Effect of HRE and *Bcl-2* on the Production of Plasminogen Activator in CHO cells

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Abstract

CHO (Chinese hamster ovary) cells were transfected with plasmids containing both *cis*-acting HRE (hypoxia response element) and CMV-promoter that controls tissue-type plasminogen activator (t-PA). CHO cells with HRE produced 16.2 fold higher t-PA concentration than CHO cells without HRE. It was noted that hypoxia strongly induced CHO cell apoptosis, which resulted in decrease of cell viability and protein production. In this study, by introducing *Bcl-2*, anti-apoptotic gene, we tried to recover cell viability and increase the protein production. When batch culture of both control cells without transfection of *Bcl-2* and cells transfected with *Bcl-2* were performed in the absence of $CoCl_2$, hypoxia mimic condition, the cells with *Bcl-2* were effected specific cell growth rates, maximum cell density. Immunoblotting assay showed Bcl-2 was recombinant with HRE dependent t-PA expression cassette, and their expression level was depended on hypoxia. By introducing *Bcl-2*, both cell viability and maximum cell density could be increased.

Introduction

Animal cells have merits for production of bio-pharmaceutical proteins. But, the production cost in this system is higher than in microorganism or plant cells. Also, animal cells are inevitably involved in apoptosis from various stresses under natural culture condition, such as hyperoxia, hypoxia, glutamine deprivation, glucose deprivation and serum limitation (1). Apoptosis affects both cell viability and protein productivity. Among the stresses, hypoxia induces a transcription factor, HIF-1, and activates hypoxia dependent genes by binding to HRE within control region (2). CHO cell lines expressing t-PA were developed by introducing HRE ahead of CMV promoter. In this work, the effect of Bcl-2 was investigated for cell growth and protein production. It was reported that cell death from hypoxia was prevented by introducing antiapoptotic gene, *Bcl-2,* which does not involve an oxidative reactive pathway (1,3). The effect of Bcl-2 was also investigated for the increase of cell viability in hypoxia condition.

Materials and Methods

Cell culture and stable cell line

Dihydroforate reductase (*dhfr*)-deficient CHO (CRL-9096, ATCC) cells were maintained with Iscoves modified Dulbelccoss medium (Gibco-BRL) containing 4 mM L-glutamine, 3.024 g/L sodium bicarbonate, 0.1 mM hypoxanthine, and 0.016 mM thymidine and supplemented with 10 % fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin in a humidified atmosphere of 5 % CO_2 in air at 37 °C. Plasmids containing *t-PA* gene and *dhfr* gene or *Bcl-2* gene were transfected into CHO *dhfr* cells using lipofectamine (Gibco-BRL) according to the manufactures instructions. Two days after transfection, resistant cells were selected with 600 μg/ml of G418 (Gibco-BRL) for two weeks, then adapted to growth media. Regarding recombinant gene amplification, cells were cultured in MEM-α medium (Gibco-BRL) without deoxy- and ribonucleotieds, and adapted to by adding 20 μM methotrexate (MTX) (Sigma), a inhibitor of *dhfr* protein in *de novo* pathway.

Construction of t-PA expression vector containing HRE

A synthetic HRE duplexes (2) are designed to make a consensus sequence for binding of HIF-1, 5-TACGTGCT-3, including 5-GATC-3 identified to cohesive end of *Bgl*II and *BamH*I and also two restriction enzyme sites, *EcoR*V and *Sma*I. A HRE fragment was cloned into both pSV-dhfr (ATCC 37146) and pCIneo (Promega) digested with *Bam*HI, *Bgl*II respectively. The resulting recombinant plasmids were designated as pSV-dhfr/HRE and pCIneo/HRE, respectively. Dhfr-HRE fragment, a *Pvu*II/*Eco*RV fragment of pSV-dhfr/HRE vector, was then subcloned into pCIneo/HRE digested with *Eco*RV. The resulting plasmid was designated as pCI/dhfr/2HRE, where each HRE was neighboring reversely. To construct a control vector, a blunted dhfr gene expression cassette from pSV-dhfr digested with *Bam*HI/Klenow/*Pvu*II one by one was ligated into blunted pCIneo digested with *Bgl*II/Klenow in the order of pCI/dhfr. About 1.7-kilobase fragment of t-PA cDNA was obtained from pETPFR (US patent 4,766,075, ATCC 40403), which contained t-PA cDNA from human melanoma cell, by PCR using specific primers (sense primer; 5-GAC GCT GTG AAG CAA TCA TG-3, antisense primer; 5-GAG GAG TCG GGT GTT CCT GGT CA-3) and cloned into the pCR 2.1 vector (Invitrogen) (pCR 2.1/t-PA). The fragment of t-PA gene digested with *Spe*I and *Xho*I was ligated into XbaI/XhoI-digested pCI/dhfr, and pCI/dhfr/2HRE, respectively. The resulting recombinant plasmids was designated as pCI/dhfr/t-PA, and pCI/dhfr/2HRE-t-PA, respectively. *Bcl-2* gene donated from Lee (3) was cloned into pCIneo expression vector (Promega) at *EcoR*I site.

Gel electrophoresis and Immunoblotting of t-PA

To determine the level of t-PA and Bcl-2, cells were incubated with a concentration of 5×10^6 cells in 100 mm dish for 24 hrs and then harvested after 24 hrs with various $CoCl_2$ treatment. Cells were washed 3 times with ice-cold PBS. The pellets were lysed in lysis buffer (50 mM tris-HCl, pH 7.5, 5 mM $MgSO_4$, 0.5 mM EGTA, 0.1% 2-mercaptoethanol, 10 μg/ml leupeptin and aprotinin, and 1 mM PMSF) containing 1%

Nonidet P-40 and placed on ice for 1 hr. Cellular debris was removed by centrifugation in a microfuge at 10,000 x g for 20 min. The supernatants were prepared for SDS-PAGE by addition of 5×SDS sample buffer and boiled for 10 min. Samples (20 μg protein) were resolved in 7.5 and 12.5 % acrylamide gels at 120 V for 1.5 h for t-PA, Bcl-2 protein, respectively and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, pore size = 0.2 mm). On transferal to a PVDF membrane, immunoblotting was performed using rabbit anti-human t-PA antibody (ICN, 1/250). After rocking for overnight at 4°C, the membrane was washed with Tris-buffered saline (TBS) containing 0.1% tween-20. The membrane was washed 3 times with TBS containing 0.1% tween-20 (TTBS) and HRP-conjugated goat anti-rabbit IgG (Santa Cruz, 1/2000) was added. The antigen-antibody complexes were visualized using chemiluminescence (Luminol reagents, Santa Cruz).

Results and Discussion

We developed two cells producing t-PA protein, that controlled by CMV promoter. A CHO cell line (CHO/pCI/2HRE-t-PA) was activated by HRE on the condition of hypoxia while the other cells line (CHO/pCI/t-PA) was not. CHO/pCI/2HRE-t-PA cells produced more 16 folds t-PA than CHO/pCI/t-PA on repeated fed batch cultures in cell immobilization. HRE only, however, could not resolve apoptosis of CHO cells caused by hypoxia. Apoptosis affected cell viability and protein production. In order to resolve apoptosis caused by hypoxia, we transfected both cells with *Bcl-2*, an anti-apoptotic gene.

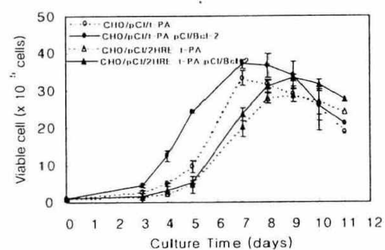

Fig. 1. Profiles of cell growth. Anchoraged cells (1×10^5 cells) was cultured with 3 ml MEM-α medium.

Fig. 1 shows the cell viability of both cells transfected with or without *Bcl-2*. *Bcl-2* gene affected specific cell growth rates and maximum cell density. CHO/pCI/t-PA cells grow faster than CHO/pCI/2HRE-t-PA cells in both case with *Bcl-2* or without *Bcl-2* gene. On transfection with *Bcl-2*, Specific growth rates of CHO/pCI/t-PA cells are higher than those of CHO/pCI/2HRE-t-PA cells. Also maximum cell density was affected with *Bcl-2* gene in both cells. Fig. 2 shows immunoblots against *Bcl-2* protein in both cells which were overexpressed with Bcl-2 (1-4 lane). CHO/pCI/2HRE-t-PA cells were selected more strictly than CHO/pCI/t-PA. Because *Bcl-2* gene was transfected with the same expression vector as t-PA (pCI-neo), both cells were selected at different level with G418. With $CoCl_2$ treatment for 1 day (5-8 lane). CHO/pCI/2HRE-t-PA cells were strongly activated with Bcl-2 (lane 6). It was supposed that *Bcl-2* gene be recombinated with t-PA expression vector depending HRE enhancer. Lane 6 and 8 shows that Bcl-2 was overexpressed on hypoxia condition.

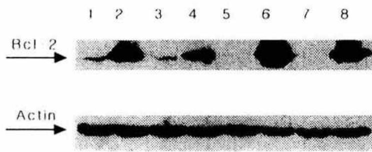

Fig. 2. Immunoblotting of Bcl-2. Cells were treated with $CoCl_2$ for 24hrs. Cell lysates were loaded by 20 μg into each well 1, 5 lane: pCI-2HRE-t-PA; 2, 6 lane: pCI-2HRE-t-PA pCI-Bcl-2; 3, 7 lane: pCI-t-PA; 4, 8 lane: pCI-t-PA, pCI-Bcl-2; 1-4 lane: untreated with $CoCl_2$; 5-8 lane: treated with 200 μM $CoCl_2$.

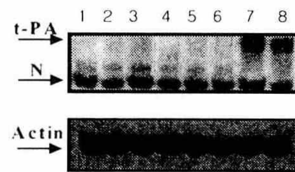

Fig. 3. Immunoblotting of t-PA. Cells were treated with $CoCl_2$ for 24hrs. Cell lysates were loaded by 20 μg into each well.1, 5 lane: pCI-t-PA, 2, 6 lane: pCI-t-PA pCI-Bcl-2, 3, 7 lane: pCI-2HRE-t-PA, 4, 8 lane: pCI-2HRE-t-PA pCI-Bcl-2, 1-4lane: untreated with $CoCl_2$, 5-8 lane: treated with 200 μM $CoCl_2$.

Fig. 3 shows immunoblot against t-PA protein. The low t-PA expression in CHO/pCI/2HRE-t-PA cells indicated that *Bcl-2* might exchange t-PA by recombinant mechanism. Although exchanged with *Bcl-2*, t-PA within CHO/pCI/2HRE-t-PA cell was activated with $CoCl_2$ treatment (7-8 lane). Due to this exchange, the cells transfected with *Bcl-2* (CHO/pCI/2HRE-t-PA pCI/Bcl-2) (8 lane) produced less amount of t-PA than those without transfection of *Bcl-2*. It remains to be solved whether the *Bcl-2* transfection into t-PA producing cells would protect cell death from hypoxia.

References

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