caspase-3 is synthesized as an inactive proenzyme (32 kDa) that is processed in cells undergoing apoptosis. The processed form consists of large (17 kDa) and small subunits (12 kDa) which associate to form an active enzyme. This antibody recognizes only the cleaved large fragment as shown in STS lanes. In control cells, bands of active caspase-3 were hardly detected in each treatment. Staurosporine and TRAIL triggered rapid production of active caspase-3 fragment and reached to the maximum at 4-8 hr. Notably, the precursor fragments of active caspase-3 (19 kDa) were also detected in TRAIL treatment. On the other hand, the cleaved form increased gradually and to less extent in ER stress-induced apoptosis. Continued incubation upto 48 hr with ER stress inducers showed further increase of cleaved caspase-3 (Data not shown). The equal amounts of proteins were applied as shown in the low panel (Only tunicamycin exposure was shown as a typical result).

In our system, total cell death was assayed by 96-well plate based WST-8 and LDH release methods. To further analyze the cell death mechanism of the chemicals, we established one dish-based apoptosis assays for detail characterization of Hela cells exposed to the various apoptosis inducers. This approach should provide us comparable and reliable data even among the multiple assays. Although all the chemicals used in the assessment of the assay system was apoptotic, anti-apoptotic activities would be easily detected by co-treatment of the test chemicals and the apoptosis inducers.

Interestingly, dioxins seems to have both anti-apoptotic and apoptotic properties. In rat hepatocytes treated with UV-irradiation, TCDD showed anti-apoptotic activities by hyperphosphorylating p53[3]. In recent publications, TCDD act as anti-apoptotic agents in a normal human mammary cell line[4], however, the chemical induces apoptosis in mouse T cells[5]. We are planning to screen dioxins and dioxin-related chemicals using the apoptosis assay system described here.

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