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Over-expression of the X-linked inhibitor of apoptosis protein (XIAP) delays serum deprivation-induced apoptosis in CHO-K1 cells

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Serum deprivation inhibits cell growth and initiates apoptosis cell death in mammalian cell cultures. Since apoptosis is a genetically controlled cell death pathway, over-expression of anti-apoptotic proteins may provide a way to delay apoptosis. This study investigated the ability of the X-linked inhibitor of apoptosis protein (XIAP) to inhibit apoptosis induced by serum deprivation. Study includes evaluation of the ability of XIAP to prolong culture period and its effect on cell proliferation in serum-deprived media. The full length human XIAP was introduced into CHO-K1 cell lines and the effects of XIAP over-expression on the inhibition of apoptosis induced by serum-deprived conditions were examined. In batch cultures, cells over-expressing XIAP showed decreased levels of apoptosis and a higher number of viable cell under serum-deprived conditions compared to the control cell lines. The viability of control cells dropped to 40% after 2 days of serum deprivation, the XIAP expressing cells still maintained at a viability higher than 90%. Further investigation revealed that the caspase-3 activity of the CHO-K1 cell line was inhibited as a result of XIAP expression.

[**Key words:** XIAP; CHO-K1; Serum deprivation; Apoptosis; Mammalian cell culture; Flow cytometry]

Over the past decades, mammalian cells have been widely used to produce complex proteins for both therapeutic and diagnostic use. Chinese hamster ovary (CHO) cells, one of the commercially popular cell lines, are the choice for the mass production of various recombinant proteins. In general, cells face the risk of encountering numerous insults present in a bioreactor, such as mechanical agitation, nutrient depletion, hydrodynamic stress, toxin accumulation, hypoxia and viral infection (1). Upon these stresses, cells tend to undergo apoptosis cell death. Therefore, much effort has been expended to improve process efficiency via metabolic engineering, metabolic pathway analysis and optimization of the scale-up cultures (2–7).

Mammalian cell culture requires growth factors and hormone that present in the serum to enhance cell growth and to retain its cellular functions (8). However, the use of serum has several disadvantages, including high cost, lot-to-lot variations, contamination of infectious agents, such as virus and prions. Also, the presence of various protein components in the serum complicates the downstream purification process, whereby the removal of serum proteins from secreted bioproducts can be laborious, time-consuming and costly. Hence, for effective downstream processing and purification of cellular products, works on development of a serum-free culture that can be utilized for effective industrial-scale production of biopharmaceuticals are being explored continuously and extensively.

Serum withdrawal not only inhibits cell growth, but also triggers apoptosis cell death. Apoptotic cell death causes premature termination of cell culture duration, particularly in high cell density culture (9). Studies have shown that serum deprivation induces apoptosis in a wide range of cell lines, including hybridomas, myelomas, CHO, and BHK cells (10–13). Much attention is now being devoted to strategies for controlling and limiting cell death, either through manipulation of media supplementation or alteration of intracellular biochemistry using genetic engineering (14,15). Researchers have found that the introduction of mitogenic factors, such as insulin, transferin and insulin-like growth factor, can prolong the viability of cells cultured in serum-free media. The addition of basic fibroblast growth factor and insulin synergistically promoted the growth of CHO cells under serum-free conditions (16). There have also been other attempts to reduce apoptosis in mammalian cell-culture systems by utilizing amino acid mixtures or chemical apoptosis inhibitors such as N-acetylcysteine (NAC), bonkrekic acid, z-VAD.fluoromethly ketone (FMK), or 7-amino-4-trifluoromethyl coumarin (AFC) (17).

Studies on anti-apoptotic proteins can suppress apoptosis effectively, thereby increasing the production of the bioproducts. Upregulation of anti-apoptosis engineering using anti-apoptotic proteins

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such as bcl-2 and bcl-xL had shown to improve the productivity of recombinant proteins by suppressing apoptotic cell death (11–13,18). Apoptosis is a programmed cell death mode where caspases are the key effector of apoptosis (19). Hence, inhibition of caspases activity is one of the effective methods to inhibit apoptosis cascade. The inhibitor of apoptosis protein (IAP) is a family of anti-apoptotic proteins that regulate apoptosis, where XIAP (X-linked IAP) is the star player of IAP family. XIAP inhibits the caspase activity through direct binding of its baculoviral IAP repeat (BIR) domains to caspases (20). It has been evaluated as one of the key regulators of apoptosis, not only that it is the most potent inhibitor of apoptosis, it is also the best-characterized member of IAP family. Originally, the function of XIAP was assigned to inhibit apoptosis, but it was then discovered to be involved in a number of diverse cellular processes, including cell cycling, ubiquitylation and receptor-mediated signaling (21), making it a multi-functional protein.

Previously, Sauerwald et al. (22) reported that the over-expression of a XIAP variant, which retains the BIR domain but without the C-terminal RING domain, provided protection to CHO cells exposed to Sindbis virus, etoposide and spent medium. It was then found that this XIAP variant provides a consistent enhancement in the viable cell density in lactate supplemented cultures (23). While Kim et al. (24) reported that the over-expression of XIAP could not inhibit the sodium butyrate-induced apoptosis effectively in CHO cells, Kaufmann's group reported that co-expression of both XBP-1 and XIAP genes resulted in a higher titer of IgG in a serum-free medium (25). To date, there is no attempt to study the effect of XIAP over-expression on delaying the cell death and cell proliferation under serum deprived condition. In this study, we focused on the effect of the full length XIAP on the suppression of the cell proliferation and apoptosis under serum-deprived conditions.

MATERIALS AND METHODS

Cell line and cell maintenance The CHO-K1 cells were routinely cultured in Ham's F12 medium supplemented with 2 mM L-glutamine (Gibco, USA) and 10% FBS (Gibco, USA) and further cultured in serum-deprived medium. For MCF-7 cell line, cells were maintained in Dulbecco's modified Eagle's media containing 4.5 gm/l glucose (Sigma-Aldrich, UK) and 10% FBS (Gibco, USA). All cell lines were incubated at 37 °C in a humidified chamber at a fixed setting of 5% CO_2.

Transfection The pcDNA3-myc-XIAP mammalian expression vector was kindly provided by Dr. Takeo Namuro (Department of Urology, Oita Medical University, Japan). Construction of the plasmid, pcDNA3-myc-XIAP was described previously. The vector contains a 1.5 kb human XIAP coding region and resistance genes for ampicillin and neomycin. The myc tag was used for the detection of the XIAP protein expression (26). Transfection was conducted in a 6-well plate using the GeneJuice Transfection Reagent (Novagen, USA), as recommended by the manufacturer. Transfected cells were selected in 800 μg/ml G418 sulfate (Gibco, USA) selection medium for 2 weeks. Cell cloning by limiting dilution was conducted to select stable transfectant clones.

MTT assay During screening of potential clones, relative cell viability was assessed by adding 20 μl of 0.5 g/l MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide, a tetrazole] (Sigma, USA) solution to each well. After 4 hour of incubation, 100 μl of DMSO was added to the wells and further incubated for 30 min. Absorbance at 570 nm was measured by μ-Quant ELISA microplate reader (Bio-tek Instruments, USA).

Analysis of XIAP expression Potential clones were propagated in 6-well culture plates and harvested when the cell density reached 90% confluent. Harvested cells were fixed with 100 μl Cytofix/Cytoperm Fixation/Permeabilization solution (BD, USA) and incubated on ice for 20 min. One μl of 150 μg/ml anti-XIAP antibody (BD, USA) was added and incubated on ice for 30 min. Cells were washed after incubation and 1 μl of 0.5 mg/ml FITC-conjugated goat anti-mouse antibody (BD, USA) was added and incubated on ice for 20 min in dark. XIAP-FITC fluorescence was measured by the FACS Calibur System (BD, USA), while XIAP expression was analyzed using the Cell Quest Software.

Cell viability analysis Batch cultures were sacrificed at 24 h intervals and cell viability was determined by using the trypan blue exclusion method. Cell suspensions were mixed with 0.4% trypan blue solution (Sigma, USA) at a 1:1 dilution. Dead and viable cells were identified and the percentage was calculated. Cell density was measured using a haemacytometer slide and cell viability was determined by dividing the number of viable cells by the total number of cells.

Fluorescent microscopic analysis of apoptosis Apoptosis/necrosis count was determined using acridine orange (AO)/propidium iodide (PI) dual stain fluorescent microscopy. Cells were stained with 10 μl of AO/PI dual dye solution (15 μg/ml AO, 15 μg/ml PI) (Sigma, USA) for 2 min. Approximately 10 μl of stained cells were loaded onto a glass slide and observed under an inverted fluorescence microscope (Nikon, Japan) using a 10x objective lens. Cell images were captured using the ESI-Element software. The cell population was classified into 4 categories: viable (green cells with diffused chromatin), early apoptotic (green cells with condensed chromatin), late apoptotic (red cells with condensed chromatin) and necrotic (red cells with non-condensed chromatin) (12).

Measurement of Caspase-3 Activity Cell lysates were prepared from 6-well culture plates. In brief, caspase-3 activity in the extract of approximately 1×10^6 cells was measured using the Caspase-3/CPP32 colorimetric assay kit (Biovision, USA). The caspase substrate DEVD-*p*NA (4 mM, 5 μl) was added to the samples and incubated at 37 °C for 2 hr. The enzyme-catalyzed release of *p*NA was quantified at 400 nm using the μ-Quant ELISA microplate reader (Bio-tek Instruments, USA).

Cell cycle analysis Harvested cells were centrifuged and pellets were resuspended thoroughly in remnant. Cells were then fixed with 500 μl of ice-cold 70% (v/v) ethanol and stored at -20 °C until analysis (at least 2 hours). Fixed cells were washed twice with ice-cold PBS washing buffer (2% BSA, 0.1% Azide-EDTA), resuspended in 1 ml of PBS staining buffer (0.1% Triton X-EDTA) containing 100 mg/ml RNase A, and incubated for 30 min. Cells were then stained with 200 μl of 50 μg/ml PI solution (Sigma, USA) in dark for 15 min. DNA fluorescence was measured by using the FACS Calibur System (BD, USA), while sub populations of DNA distribution histograms were analyzed using the Cell Quest Software. Cell debris and aggregates were excluded by appropriate gating.

RESULTS

Selection of stable XIAP expression clones The effect of XIAP over-expression on the suppression of apoptosis was investigated by transfecting the CHO-K1 cells with the pcDNA-myc-XIAP plasmid. Stable transfectants were selected in G418 selection medium and limiting dilution was performed to select high XIAP expresser clones. Further selection was conducted by exposing 30 isolated clones in medium without serum supplementation for 3 days. Ten most potential clones were then evaluated by MTT assay and the results are shown in Table 1. Three clones (Clones 3, 5 and 25) showing the highest viability were selected for the evaluation of XIAP expression.

Flow cytometric analysis using anti-XIAP antibody conjugated to FITC was performed to confirm the XIAP expression in the selected clones. The evidence of the over-expression of XIAP in transfected population of CHO-K1 cells is shown in fluorescence histogram profiles (Fig. 1). This analysis gives a clear evidence that the selected clones (Clones 3, 5 and 25) (Fig. 1B-D) displayed higher levels of XIAP protein compared to the negative control (parental CHO-K1) (Fig. 1A). MCF-7 breast cancer cell was used as the positive control (Fig. 1E) in this analysis. An increase in fluorescence intensity was observed and the whole cell population of CHO-K1-XIAP cells was shifted to the right compared to the negative control. Stable CHO-K1-XIAP clone (Clone 5) displaying the highest expression level of XIAP was selected for further characterization. Approximately 84% of the cell population in clone 5 (Fig. 1C) was found to be expressing the XIAP stably after being passaged continuously in batch culture for 3 months.

XIAP expression enhances survivability of CHO-K1 cells Both CHO-K1-XIAP and the control (parental CHO-K1) cell line were plated

TABLE 1. Relative viability of the potential clones expressing XIAP.

Clone	Relative Cell Viability (% of control)
1	3.4
2	38.3
3	141.3
5	119.3
7	95.9
11	91.1
12	56.7
15	57.1
20	97.1
25	98.2

After the clones were exposed in serum-deprived media for 3 days, the viability was measured using MTT assay. The relative cell viability (% of control) represents the percentage absorbance relative of the pcDNA-myc-XIAP transfected clones to the parental cells.