Qing Jiang¹Yuanli Sun¹Zilong Guo¹Mingpeng Fu¹Qiang Wang²Huifen Zhu¹Ping Lei¹Guanxin Shen¹

Research Article

Overexpression of GRP78 enhances survival of CHO cells in response to serum deprivation and oxidative stress

Chinese hamster ovary (CHO) cells are regarded as one of the most commonly used mammalian hosts, which decreases the productivity due to loss in culture viability. Overexpressing antiapoptosis genes in CHO cells was developed as a means of limiting cell death upon exposure to environmental insults. Glucose-regulated protein 78 (GRP78) is traditionally regarded as a major ER chaperone that participates in protein folding and other cell processes. It is also a potent antiapoptotic protein and plays a critical role in cell survival, proliferation, and metastasis. In this study, the impact of GRP78 on CHO cells in response to environmental insults such as serum deprivation and oxidative stress was investigated. First, it was confirmed that CHO cells were very sensitive to environmental insults. Then, GRP78 overexpressing CHO cell line was established and exposed to serum deprivation and H₂O₂. Results showed that GRP78 engineering increased the viability and decreased the apoptosis of CHO cells. The survival advantage due to GRP78 engineering could be mediated by suppression of caspase-3 involved in cell death pathways in stressed cells. Besides, GRP78 engineering also enhanced yields of antibody against transferrin receptor in CHO cells. GRP78 should be a potential application in the biopharmaceutical industries.

Keywords: Apoptosis / ER stress / Glucose-regulated protein 78 / Oxidative stress / Serum deprivation

Received: October 26, 2015; *revised:* May 13, 2016; *accepted:* June 14, 2016

DOI: 10.1002/elsc.201500152

¹Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Hubei, China

²Department of Immunology, Medical College of Wuhan University of Science and Technology, Hubei, China

1 Introduction

Chinese hamster ovary (CHO) cells are frequently used in biomedical research for heterologous protein expression. They have been the number one production cell line in the past 30 years and continue to remain the industry's premier workhorse for therapeutic protein production [1]. However, despite their straightforward adaptation for growth in serum-/protein-free media and their ability to grow to very high densities in suspension cultures, a substantial percentage of CHO cells are found to die by apoptosis during standard bioreactor operation [2, 3].

It poses a major limiting factor in the production of recombinant proteins in cell cultures [3]. This may partly explain why the volumetric yields of protein produced using CHO cells are relatively lower than what can be achieved using microbial host systems.

To solve the dilemma of desired serum-free cultivations resulting in increased apoptosis, a variety of physiological and metabolic engineering strategies have been made to enhance productivity. For instance, some energy metabolism pathways [4–6] have been engineered to limit lactate production levels in culture to enhance viability of CHO cells. Antiapoptosis engineering of CHO cell lines [7–9] have been well characterized to understand cell cycle mechanisms and to prolong culture.

In addition to focusing on cell death protection, how to increase the quality of produced proteins by CHO cells is also our concern. Protein folding conferred by the hosts dictates the properties of the products. Since heterologous protein production places abnormal ER stress on host cell, the unfolded protein response (UPR) would be activated to facilitate the correct folding of heterologous proteins and degradation of misfolded proteins [10]. As a master regulator of UPR, glucose-regulated

Correspondence: Dr. Ping Lei (adaleip@hust.edu.cn), Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030 Hubei, China

Abbreviations: CHO, Chinese hamster ovary; CHO-GRP78, CHO modified by GRP78; FCM, flow cytometry; GRP78, glucose-regulated protein 78; MFI, mean fluorescence index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIT-GRP78, NIT-1 modified by GRP78; RPMI, Roswell Park Memorial Institute; TFR-Ab, antibody against transferrin receptor; UPR, unfolded protein response

protein 78 (GRP78) is one of the best-characterized ER chaperone proteins. It controls the processing and maturation of secretory proteins [11]. Furthermore, when misfolded proteins accumulate in the ER, GRP78 binds to them, thereby leading to the UPR in ER [12] to confer correct folding of newly made proteins. Besides that, GRP78 was reported to promote cell proliferation, suppress apoptosis [13], and is associated with aggressive growth and invasive properties of cancer cells [14, 15]. We also reported that insulin-secreting insulinoma cells NIT-1 modified by GRP78 (NIT-GRP78) showed prolonged survival against different insults [16]. And a significant increase of insulin concentration could be observed in diabetic mice received NIT-GRP78 cells [17].

Given the roles of GRP78 in protein folding and in survival promotion, we present here an analysis of GRP78 engineering on the survival characteristics of CHO cells adapted for suspension growth in serum-free media and on the yields of antibody product. Data showed that GRP78-based engineering could protect CHO cells from insults including serum withdrawal and oxidative stress. In the meantime, GRP78 engineering also increased yields of antibody against transferrin receptor (TfR-Ab) and improved cell viability. This study gained an understanding of apoptosis in CHO in order to develop strategies to delay the onset of apoptosis and concomitantly prolong cell culture and provided a basis for the potential application of GRP78 in the biopharmaceutical industries.

2 Materials and methods

2.1 Cell culture

The Chinese hamster ovary (CHO) cells, human stomach adenocarcinoma (AGS) cell line, and human hepatoma cell line HepG2 (China Center for Type Culture Collection, Wuhan, P. R. China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 and DMEM medium (Gibco BRL, NY, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China) and 100 U/mL ampicillin, 100 mg/mL streptomycin in an incubator containing 5% CO₂ at 37°C (standard conditions). Serum deprivation medium means the above medium supplemented with less than 1% FBS [18].

2.2 Construction and transfection

The GRP78 cDNA was amplified by RT-PCR from total RNA prepared from AGS cells using two synthetic primers (sense: TTAGGATCC *Bam*HI ACCACCATGAAGCTCTCCCTG; antisense: CGCGGATCC *Bam*HI CTACAACATCTTTTCTGC) with RT-PCR Kit (ToYoBo, Osaka, Japan). The PCR products were digested by *Bam*HI (Fermentas, Wuhan, China) and then subcloned into the pIRES2-EGFP vector. The correct clone was identified by restriction endonuclease digestion and DNA sequencing. CHO cells were plated at a density of 3×10^5 cells/well in a 6-well plate and transfected with 3.2 µg of linear recombinant vector pIRES2-EGFP/GRP78 digested by *Bsp*TI (Fermentas) using LipofectamineTM 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h, the CHO cells were subcultured in 1:10, and selectively cultured with 600 µg/mL G418 (Sigma-Aldrich,

Shanghai, China). Culture media were changed every 3 days. After that, six positive clones were selected. But only two clones (C8 and C17) were screened for stable clones and used in this experiment. The clones maintained in the medium supplemented with 300 µg/mL G418 and named as CHO modified by GRP78 (CHO-GRP78). CHO cells were set as negative control.

2.3 RT-PCR

Total RNA were extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed using RT-PCR kit (ToYoBo). PCR was performed to amplify GRP78 using the following primers (sense: 5'-TTCGGATCCATGGAGGAGGAGGACAAGA-3'; antisense: 5'-CGCGGATCCCTACAACTCATCTTTTCTGCT-3'). As a reference, the extent of β -actin expression in the same cells was determined using two synthetic primers (sense: 5'-CTGGGGCGCCCCAGGCACCA-3'; antisense: 5'-CTCCTTAATGTCACGCACGATTTC-3'). Relative GRP78 mRNA expression was given as GRP78/ β -actin ratio. Relative amounts of mRNA were quantified using Image Gel-Pro analyzer software (Media Cybernetics, Bethesda, FL, USA).

2.4 Western blot analysis

CHO and CHO-GRP78 cells (5×10^5) were lysed in radio immunoprecipitation assay lysis buffer (Beyotime, Shanghai China) containing 1 mM PMSF (Beyotime) according to the manufacturer's instructions. Then concentrations of extracts were determined using bicinchoninic acid protein assay kit (Beyotime). The supernatants were clarified by centrifugation for 30 min at 2500 rpm and concentrated by the 30-kDa MW cutoff ultrafiltration membranes (Millipore, Billerica, MA, USA).

Samples were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked in 5% skimmed milk for 1 h at 37°C and then incubated with rabbit anti-GRP78 polyclonal antibody (1:500 dilution, Abcam, Cambridge, MA), rabbit anticaspase-3 (1:1000 dilution, CST, Boston, USA), and rabbit anticlaved caspase-3 (1:1000 dilution, CST) overnight at 4°C followed by incubation with peroxidase-labeled goat anti-rabbit IgG antibody (1:2000 dilution, ProteinTech Group, Wuhan, China) for 1 h at 37°C. Proteins were detected using enhanced chemiluminescence (ECL) kit (Tiangen, Beijing, China). β -Actin (1:500 dilution, Santa Cruz, CA) signals were used to normalize the GRP78 signals. Relative amounts of protein were quantified using Image Gel-Pro analyzer software.

2.5 Sample collection and analysis

CHO or CHO-GRP78 cells were transiently transfected with plasmid encoding for the tetravalent antibody against TfR (pOptiVECTM-TOPO[®]/TfR-Ab) and then cultured in SFM4CHOTM medium (Thermo, MA, USA). At regular time points, supernatants supposed to contain Ab product were collected and, after removal of particulates, stored at -80°C for the following ELISA and TfR binding assays. Cell viability was

detected by propidium iodide (PI, KeyGen, Nanjing, China) staining. Viable cells were counted in triplicate for each well with the Trypan blue exclusion method.

2.6 ELISA assay

Wells were coated with 100 μ L anti-human IgG (dilution at 1:200, Meridian, TN, USA) in 0.05 M carbonate–bicarbonate buffer (pH 9.6) overnight at 4°C. Two hundred microliters blocking buffer (2% BSA in the PBST) was added to each well and incubated for 1 h at 37°C. After washing three times with 300 μ L 0.05% PBST, 100 μ L sample was added into the wells at 37°C for 2 h and then rewashed for five times. Afterwards, 100 μ L HRP-conjugated goat anti-human antibody (1:3000; Thermo) was added to incubate at 37°C for 30 min. The color was developed by incubating with 100 μ L freshly prepared substrate solution (composed of 10 mL pH 5.0 phosphate-citrate buffer, 4 mg O-phenylenediamine, and 30 μ L 30% H₂O₂) at 37°C for 15 min in the dark. Finally, 50 μ L of 2 M H₂SO₄ was supplemented to terminate the reaction. OD value was read using a microplate reader (TECAN, Männedorf, Switzerland) under 490 nm wavelength.

2.7 Flow cytometric analysis

CHO and CHO-GRP78 cells (3×10^6) were fixed with 4% paraformaldehyde at 4°C for 30 min. Half of the cells were permeabilized with saponin solution (1% saponin + 1% BSA + PBS; Sigma) at 4°C for 15 min. After blocked with blocking solution (10% goat serum + 1% BSA + PBS) at 37°C for 30 min, all cells were incubated with anti-GRP78 antibody for 30 min at 4°C, then with goat anti-rabbit PE-conjugated secondary antibody for 30 min at 4°C in the dark. Finally, cells were resuspended in ice-cold washing solution (1% BSA + PBS) and analyzed by FACS Calibur (BD Biosciences, CA, USA).

For TfR binding assays, 3×10^5 HepG2 cells were incubated with 350 μ L Ab-contained supernatant for 1 h at 4°C after blocked, followed by incubation with goat anti-human Allophycocyanin (APC)-conjugated secondary antibody (Zoman Biotechnology, Beijing, China) for 30 min at 4°C in the dark. Finally, cells were resuspended in ice-cold washing solution and analyzed by FACS Calibur (BD Biosciences).

2.8 Immunocytochemistry

CHO and CHO-GRP78 cells grown to approximately 50% confluence on chamber slides were washed thrice with PBS and fixed with 4% paraformaldehyde at 4°C for 30 min. Then, the cells were permeabilized with saponin solution at 4°C for 20 min and rinsed twice with ice-cold PBS to detect intracellular protein. For detection of GRP78, the cells were stained with a 1:100 dilution of anti-GRP78 antibody and a 1:100 diluted goat anti-rabbit PE-conjugated secondary antibody (ProteinTech Group). After that, cells were stained with hoechst33342 (5 μ g/mL, Beyotime) for 10 min at room temperature. The chamber slides were placed on glass slides and observed under the fluorescence microscope (LSM 710 and ConfoCor 3, Zeiss, Germany).

2.9 Apoptosis assay

CHO and CHO-GRP78 cells were seeded into 12-well plate at a density of 1.5×10^5 cells per well and incubated under the standard conditions for 24 h prior to twice PBS washing and transferring to serum-free culture supplemented with or without H₂O₂. In order to ensure that other nutrient limitations were not experienced, cells were fed fresh medium every 48 h. Apoptotic assays were performed by starving cells as above for 24 h, 48 h, or longer time. Then cells were stained with Annexin V and PI (KeyGen) and analyzed by a FACS Calibur flow cytometer. Annexin V/PI double-negative represent cell viability, Annexin V-negative/PI-positive staining is regarded as primary necrosis, and the others are considered as apoptosis.

2.10 MTT assay

To assess the cell survival in the presence of H₂O₂ (Zhongtian, Wuhan, China), the cell viability was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). After being seeded at a density of 0.6×10^5 cells/mL onto 96-well plates and cultivated for 24 h, the cells were cultured with serum-free medium for 12 h to synchronize and then exposed to H₂O₂ (Zhongtian) at increasing concentration from 0 to 600 μ M for 4 h. The MTT assay was performed as manufacture's recommendation. The amount of formazan was determined by the absorbance at 490 nm. The surviving cells were relatively represented as the percentage of MTT reduction.

2.11 Statistical analysis

Results were expressed as means $\pm$ SDs from three independent experiments. Data were analyzed by Student's *t*-test for data of two groups.

3 Results

3.1 CHO cells are sensitive to serum deprivation and oxidative stress

Prior to assess the effect of GRP78 engineering on CHO cells, the viability of stressed cells was determined. CHO cells were cultivated on serum-free RPMI 1640 supplemented with or without H₂O₂ for extended period. A small fraction of cells were taken at serial time points and analyzed for cell viabilities. Data showed that decreasing of viability of cells cultured in serum-free media was already evident at 48 h of incubation and going on for long-time cultivation. Within 4 days, nearly one-third of serum-deprived cells died. After 12 days, cells had a viability of 18.35%, whereas those exposed to H₂O₂ were only 2.70% (Fig. 1). Results confirmed that CHO cells were extremely sensitive to their environment.

Figure 1. Viability of Chinese hamster ovary (CHO) cells in response to serum deprivation and oxidative stress. CHO cells were seeded in 12-well plates at a density of 1.5×10^5 cells per well and grown for 24 h. Then cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, serum free medium with or without $200 \mu\text{M H}_2\text{O}_2$. In order to ensure that other nutrient limitations were not experienced, cells were fed fresh medium every 48 h. Cells were taken every 2 days and analyzed for cell viability using Annexin V/PI double staining. Error bars represent SD for each measurement.

3.2 Establishment of GRP78-engineered CHO cells

Previous antiapoptosis engineering studies exemplified appositive impact of GRP78 on the viability of transgenic NIT-1 cells [16]. In order to validate this effect for CHO cells, GRP78-engineered cells needed to be established. First, the full length of GRP78 was amplified from AGS cells by RT-PCR and then subcloned into pIRES2-EGFP eukaryotic expression vector to construct pIRES2-EGFP/GRP78. DNA sequencing confirmed the in-frame insertion of GRP78 cDNA into pIRES2-EGFP plasmid and the introduction of initial codon, terminal codon, Kozak translation initiation site, and restriction sites of *Bam*HI into the sequence. The DNA and the predicted amino acid sequence of GRP78 were in agreement with NM005347 in GenBank database. Restriction digestion mapping showed the presence and right connection direction of GRP78 in pIRES2-EGFP/GRP78 (data not shown). Second, pIRES2-EGFP/GRP78 was stably transfected into CHO cells (CHO-GRP78). In order to confirm the expression of GRP78 gene, we performed GRP78-directed western blot analysis. Western blot showed there was a thicker protein band with a molecular weight of about 78 kDa emerged in the total extract of two CHO-GRP78 transfectants (clone C8 and C17). And the expression levels of GRP78 in these two transfectants were statistically higher than the endogenous GRP78 expression. Given that C17 clone showed higher expression of GRP78 than C8 clone, C17 clone was chosen to perform the following experiments (Fig. 2A). RT-PCR also confirmed the upregulated expression of GRP78 in CHO-GRP78 cells (Fig. 2B).

ER stress or ectopic expression of GRP78 leads to localization of a subfraction of GRP78 on the cell surface [19]. It was reported that GRP78 might also promote cell proliferation from the cell surface [20]. To verify the localization of overexpressed GRP78, flow cytometry (FCM) and immunocytochemistry were performed. FCM suggested that only 0.1%

CHO-GRP78 cells expressed membrane-bound GRP78, which showed no difference with CHO cells (0.1 vs. 0.2%). Immunocytochemistry confirmed no detectable membrane-bound GRP78 fluorescence on CHO-GRP78 cells. After being permeabilized with saponin, 82.1% CHO-GRP78 cells were GRP78-positive, and 70.5% CHO-GRP78 cells were GRP-GFP-positive. GRP78-positive value of CHO-GRP78 cells was much higher than that of CHO cells (82.1 vs. 48.6%), as well as the mean fluorescence index (MFI). GRP78 fluorescence distributed to the periphery and in the perinuclear regions of the CHO-GRP78 cells, but rare on the cell surface (Fig. 2C and D).

To test the level of secreted GRP78 in the supernatant, cells were deprived with serum for 24, 48, or 72 h. Immunoblotting showed that an obvious band emerged in the supernatant of 24-h serum-deprived CHO-GRP78 cells and the band intensity increased as deprivation times went by (at 48 and 72 h time points). For control CHO cells, very weak band emerged after 24 h deprivation, but an obvious one presented after 48 and 72 h deprivation (Fig. 2E). These data suggested that GRP78 predominantly expressed in cytoplasm but rarely on the membrane. Upon stimuli, GRP78 could be secreted into extracellular space.

3.3 GRP78 engineering protected cells against serum deprivation induced apoptosis

Cells in culture require numerous growth factors that are often readily provided by serum supplementation. Some of these growth factors act as mitogens inducing cell proliferation, others act as survival factors actively suppressing apoptosis in cultured cells [21]. Serum withdrawal has been a classical method to induce apoptosis [18]. In order to determine the effect of GRP78 on this insult, CHO cells were cultivated in serum-free RPMI 1640 for short-term deprivation studies (24–48 h). Data showed that more CHO-GRP78 cells survived in serum-free media after short-term incubation ($p < 0.05$ for 24 h and $p < 0.01$ for 48 h, Fig. 3A). These results indicated that GRP78 engineering could enhance the viability of CHO cells cultured in serum-free media. To assess if the viability enhancement ability of GRP78 was due to antidamage effects, CHO-GRP78 cells were further analyzed for apoptosis and necrosis assay by Annexin V/PI double staining. Correlating with an increase in viability, cells also showed a dramatic reduction in the percentage of cells that initiate apoptosis and necrosis programs (Fig. 3B).

The potential contribution of caspase-dependent pathway to apoptosis under ER stress was investigated by assessing cleaved caspase-3 levels. Data showed the levels of cleaved caspase-3 in CHO-GRP78 cells were relatively lower than those in negative control both after 24 and 48 h serum deprivation ($p < 0.01$, Fig. 3C).

3.4 GRP78 engineering improved the viability of cells exposed to H_2O_2

In addition to nutrient and growth factor limitations, another problem associated with typical cell culture operations is oxidative stress during late stages of cultures. Oxidative stress is

Figure 2. Overexpression and localization of glucose-regulated protein 78 (GRP78). (A) Cytoplasmic extracts prepared from Chinese hamster ovary (CHO) and CHO modified by GRP78 (CHO-GRP78; clones 8 and 17) cells were separated on 12% SDS-PAGE and immunoblotted using anti-GRP78 antibody and anti- β -actin antibody. The GRP78 band (78 kDa) and the β -actin (43 kDa) were indicated (left). Right, densitometric analysis of GRP78 levels of the western blots. (B) GRP78 mRNA levels detected by real-time PCR (inserted) and densitometric analysis of GRP78 levels of the RT-PCR. (C) Localization of GRP78 detected by flow cytometry (FCM; left). Saponin-treated cells were used to detect the total GRP78 level and nonpermeabilized cells for membrane bound GRP78. The right bar graphs represented FCM analysis of the percentage of GRP78 positive cells and mean fluorescent index (MFI) of GRP78. (D) Localization of GRP78 detected by immunocytochemistry. (E) Concentrated supernatant from serum deprived CHO and CHO-GRP78 cells were transferred on 12% SDS-PAGE and immunoblotted using anti-GRP78 antibody.

also an important contributor proved to induce ER stress cascade processes [22–24]. In order to elucidate whether GRP78 engineering could provide protection for CHO cells exposed to oxidative stress, cells were exposed to H_2O_2 at an increasing concentrations from 0 to 600 μM for 4 h. Data from MTT assay showed that as the concentration of H_2O_2 increased, the percentage of MTT reduction decreased. These suggested that H_2O_2 decreased the viability of CHO cells in a dose-dependent

manner. H_2O_2 at concentration of 200 and 400 μM led to more CHO-GRP78 cells survived from stress than control CHO cells ($p < 0.05$ and $p < 0.001$), but H_2O_2 at concentration of 600 μM did not (Fig. 4A). The levels of cleaved caspase-3 in CHO-GRP78 cells were also increased when cells were exposed to H_2O_2 at concentration of 400 and 600 μM and peaked at 400 μM , but these values were statistically lower than those in CHO cells ($p < 0.05$). However, there are no changes of cleaved

Figure 3. Glucose-regulated protein 78 (GRP78) engineering protected cells from serum deprivation induced damage. (A) Percent viability of serum-deprived Chinese hamster ovary (CHO) and CHO modified by GRP78 (CHO-GRP78) cells were detected by labeling with Annexin V and PI using flow cytometry (FCM). (B) Bar graph represented FCM analysis of the percentage of necrosis and apoptosis cells at 24 and 48 h time points. (C) Level of cleaved caspase-3 in serum-deprived cells was detected by WB (left). The upper right bar graph represented densitometric analysis of GRP78 levels of the western blots and the lower right of cleaved caspase-3. Data were mean values $\pm$ SD, $n > 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, nonsignificant difference versus negative control.

caspase-3 induced by H_2O_2 (200 μ M) in CHO and CHO-GRP78 cells (Fig. 4B).

3.5 GRP78 engineering enhanced yields of TfR-Ab and improved cell viability

To elucidate whether GRP78 engineering could increase the yields of protein product, CHO and CHO-GRP78 cells were transiently transfected with plasmid encoding for the antibody against TfR. ELISA assay showed that the concentrations of TfR-Ab in the supernatant of CHO-GRP78 cells were higher than those in CHO cells at any time points. Concentration of TfR-Ab in the supernatant of CHO-GRP78 cells gradually increased to reach a peak (124.82 ng/mL) at the seventh day (Fig. 5A), compared with no obvious changes in the supernatant of CHO cells. Flow cytometric analysis confirmed antibody produced by CHO-GRP78 cells showed higher MFI when binding with TfR⁺ HepG2 cells, than that by CHO cells (Fig. 5B), even when the Ab concentrations in these two groups showed no difference at the

Figure 4. Glucose-regulated protein 78 (GRP78) engineering protected Chinese hamster ovary (CHO) cells against injury induced by H_2O_2 . (A) Cells synchronized in serum-free medium were treated with H_2O_2 at increasing concentration for 4 h and then cultured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL) for 4 h. Bar graph represented the percentage of viable cells assayed by MTT. (B) The level of cleaved caspase-3 in H_2O_2 -treated cells was detected by western blot (upper). The lower left bar graph represented densitometric analysis of GRP78 levels of the western blots and the lower right of cleaved caspase-3. Data were mean values $\pm$ SD, $n > 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, nonsignificant difference versus negative control.

11th day (Fig. 5A and B). Further studies (Fig. 5C and D) manifested that Ab-produced CHO-GRP78 cells had much higher viability than CHO cells when cultured in serum-free SFM4CHO media. The difference between them was already evident at third day of incubation and going on for long-time cultivation. At 11th day, CHO-GRP78 cells had a viability of 33.87%, whereas CHO cells only had 3.23 % (Fig. 5C). The results confirmed that GRP78 engineering could enhance yields and quality of protein products and improved cell viability.

4 Discussion

One of the major challenge in using CHO and other mammalian cell lines as recombinant protein production hosts is that the volumetric yields of protein produced from processes using these cells are relatively low. Great efforts, including increasing cell density, longer culture durations, medium optimization, process monitoring and control, screening, and CHO-dhfr^{-/-} cell

Figure 5. Glucose-regulated protein 78 (GRP78) engineering enhanced yields of TfR-Ab and improved cell viability. Chinese hamster ovary (CHO) and CHO modified by GRP78 (CHO-GRP78) cells were seeded in 12-well plates at a density of 2×10^5 cells per well and transiently transfected with pOptiVEC™.TOPO®/TfR-Ab, followed by culturing with SFM4CHO™ medium. (A) The concentration of TfR-Ab in the supernatant was detected by ELISA assay. (B) The quality of TfR-Ab in the supernatant was evaluated by its binding ability with TfR⁺ HepG2 cells using flow cytometry (FCM). The bar graphs represented FCM analysis of mean fluorescence index (MFI). (C) Bar graph represented FCM analysis of the percentage viability of CHO and CHO-GRP78 cells. (D) The viable cell numbers of CHO and CHO-GRP78 cells were counted after Trypan blue staining. Data were mean values $\pm$ SD, $n > 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus negative control.

line development [1, 5], have been made and significant improvements have been achieved over the past 30 years. Although CHO cells can be cultured for prolonged time periods, the long-term continuous culture may still adversely affect the structure and function, leading to cellular suicide or apoptosis [25]. Many cell lines undergo apoptosis in response to physiological and nonphysiological factors, such as nutrient limitation, ionizing radiation, and oxidative stress [26], which negatively impact the overall production. Genetic modification to confer apoptosis resistance is a strategy for apoptosis inhibition in culture

systems to limit cell death upon exposure to environmental insults [9, 27]. Apoptosis-suppressing genes, such as bcl-2/bcl-xL, DN-Caspase9, XIAP, CrmA, etc., have been explored to engineer CHO cells and they displayed their cell death protective capacity [8, 27–29].

As a central monitor and marker of ER stress, GRP78 relieves ER stress and thus protects the cells from apoptosis through preferential binding with misfolded and/or unfolded proteins that accumulate and aggregate in ER lumen under stress conditions, such as nutrient deprivation, hypoxia, alterations in

glycosylation status, disturbances of calcium flux, and so on [30–32]. Our previous report confirmed the antiapoptosis effect of GRP78 on insulinoma NIT cells [16]. In order to check this effect on CHO cells, GRP78-modified CHO cells were established. Western blot and RT-PCR confirmed the upregulated expression of GRP78 in CHO-GRP78 cells. It was reported that ectopic expression of GRP78 on cell membrane could act as receptor to bind with ligands to transduce signals associated with cell survival, proliferation, and metastasis [33–35]. However, our results from immunofluorescent staining showed that GRP78 was scarcely expressed on the cell membrane. GRP78 predominantly resided intracellularly. But a small amount of GRP78 could be secreted into extracellular matrix by cells upon stimuli [36,37]. Our result showed that GRP78 was a stress responsive element that could be secreted into extracellular space upon stimuli. Hence, the principal role of GRP78 as a molecular chaperone is a matter of our study.

Serum deprivation has been a classical method to induce stress-associated apoptosis [18]. Our data suggested that up-regulated expression of GRP78 protected CHO from cell death upon serum deprivation and GRP78 could be employed to selectively modulate the destructive effects of prolonged ER stress. Furthermore, oxidative stress is closely related to ER stress [22] and induction of it plays a key role in serum deprivation induced apoptosis [23]. Our previous studies also reported that streptozotocin or cytokines-treated NIT-GRP78 cells showed decreased secretions of NO and increased activities of superoxide dismutase, which could protect cells against harmful effects of superoxide free radicals [16]. In this article, data showed GRP78 did protect CHO from cell death in response to H_2O_2 -induced oxidative stress and the statistical difference of survival rate between CHO-GRP78 and control cells was more significant as the concentration of H_2O_2 increased from 0 to 400 μM . This suggests the more powerful the stress in a range, the more evident the GRP78 survival advantage. However, the advantage disappeared at 600 μM . It might be that high concentrations of H_2O_2 were very toxic even for shorter incubations. Upon such severe stress, GRP78 could not modulate homeostasis anymore and stressed cells underwent apoptosis or death through the activation of mediators of the stress-induced apoptosis.

Mediators involved in the ER stress-induced apoptosis included caspase-9,12, JNK and the transcription factor CHOP [38]. NF- κ B, JNK/SAPK, and p38 MAPK pathways were all suggested to be involved in oxidative stress activated signaling pathway [39]. ER stress and oxidative stress are primary causes of cell death during the decline phase of batch culture. It is worth of research on molecules coupling these processes. Hitomi et al. reported that caspase-3 was mainly activated by ER stress and might be important in ER stress and serum withdrawal induced apoptosis [18,40]. To assess the contribution of caspase-3 in GRP78 survival advantage, cleaved caspase-3 was measured. Data suggested that serum deprivation and oxidative stress promoted the expression of cleaved caspase-3. But GRP78 overexpression could rescue CHO cells from these stresses-induced apoptosis through downregulation of cleaved caspase-3. It was hypothesized that, through forming complex with caspases-7 and caspase-12 in the perinuclear/ER region [41], GRP78 inhibited the activation of cytoplasmic caspase-3 that was mediated indirectly by activated ER-resident caspase-12 [40]. This

decreased caspase-3 activity, clearly increased GRP78-mediated starvation and oxidative survival. Thus, one mechanism for the protective effect of GRP78 might be that its overexpression could suppress the activation of caspase-3-mediated cell death pathways in stressed cells. Further investigation into this mechanism was going to be carried out.

Despite the apoptosis-resistance effect of GRP78-based engineering on CHO cells, it was uncertain whether GRP78 engineering has an impact on product yields. It was reported that antiapoptosis engineering can probably not keep up with expectations. For instance, Meents et al. [8] tested the impact of antiapoptosis determinants bcl-2/bcl-xL on productivity of CHO-DG44 cells. They found bcl-2 overexpression dramatically decreases the specific productivity of various secreted and heterologous proteins. This may result from shifts in intracellular mitochondria concentrations. Given these, GRP78-mediated productivity of our genetically engineered antibodies had to be investigated. ELISA and FCM showed that GRP78 engineering could enhance the yields and quality of genetically engineered Ab. This high productivity might result from the essential role of GRP78 in regulating the UPR. Since heterologous protein production places abnormal ER stress on host cell, UPR would be activated in response to such stress in CHO cells to facilitate the correct folding of heterologous proteins and degradation of misfolded proteins [10,42]. As a master regulator of UPR, GRP78 controls the processing and maturation of secretory proteins [11]. Furthermore, when misfolded proteins accumulate in the ER, they would be bound by GRP78 and thus to disrupt its interaction with proximal stress sensors: inositol-requiring 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), thereby leading to the UPR in ER [12,43–45] to confer correct folding of newly made proteins. So, GRP78 engineering could improve the yields and quality of TfR-Ab by enhancing the ER protein folding capacity. It is suggested that GRP78 engineering might promote productivity of antibody by maintaining ER function and integrity, ensuring protein folding and protecting cells from misfolded protein toxicity. However, the mechanisms underlying this still need further investigation. The above analysis described here enabled our group to develop apoptosis-resistant CHO cell lines that may facilitate the productivity enhancement of our secreted protein products.

Practical application

This study engineers CHO cells with apoptosis-suppressing genes GRP78 and gains an understanding of apoptosis in CHO in order to develop strategies to delay the onset of apoptosis and concomitantly prolong cell culture. The analysis described here enabled our group to develop apoptosis-resistant CHO cell lines that may facilitate the productivity enhancement of our secreted protein products. GRP78 may be a potential application in the biopharmaceutical industries to prolong culture time of CHO cells.

This work was supported by the Natural Science Foundation of China (no. 81102219); Hi-tech research and development program of China (no. 2012AA02A307), the fundamental research funds for

the central universities of China (no. 2015TS124), and the major research project of Hubei Provincial Department of Education of China (no. D20131103).

The authors have declared no conflict of interest.

5 References

- [1] Jayapal, K. P., Wlaschin, K. F., Yap, M. G. S., Hu, W.-S., Recombinant protein therapeutics from CHO cells—20 years and counting. *Chem. Eng. Prog.* 2007, 103, 40–47.
- [2] Singh, R. P., Al-Rubeai, M., Gregory, C. D., Emery, A. N., Cell death in bioreactors: A role for apoptosis. *Biotechnol. Bioeng.* 1994, 44, 720–726.
- [3] Moore, A., Donahue, C. J., Hooley, J., Stocks, D. L. et al., Apoptosis in CHO cell batch cultures: Examination by flow cytometry. *Cytotechnology* 1995, 17, 1–11.
- [4] Irani, N., Wirth, M., van Den Heuvel, J., Wagner, R., Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction. *Biotechnol. Bioeng.* 1999, 66, 238–246.
- [5] Wlaschin, K. F., Hu, W. S., Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. *J. Biotechnol.* 2007, 131, 168–176.
- [6] Jeong, D. W., Cho, I. T., Kim, T. S., Bae, G. W. et al., Effects of lactate dehydrogenase suppression and glycerol-3-phosphate dehydrogenase overexpression on cellular metabolism. *Mol. Cell. Biochem.* 2006, 284, 1–8.
- [7] Mastrangelo, A. J., Hardwick, J. M., Zou, S., Betenbaugh, M. J., Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. *Biotechnol. Bioeng.* 2000, 67, 555–564.
- [8] Meents, H., Enenkel, B., Eppenberger, H. M., Werner, R. G. et al., Impact of coexpression and coamplification of sICAM and antiapoptosis determinants bcl-2/bcl-x(L) on productivity, cell survival, and mitochondria number in CHO-DG44 grown in suspension and serum-free media. *Biotechnol. Bioeng.* 2002, 80, 706–716.
- [9] Laken, H. A., Leonard, M. W., Understanding and modulating apoptosis in industrial cell culture. *Curr. Opin. Biotechnol.* 2001, 12, 175–179.
- [10] Mori, K., Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000, 101, 451–454.
- [11] Luo, B., Lee, A. S., The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. *Oncogene* 2013, 32, 805–818.
- [12] Hetz, C., The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 2012, 13, 89–102.
- [13] Rao, R. V., Peel, A., Logvinova, A., del Rio, G. et al., Coupling endoplasmic reticulum stress to the cell death program: Role of the ER chaperone GRP78. *FEBS Lett.* 2002, 514, 122–128.
- [14] Kerr, J. F., Wyllie, A. H., Currie, A. R., Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 1972, 26, 239–257.
- [15] Al-Rubeai, M., Singh, R. P., Apoptosis in cell culture. *Curr. Opin. Biotechnol.* 1998, 9, 152–156.
- [16] Wang, M., Zhao, X. R., Wang, P., Li, L. et al., Glucose regulated proteins 78 protects insulinoma cells (NIT-1) from death induced by streptozotocin, cytokines or cytotoxic T lymphocytes. *Int. J. Biochem. Cell Biol.* 2007, 39, 2076–2082.
- [17] Wang, M., Wang, P., Liu, Y. Q., Peng, J. L. et al., The immunosuppressive and protective ability of glucose-regulated protein 78 for improvement of alloimmunity in beta cell transplantation. *Clin. Exp. Immunol.* 2007, 150, 546–552.
- [18] Schamberger, C. J., Gerner, C., Cerni, C., Caspase-9 plays a marginal role in serum starvation-induced apoptosis. *Exp. Cell Res.* 2005, 302, 115–128.
- [19] Zhang, Y., Liu, R., Ni, M., Gill, P., Lee, A. S., Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. *J. Biol. Chem.* 2010, 285, 15065–15075.
- [20] Misra, U. K., Deedwania, R., Pizzo, S. V., Activation and cross-talk between Akt, NF-kappaB, and unfolded protein response signaling in L-N prostate cancer cells consequent to ligation of cell surface-associated GRP78. *J. Biol. Chem.* 2006, 281, 13694–13707.
- [21] Cotter, T. G., al-Rubeai, M., Cell death (apoptosis) in cell culture systems. *Trends Biotechnol.* 1995, 13, 150–155.
- [22] He, S., Yaung, J., Kim, Y. H., Barron, E. et al., Endoplasmic reticulum stress induced by oxidative stress in retinal pigment epithelial cells. *Graefes Arch. Clin. Exp. Ophthalmol.* 2008, 246, 677–683.
- [23] Zhuge, J., Cederbaum, A. I., Serum deprivation-induced HepG2 cell death is potentiated by CYP2E1. *Free Radic. Biol. Med.* 2006, 40, 63–74.
- [24] Kim, S. J., Jung, H. J., Choi, H., Lim, C. J., Glutaredoxin 2a, a mitochondrial isoform, plays a protective role in a human cell line under serum deprivation. *Mol. Biol. Rep.* 2012, 39, 3755–3765.
- [25] Wei, Y. Y., Naderi, S., Meshram, M., Budman, H. et al., Proteomics analysis of Chinese hamster ovary cells undergoing apoptosis during prolonged cultivation. *Cytotechnology* 2011, 63, 663–677.
- [26] Yang, E., Korsmeyer, S. J., Molecular thanatopsis: A discourse on the BCL2 family and cell death. *Blood* 1996, 88, 386–401.
- [27] Arden, N., Betenbaugh, M. J., Life and death in mammalian cell culture: Strategies for apoptosis inhibition. *Trends Biotechnol.* 2004, 22, 174–180.
- [28] Pan, G., O'Rourke, K., Dixit, V. M., Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J. Biol. Chem.* 1998, 273, 5841–5845.
- [29] Sauerwald, T. M., Betenbaugh, M. J., Oyler, G. A., Inhibiting apoptosis in mammalian cell culture using the caspase inhibitor XIAP and deletion mutants. *Biotechnol. Bioeng.* 2002, 77, 704–716.
- [30] Chen, L., Xu, S., Liu, L., Wen, X. et al., Cab45S inhibits the ER stress-induced IRE1-JNK pathway and apoptosis via GRP78/BiP. *Cell Death Dis.* 2014, 5, e1219.
- [31] Johnson, G. G., White, M. C., Wu, J. H., Vallejo, M. et al., The deadly connection between endoplasmic reticulum, Ca²⁺, protein synthesis, and the endoplasmic reticulum stress response in malignant glioma cells. *Neuro Oncol* 2014, 16, 1086–1099.
- [32] Chaudhari, N., Talwar, P., Parimisetty, A., Lefebvre d'Hellencourt, C. et al., A molecular web: Endoplasmic

- reticulum stress, inflammation, and oxidative stress. *Front. Cell. Neurosci.* 2014, 8, 213.
- [33] Zhang, L. H., Zhang, X., Roles of GRP78 in physiology and cancer. *J. Cell. Biochem.* 2010, 110, 1299–1305.
- [34] Misra, U. K., Gonzalez-Gronow, M., Gawdi, G., Hart, J. P. et al., The role of Grp 78 in alpha 2-macroglobulin-induced signal transduction. Evidence from RNA interference that the low density lipoprotein receptor-related protein is associated with, but not necessary for, GRP 78-mediated signal transduction. *J. Biol. Chem.* 2002, 277, 42082–42087.
- [35] Misra, U. K., Gonzalez-Gronow, M., Gawdi, G., Wang, F. et al., A novel receptor function for the heat shock protein Grp78: Silencing of Grp78 gene expression attenuates alpha2M*-induced signalling. *Cell. Signal.* 2004, 16, 929–938.
- [36] Delpino, A., Castelli, M., The 78 kDa glucose-regulated protein (GRP78/BIP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. *Biosci. Rep.* 2002, 22, 407–420.
- [37] Kern, J., Untergasser, G., Zenzmaier, C., Sarg, B. et al., GRP-78 secreted by tumor cells blocks the antiangiogenic activity of bortezomib. *Blood* 2009, 114, 3960–3967.
- [38] Placido, A. I., Oliveira, C. R., Moreira, P. I., Pereira, C. M., Enhanced amyloidogenic processing of amyloid precursor protein and cell death under prolonged endoplasmic reticulum stress in brain endothelial cells. *Mol. Neurobiol.* 2015, 51, 571–590.
- [39] Evans, J. L., Goldfine, I. D., Maddux, B. A., Grodsky, G. M., Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* 2003, 52, 1–8.
- [40] Hitomi, J., Katayama, T., Taniguchi, M., Honda, A. et al., Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci. Lett.* 2004, 357, 127–130.
- [41] Reddy, R. K., Mao, C., Baumeister, P., Austin, R. C. et al., Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: Role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.* 2003, 278, 20915–20924.
- [42] Rao, R. V., Bredesen, D. E., Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Curr. Opin. Cell Biol.* 2004, 16, 653–662.
- [43] Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. et al., Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* 2000, 2, 326–332.
- [44] Lee, A. S., The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods* 2005, 35, 373–381.
- [45] Kimata, Y., Kohno, K., Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Curr. Opin. Cell Biol.* 2011, 23, 135–142.