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Using Bcl-xL anti-apoptotic protein for altering target cell apoptosis

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Abstract

Background: Altering target cell apoptosis is one of the challenging ideas of biotechnological applications. There are several applications of over expressing Bcl-xL anti-apoptotic protein from recombinant protein production to DNA vaccination strategies. The aim of the present study is to evaluate the anti-apoptotic efficacy of Bcl-xL expressing dual promoter plasmid system as a candidate to be used for recombinant protein production and DNA vaccination approaches. For this purpose, Bcl-xL anti-apoptotic protein gene was inserted in a dual expressing vector system in frame with EGFP (enhanced green fluorescence protein) after IRES (internal ribosomal site). The plasmid has a multiple cloning site after CMV (cytomegalovirus promoter) left empty to be inserted a biopharmaceutical protein gene region or DNA vaccine antigens. **Results:** In order to determine the anti-apoptotic efficacy of Bcl-xL inserted dual expressing vector, BHK-21 cells were transfected both with this plasmid and empty vector as control. Apoptosis was stimulated by several apoptosis inducing agents and serum deprivation in the transfected cells for 48 hrs. Cells expressing Bcl-xL protein in frame with EGFP were determined by flow cytometry as an indicator of cell viability. Additionally, apoptosis were determined by intracellular cleaved Casp 3 staining in Bcl-xL expressing EGFP positive cells. The dual expression plasmid bearing Bcl-xL anti-apoptotic protein prolonged the cell survival rate and protected cells from apoptosis upon apoptosis induction by doxorubicin and camptothecin in which the anti-apoptotic efficacies are inhibited through over expressing of Bcl-xL. pIRES2EGFP/Bcl-xL transfected cell ratio was significantly higher compared to empty vector transfected cells ($P < 0.001$). In contrast, apoptotic cell ratio was significantly lower in pIRES2EGFP/Bcl-xL transfected cell population compared to empty vector transfected cells ($P < 0.001$). **Conclusion:** In conclusion, it was shown that *in vitro* transient expression of Bcl-xL efficiently inhibited apoptosis induced by serum deprivation, doxorubicin and camptothecin. Thus, the dual expression plasmid bearing Bcl-xL anti-apoptotic protein could be a good candidate for recombinant protein production and DNA vaccination applications.

Keywords: animal cell biotechnology, apoptosis, Bcl-xL anti-apoptotic protein

INTRODUCTION

Altering target cell apoptosis is one of the challenging ideas of biotechnological applications. Over expression of anti-apoptotic proteins in biopharmaceutical protein production using animal cells as host increases the yields (Al-Rubeai and Singh, 1998; Kim and Lee, 2000; Kim et al. 2009; Han et al. 2011; Kim et al. 2011) as well as enhances the host immune response elicited against the vaccine antigen in DNA vaccination (Kim et al. 2004; Kim et al. 2005; Huang et al. 2007). In addition, Bcl-xL over expression efficiently inhibits neuronal cell death (Blömer et al. 1998), protects cardiac tissue from ischemia (Huang et al. 2003; Huang et al. 2005), inhibits dendritic cell death in melanoma (Kim et al. 2005) and also inhibits lymphocyte cell death in SARS (Yang et al. 2005). In contrast, most of the

cancer therapeutics must induce apoptosis to kill the cancer cells which become resistant to apoptosis through Bcl-xL over expression (Brunelle and Zhang, 2010). Another application of using Bcl-xL anti-apoptotic protein is the expressing membrane associated proteins fused with Bcl-xL can be purified easily and cost effectively (Thai et al. 2005).

During biopharmaceutical production due to the oxygen, nutrient limitations, exposure to shear stress, using serum free medium and viral infections can result in apoptosis induction (Al-Rubeai and Singh, 1998; Mastrangelo et al. 2000; Figueroa et al. 2004). To increase the productivity of the recombinant protein, apoptosis is suppressed by anti apoptotic proteins like Bcl-2 and Bcl-xL (Al-Rubeai and Singh, 1998; Mastrangelo et al. 2000) in Chinese hamster ovary (CHO) and Baby hamster kidney (BHK) cells which are commercially valuable cells and mostly used as host for recombinant protein production (Andersen and Krummen, 2002; Figueroa et al. 2003; Figueroa et al. 2004; Kim et al. 2009). Over expression of Bcl-2 and Bcl-xL results in similar protection in both CHO and BHK cells under various stress conditions like Sindbis virus infection, serum and glucose deprivation and ammonia toxicity (Figueroa et al. 2003).

The consequences of Bcl-xL over expression have been widely studied from different aspects. A recent study has demonstrated that Bcl-xL over expression not only inhibits apoptosis but also regulates cell proliferation and survival (Baik and Lee, 2009). Another anti-apoptotic protein Aven, acts in combination with Bcl-xL to enhance the function of Bcl-xL and shows protection against Sindbis virus infection (Figueroa et al. 2004) through blocking DNA damage-induced apoptosis by stabilizing Bcl-xL (Kutuk et al. 2010), XIAP (X-linked inhibitor of apoptosis protein) delays serum deprived apoptosis in CHO cells (Liew et al. 2010).

Apart from apoptosis, autophagy can also occur through nutrient deprivation (Zustiak et al. 2008; Kim et al. 2009). Bcl-xL over expression inhibits both apoptosis and autophagy in erythropoietin producing CHO cell culture (Kim et al. 2009). Also, the over expression of Bcl-xL delays autophagy and apoptosis in hyper osmotic CHO cell cultures resulting from NaCl addition in both batch culture and nutrient supplementation in a fed-batch cultures and also increased erythropoietin production (Han et al. 2011). It has been shown that Bcl-xL over expression both inhibits dimethyl sulfoxide (DMSO) induced and Na-butirat induced apoptosis (Kim and Lee, 2000; Kim et al. 2011) which are used to increase the specific productivity of foreign proteins in mammalian cells.

The aim of the present study is to determine the anti-apoptotic efficacy of the Bcl-xL expressing dual promoter plasmid designed to be used in biotechnological applications like recombinant protein production through transient gene expression and DNA vaccine applications for long-term specific antigen expression after in vivo transfection against several infectious agents or several types of cancer. The over expressing of Bcl-xL using CHO cells enhanced the transient gene expression levels of a therapeutic human fusion protein (Majors et al. 2008); long term antigen expression in DNA vaccine transfected cells expressing Bcl-xL increased the life span of the DNA vaccine transfected cells and antigen specific $CD8^+$ T cell responses (Kim et al. 2004; Kim et al. 2005; Huang et al. 2007).

For this purpose, apoptosis were induced by doxorubicin, camptothecin and by serum deprivation in pIRES2EGFP-Bcl-xL plasmid transfected BHK-21 cells. Apoptosis can be induced by different ways due to intrinsic and extrinsic pathways, as well as several chemical agents. In most of the studies apoptosis was induced by serum deprivation (Lindenboim et al. 1995; Akiyama et al. 2002). Doxorubicin is an intercalating agent and camptothecin is a topoisomerase I inhibitor (Kaufmann and Earnshaw, 2000) used to induce apoptosis in the treatments against several types of cancer including breast and colon in which the efficacies are inhibited through over expressing of Bcl-xL anti apoptotic protein (Fiebig et al. 2006; Park et al. 2007; Brunelle and Zhang, 2010).

MATERIALS AND METHODS

Constructing Bcl-xL bearing dual expression plasmids

A dual promoter pIRES2EGFP (Clontech, USA) vector was used for the plasmid construction.

Briefly, Bcl-xL anti-apoptotic protein gene (GeneBank accession no: AAC53459.1) was amplified from the total RNA of B6 MC57 mice kidney by RT-PCR with the following primers, 5'-

CCACAACCATGGTGTCTCAGAGCAACCGGGAGC-3' (33 nt, forward primer, contains Bst-XI restriction site), 5'-CCATGGTTGTGGCCTTCCGACTGAAGAGTGAGCCC-3' (35 nt, reverse primer, contains Bst-XI restriction site). Thereafter, Bcl-xL gene was inserted into pGEMT-Easy (Promega, USA) vector system according to the manufacturer's protocol. Thereafter, pIRES2EGFP and pGEMT/Bcl-xL vectors were then digested with Bst-XI and digested Bcl-xL was inserted into pIRES2EGFP to obtain pIRES2EGFP/Bcl-xL in frame with EGFP. The schematic presentation of the Bcl-xL bearing dual expression vector is shown on Figure 1. The orientation and the sequence of inserts and final DNA vaccine were determined by automatic sequencing using the ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and the BioEdit genetic analyzer.

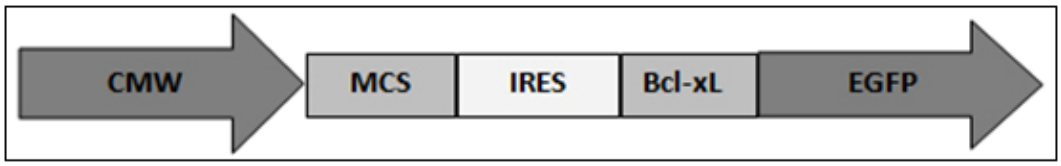

Fig. 1 Schematic presentation of the Bcl-xL bearing dual expression plasmid. CMV, immediate early *promoter* of *cytomegalovirus*; MCS, molecular cloning site for inserting recombinant protein gene regions or DNA vaccine gene regions; IRES, internal ribosomal entry site; Bcl-xL antiapoptotic protein expressed in frame with enhanced green fluorescent protein, EGFP.

Determining IC_{50} values of doxorubicin and campothesine

BHK-21 cells were maintained in Minimum Essential Medium (MEM) (Gibco, USA) supplemented with 10% FBS (HyClone, USA) 2 mM L-glutamine (Gibco, USA), 1 x penicillin-streptomycine solution (100 U/mL penicillin, 100 µg/mL streptomycine, Sigma, USA), 0.1 mM non-essential aminoacids (Gibco, USA), 1 mM sodium pyruvate (Gibco, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37ºC.

To determine the IC_{50} values of apoptosis inducing agents several concentrations of doxorubicin (ranging from 0.25 µg/mL to 10 µg/mL) and campothesine (ranging from 12.5 ng/mL to 200 ng/mL) were used. BHK-21 cells were seeded on 96 well plates at a concentration of 6 x 10^4 cells/mL after overnight incubation and treated with different concentrations of doxorubicin and campothesine for 48 hrs. Cell viability and IC_{50} values were determined by MTT assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product. After discarding the exposure medium, 0.5 mg/mL of MTT was added to each well and incubated at 37ºC, and 5% CO_2 for 4 hrs. After that, 200 µL dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan salts. The absorbance was immediately determined at 570 nm using a single beam UV-visible spectrophotometer (Molecular Devices, USA).

Bcl-xL expression upon transient transfection

BHK-21 cells (1 x 10^4 cell/mL) were incubated in 24 well tissue culture plates overnight prior to *in vitro* transfection experiments to reach approximately 80-90% confluency at the time of transfection. Cells were transfected using lipofectamine-2000 (Invitrogen, USA) following the manufacturer's protocol with 0.5 µg DNA/2 µL lipofectamine 2000 (Invitrogen, USA) complex in serum free Opti-MEM (Invitrogen, USA) medium with empty vector (PIRES2EGFP) and Bcl-xL bearing plasmid (pIRES2EGFP-Bcl-xL) for 4 hrs. Subsequently, Opti-MEM containing DNA-lipofectamine complex were discarded and MEM cell maintenance medium described above were added. After the transfection, cells were incubated for 17-20 hrs prior to treatment with IC_{50} of apoptosis inducing agents for a following 48 hrs.

Measurement of apoptosis after treatment with apoptosis inducing agents

Upon 48 hrs treatment with apoptosis inducing agents the cells expressing EGFP, transfected with empty vector (PIRES2EGFP) and Bcl-xL bearing plasmid (pIRES2EGFP-Bcl-xL) were determined by flow cytometry. Apoptotic cells were also measured by intracellular staining of cleaved caspase-3 by flow cytometry in the EGFP expressing transfected cells. Briefly, cells were fixed and permeabilized by

Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences, USA). Cells were stained with anti-cleaved Casp3 antibody (Cell Signaling, USA) and alexa flour 647 R-phycoerythrin labelled IgG secondary antibody was used for flow cytometric analysis. For each group 10000 cells were analyzed by CellQuest analyzing programme using BD FACS Aria flow cytometer (USA).

Statistical analysis

All assays were repeated at least three times to ensure reproducibility and three replicates of each group were performed in each test. The significance of difference between apoptosis inducing agents transfected with pIRES2EGFP and pIRES2EGFP/Bcl-xL plasmid were analyzed by one way analysis of variance (ANOVA) followed by Benferroni's post-hoc comparison by Prism 5.0 (Graphpad, USA).

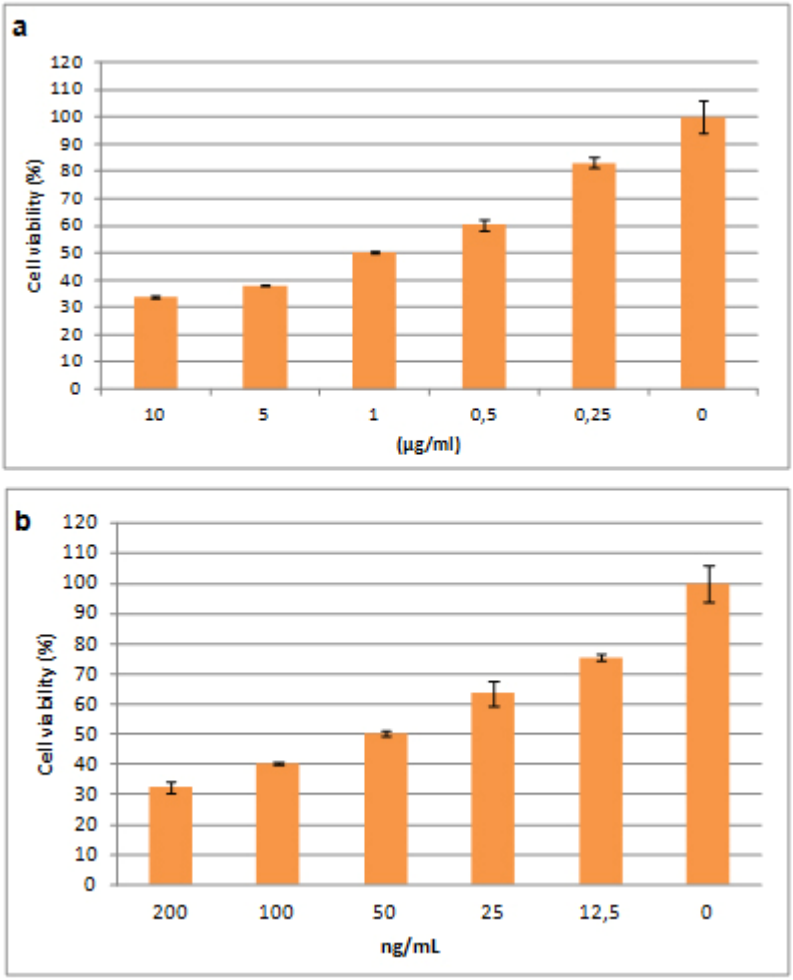

Fig. 2 (a) IC_{50} value of doxorubicin on BHK-21 cells is 1 µg/mL. (b) IC_{50} value of camptothecin on BHK-21 cells is 50 ng/mL.

RESULTS AND DISCUSSION

The dual expression plasmid bearing Bcl-xL anti-apoptotic protein was expected to prolong the cell survival rate and protect cells from apoptosis. To test this hypothesis, apoptosis was induced by doxorubicin and camptothecin in which the anti-apoptotic efficacies are inhibited through over expressing of Bcl-xL (Fiebig et al. 2006; Park et al. 2007; Brunelle and Zhang, 2010). To determine the

concentration of this agents causing 50% cell viability inhibition on BHK-21 cell line, MTT assay was performed. IC_{50} values for doxorubicin and camptothecin were found as 1 µg/mL and 50 ng/mL respectively (Figure 2a and Figure 2b). Apoptosis was induced in BHK-21 cells transfected with pIRES2EGFP, empty vector and pIRES2EGFP-Bcl-xL plasmid with the IC_{50} values of doxorubicin and camptothecin as well as serum deprivation for 48 hrs. Transfected cell ratio expressing EGFP was determined by flow cytometry 48 hrs after apoptosis induction (Figure 3). In pIRES2EGFP/Bcl-xL transfected cells, Bcl-xL is expressed as a fusion protein with EGFP thus EGFP expressing cell ratio equals Bcl-xL expressing cell ratio. In pIRES2EGFP/Bcl-xL transfected cells, transfected cell ratio was significantly higher compared to that of empty vector transfected cells ($P < 0.001$). Apoptotic cell ratios in EGFP expressing cells were determined by flow cytometry 48 hrs after apoptosis induction (Figure 4). In pIRES2EGFP/Bcl-xL transfected cells, apoptotic cell ratio was significantly lower compared to that of empty vector transfected cells ($P < 0.001$).

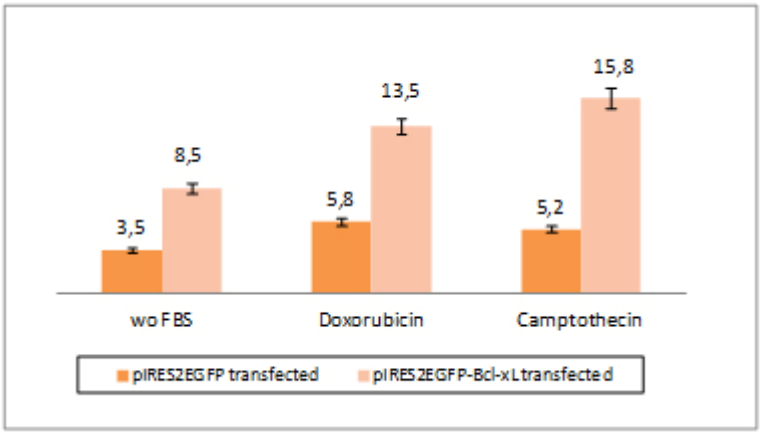

Fig. 3 48 hrs after treatment with apoptosis inducing agents and serum deprivation EGFP expressing cell ratio.

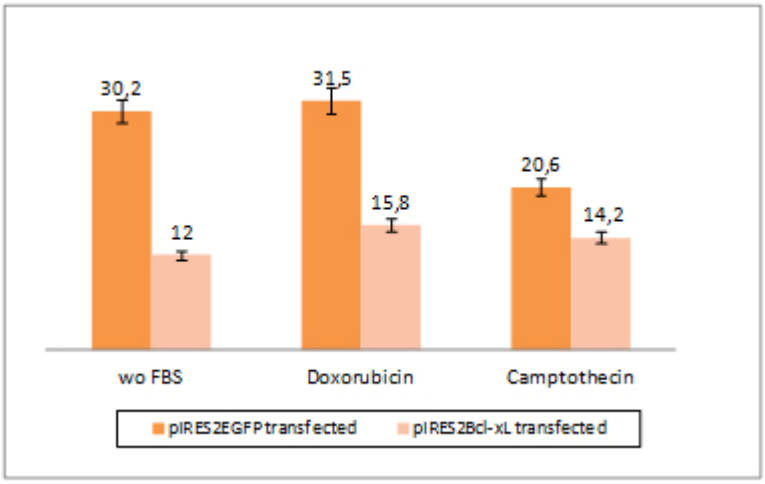

Fig. 4 48 hrs after treatment with apoptosis inducing agents and serum deprivation $Casp3^+$ apoptotic cell ratio.

Bcl-xL over expression is widely used in biotechnological applications including biopharmaceutical protein production, DNA vaccination approaches and therapeutic applications of several diseases (Al-Rubeai and Singh, 1998; Blömer et al. 1998; Kim and Lee, 2000; Huang et al. 2003; Huang et al. 2005; Kim et al. 2005; Yang et al. 2005; Kim et al. 2009; Han et al. 2011; Kim et al. 2011;). Bcl-xL has some advantages like having low molecular weight and more efficient compared to other anti-apoptotic proteins like Bcl-2 (Fiebig et al. 2006).

Bcl-xL is an anti-apoptotic protein that acts by preventing the release of mitochondrial Cytochrome C into cytosol induced by cytotoxic stimuli (Park et al. 2007). Bcl-xL over expression affects the proteins related with cell survival, cell proliferation, and repair of DNA damage thus Bcl-xL over expression affect cell growth in various ways other than apoptosis inhibition (Baik and Lee, 2009).

The growing biotechnological industry has new expectations. Engineering stable cell lines take long period of time in biopharmaceutical protein production thus transient gene expression gains importance. Bcl-xL over expression is one of the promising approaches to increase the recombinant protein expression yields through transient gene expression (Majors et al. 2008). In this study, the efficacy of Bcl-xL anti-apoptotic protein was shown by cell viability assay which are expressing EGFP fused with Bcl-xL. EGFP expressing cell ratio was 2.4 fold higher in pIRES2EGFP/Bcl-xL transfected cells compared to that of pIRES2EGFP transfected cells after serum deprivation; in doxorubicin and camptothecin exposure cells transfected with pIRES2EGFP/Bcl-xL plasmid EGFP expressing cell ratios were 2.3 and 3.0 fold higher compared to pIRES2EGFP transfected cells (Figure 3). Casp3 expressing cell ratio in EGFP transfected cells was 2.5 fold lower in pIRES2EGFP/Bcl-xL transfected cells compared to pIRES2EGFP transfected cells after serum deprivation; in doxorubicin and camptothecin exposure cells transfected with pIRES2EGFP/Bcl-xL plasmid Casp3 expressing cell ratios were 2 and 1.5 fold lower compared to pIRES2EGFP transfected cells (Figure 4). In addition, apoptosis ratio was lower in the cells transfected with Bcl-xL anti-apoptotic protein expressing DNA vaccines compared to control vector transfected cells (Blömer et al. 1998; Kim et al. 2004; Yang et al. 2005). In HEK-293 cells apoptosis ratio was 1.5 fold lower (Kim et al. 2004); in Jurkat T cells 1.7 fold lower (Yang et al. 2005); in PC12 cells 1.48 fold lower (Blömer et al. 1998). Bcl-xL over expression increases antigen specific $CD8^+$ T cell responses in human papillomavirus vaccine model (Kim et al. 2004; Huang et al. 2007).

Doxorubicin has been selected as apoptosis inducing agent because it is used as a cancer therapeutic in breast cancers and Bcl-xL over expression inhibits the efficacy of doxorubicin induced apoptosis (Fiebig et al. 2006; Park et al. 2007; Brunelle and Zhang, 2010). Therefore, in this study, in order to determine the anti-apoptotic efficacy of Bcl-xL over expression, apoptosis was induced by doxorubicin. Bcl-xL over expression inhibited apoptosis in MCF-7 breast cancer cell line (Fiebig et al. 2006). Hirsch et al. (1998), reported that the IC_{50} value of doxorubicin was 1µg/mL as in the case of this study.

The results suggest a transitory role for Bcl-xL over expression in protecting the cellular compartments from DOX invasion and in maintaining the number of mitochondria (Mhawi, 2009). Camptothecin efficacy was also inhibited by Bcl-2 and Bcl-xL over expression (El Assaad et al. 1998; Qian et al. 2009). After 48 hrs treatment with 10 µM camptothecin in MCF-7 cell line over expressing Bcl-xL, apoptosis was inhibited 3.42 fold compared to control MCF-7 (El Assaad et al. 1998). Another study showed that dexamethasone induced Bcl-xL anti-apoptotic pathway and inhibited apoptosis in solid tumor therapies conducted with camptothecin (Qian et al. 2009).

In conclusion, it was shown that *in vitro* transient expression of Bcl-xL efficiently inhibited apoptosis induced by serum deprivation, doxorubicin and camptothecin. Thus, the dual expression plasmid bearing Bcl-xL anti-apoptotic protein could be a good candidate for recombinant protein production and DNA vaccination applications to alter target cell apoptosis for higher recombinant protein yields and prolonged immune responses.

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