

Dicistronic Expression Units for Simultaneous Apoptosis Engineering and Product Expression in CHO-DG44 Cells Adapted for Growth in Serum-free Media

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SUMMARY

We have constructed dicistronic expression vectors for one-step antiapoptosis engineering and product expression. Expression units encoding the soluble intercellular adhesion molecule 1 (sICAM), a potential therapeutic for treatment of the common cold, and *bcl-2/bcl-x_l* were integrated and amplified in Chinese hamster ovary (CHO) DG44 cells adapted for anchorage-independent growth. sICAM expression was translated in a cap-dependent and survival gene expression in a cap-independent manner based on the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) element. Batch cultivations of engineered CHO-DG44 cells containing amplified transgene expression units exhibited an improved viability and a delayed onset of apoptosis compared to cell lines harboring monocistronic control constructs. *bcl-2*-mediated apoptosis protection was significantly better compared to *bcl-2*-based antiapoptosis engineering. High level expression of *bcl-2* and *bcl-x_l* are required to compensate for increased mitochondria numbers found to be associated with production cell lines grown in serum-free medium.

INTRODUCTION

Chinese hamster ovary (CHO) cell derivatives have emerged as the number one production cell line in the past decades due to their straightforward adaptation for growth in serum/protein-free media, and their compatibility with *dhfr*-based amplification protocols to increase the copy number of product gene-coding chromosomal loci (Kaufman and Sharp, 1982). State-of-the-art large-scale biopharmaceutical manufacturing is predominantly based on stirred-tank bioreactor operation using transgenic cell lines adapted for suspension growth in serum- or protein-free media. During standard bioreactor operation a substantial percentage of production cell lines die following a genetically determined program known as apoptosis or programmed cell death. Serum components have been identified as major apoptosis-protective agents and their absence in modern biopharmaceutical manufacturing results in increased sensitivity of production cell lines to programmed cell death (Zanghi et al., 1999). *bcl-2*, the prototype apoptosis suppressor and key member of the *Bcl-2* family of highly conserved pro- (for example *bcl* and *bax*) and anti-apoptotic (for example *bcl-2*, *bcl-x_l*) response regulators, has been the prime choice of antiapoptosis engineering in the biotech community. *Bcl-2* family members have been shown to modulate the caspase-9-dependent apoptosis pathway in response to a molecular rheostat localized at the outer mitochondrial membrane consisting of homo- and heterodimerized pro- and antiapoptotic *Bcl-2*-type proteins. This assembly of crucial players in the apoptosis-controlling machinery within the outer mitochondrial membrane brings these cellular power stations into the focus for antiapoptosis engineering (Folstad et al., 2000). However, biotechnologists have not yet discovered mitochondria as a potential target for improving desired characteristics of production cell lines.

MATERIALS AND METHODS

Cell Culture and Batch Cultivations

CHO-DG44/*dhfr*-⁺ adapted for growth in suspension was used as parental cell line throughout this report and cultivated like its transgenic counterparts in CHO-S-SEM-I-derived medium (Invitrogen). Viability was analyzed using trypan blue dye exclusion technology. For batch cultivations clones were plated in duplicate into cell culture flasks (T25) containing 12 ml of the appropriate medium.

Plasmid Constructs, Western Blot Analysis and Quantification of Apoptosis

The basic vector pHD, which was used throughout this study, mediates constitutive expression of desired transgenes (Meents et al., 2002b). In addition, pHD encodes the *dhfr* mini gene as selection and amplification marker. pHD-sICAM, pHD-sICAM-*bcl-2*, and pHD-sICAM-*bcl-x_l* have been described elsewhere (Meents et al., 2002b). Western blot analysis was described previously (Meents et al., 2002a). Proteins were detected using mouse monoclonal antibodies specific for *Bcl-2* and *Bcl-x_l* (Santa Cruz Biotechnology). Fragmented DNA levels were quantified using a fluorescence-based TUNEL assay (PharMingen). Cells were analyzed according to the manufacturer's protocol using a FACScan (Becton-Dickinson).

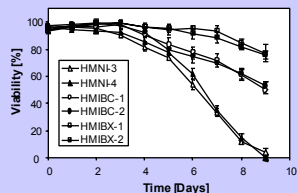
Transient Transfections and Production of Clonal Populations

Transfections were conducted using the Fugene reagent (Roche Biochemicals). Single-cell cloning was done by standard limited dilution technology. For amplification of desired chromosomal transgene, single cell lines derived from a specific mixed population were seeded into 96-well chambers that contained 200 µl standard by phosphate-thymidine-free medium supplemented with 20 nM MTX.

Flow Cytometry and Fluorescence Microscopy of Labeled Mitochondria

Transfections were conducted using the Fugene reagent (Roche Biochemicals). Single-cell cloning was done by standard limited dilution technology. For amplification of desired chromosomal transgene, single cell lines derived from a specific mixed population were seeded into 96-well chambers that contained 200 µl standard by phosphate-thymidine-free medium supplemented with 20 nM MTX. Flow cytometry and fluorescence microscopy of labeled mitochondria 0.5 × 10⁶ cells per sample were harvested, washed with PBS, and treated with 1% paraformaldehyde in PBS for 15 min at 4 °C. After a second PBS washing step the cells were ready for the fluorescence-based staining of their mitochondria. A PBS solution containing 500 µM of the mitochondria-selective MitoTracker Green FM dye (Molecular Probes) was prepared. Cells were incubated in 200 µl staining solution for 15 min at 37 °C. Subsequently, the cells were washed twice with PBS resuspended in 4 ml PBS and subjected to flow cytometric analysis. 100 µl of the remaining cell solutions were centrifuged on poly-L-lysine-coated slides at 800 × g for 5 min and examined under a fluorescence microscope (laser scanning microscope, Leica TCS).

In order to facilitate *bcl-2* and *bcl-x_l*-based antiapoptosis engineering we constructed dicistronic expression configurations which contained sICAM in the first cistron, translated in a classical cap-dependent manner and either of the two survival genes in the second cistron, translation of which is initiated in a cap-independent manner driven by an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus.



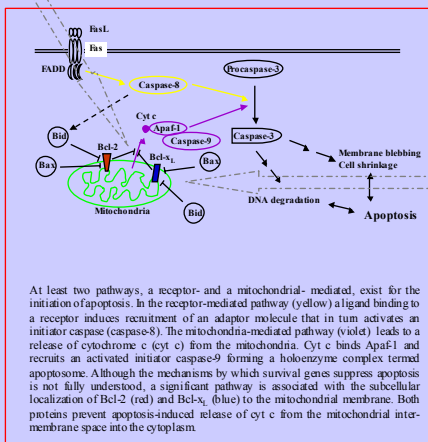
The impact of *bcl-2* and *bcl-x_l*-based antiapoptosis engineering of production cell lines has been quite diverse ranging from none to substantial protective effects. In order to evaluate whether *bcl-2* and *bcl-x_l* retain their viability-extending capacity when expressed from the second cistron we generated mixed population and amplified individual mixed populations and analyzed the two highest sICAM producers: HMNI-3/HMNI-4 encode the control vector pHD-sICAM, HMIBC-1/HMIBC-2 contains pHD-sICAM-*bcl-2* and HMIBX-1/HMIBX-2 harbor pHD-sICAM-*bcl-x_l*. All non-amplified cell clones exhibited similar viability profiles (data not shown). However, significant increases in cell viability could be observed during the decline phase of clones containing amplified transgenes. *bcl-x_l* clearly outperformed *bcl-2* with respect to cell death protection.



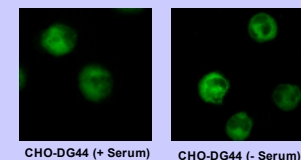
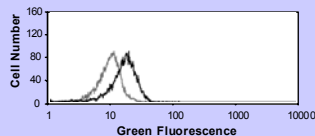
In order to confirm the amplification status of the antiapoptosis genes we performed *Bcl-2* and *Bcl-x_l*-directed Western blot analysis. High level expression of *Bcl-2* could be visualized in HMIBC-1/2, whereas in HMNI-3 and in the CHO-DG44 host cell almost no endogenous levels could be detected. Similarly, high level expression of *Bcl-x_l* could be observed in HMIBX-1/2 and only basal expression in CHO-DG44 at levels which is near the detection limit and comparable to the transgene control cell line HMNI-3.

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At least two pathways, a receptor- and a mitochondrial-mediated, exist for the initiation of apoptosis. In the receptor-mediated pathway (yellow) ligand binding to a receptor induces recruitment of an adaptor molecule that in turn activates an initiator caspase (caspase-8). The mitochondrial-mediated pathway (violet) leads to a release of cytochrome c (cyt c) from the mitochondria. Cyt c binds Apaf-1 and recruits an activated initiator caspase-9 forming a holoenzyme complex termed apoptosome. Although the mechanisms by which survival genes suppress apoptosis is not fully understood, a significant pathway is associated with the subcellular localization of *Bcl-2* (red) and *Bcl-x_l* (blue) to the mitochondrial membrane. Both proteins prevent apoptosis-induced release of cyt c from the mitochondrial intermembrane space into the cytoplasm.



The obvious requirement for a high *bcl-2* or *bcl-x_l* dosage in order to achieve significant survival impact in CHO-DG44 grown under serum-free conditions, initiated speculations whether the mitochondria content and therefore sensitivity to programmed cell death is linked to the presence of serum. With a focus on determining the serum-dependence of the specific mitochondria content, we cultivated CHO-DG44 in serum-free and serum-containing media. Following cultivation for two weeks under said conditions, the cells of both cultures were pooled for their specific mitochondria content using MitoTracker Green FM, a mitochondria-specific fluorescent dye. As MitoTracker Green FM accumulates in mitochondria in a membrane potential-independent manner it is a well-established tool for the quantification of these organelles. Mitochondria-specific staining was significantly increased in cells cultivated in the absence of serum as assessed by fluorescence microscopy and FACS-mediated analysis quantified an up to 3-fold boost in specific mitochondria content.

CONCLUSIONS

- *bcl-2*, as well as *bcl-x_l*, are only apoptosis-protective at high expression levels. It is therefore not surprising that apoptosis-suppressing and viability-enhancing characteristics are only detectable following *dhfr*-based amplification of *bcl-2* and *bcl-x_l*.
- *bcl-x_l* outperforms *bcl-2* with respect to cell death protection. If ectopic expression of this survival genes is chosen to improve cell culture performance, the use of *bcl-x_l*, rather than *bcl-2*, is highly recommended.
- The mitochondria content of production cell lines grown in serum-free media is about three-fold higher compared to serum-containing culture conditions, suggesting that increased *bcl-2* and *bcl-x_l* expression levels are required to compensate for the higher intracellular concentration of apoptosis machineries associated with these organelles.

A later event during apoptosis is the activation of endonucleases, which cleave DNA resulting in a characteristic "ladder" of DNA fragments. A method which is often used to detect fragmented DNA utilizes a reaction catalyzed by exogenous terminal deoxynucleotidyltransferase (TdT), referred to as "TUNEL" (TdT dUTP nick end labeling). Apoptosis can be quantified by using FITC-dUTP labeling of DNA breaks, followed by flow cytometric analysis. Correlating with an increase in viability amplified cell clones also showed a dramatic reduction in the percentage of cells which initiate apoptosis programs. The apoptosis-suppressing potential of *bcl-x_l* was higher compared to *bcl-2*.