Production of tissue-type plasminogen activator from immobilized CHO cells introduced hypoxia response element

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Abstract

Dissolved oxygen level of cell culture media has a critical effect on cellular metabolism, which governs specific productivity of recombinant proteins and mammalian cell growth. However, in the cores of cell aggregates or cell-immobilized beads, oxygen level frequently goes below a critical level. Mammalian cells have a number of genes induced in the lower level of oxygen, and the genes contain a common *cis*-acting element (-RCGTG-), hypoxia response element (HRE). By binding of hypoxia inducible factor-1 (HIF-1) to the HRE, promoters of hypoxia inducible genes are activated, which is a survival mechanism. In this work, to develop a CHO cell capable of producing recombinant proteins in immobilization and high density cell culture efficiently, mammalian expression vectors containing human tissue-type plasminogen activator (t-PA) gene controlled by HRE were constructed and stably transfected into the CHO cells. In Ba^{2+}-alginate immobilization culture, CHO/pCI/dhfr/2HRE-t-PA cells produced 2 folds higher recombinant t-PA activity than CHO/pCI/dhfr/t-PA cells without $CoCl_2$ treatment. Furthermore, in repeated fed batch culture, productivity of t-PA in immobilized CHO/pCI/dhfr/2HRE-t-PA cells was 121 ng/ml/day, total production of 0.968 mg/day at 11 days culture while CHO/pCI/dhfr/t-PA cells was 22.8 ng/ml/day. All these results indicate that HRE is very useful for the enhancement of protein productivity in mammalian cell cultures.

Introduction

To over-express recombinant protein in bioreactor, high-density cell culture and immobilization of cell in capsules or beads have been commonly used. But cell growth and protein production were often limited by nutrients, serum, growth factors and toxic wastes gradients due to low mass transfer coefficient. Among gradients, amount of diffusible oxygen into beads often determined the productivity of an immobilized cell (1, 2). These diffusive limitations of oxygen inside of beads depend on a multitude of factors such as the oxygen supply, the cellular uptake kinetics, and the dense matrix created by the cells themselves and by the polymeric network of the gel. In this study,

to develop CHO cell line producing recombinant protein through immobilization and high density cell culture, we designed mammalian expression vectors containing diverse copy number of HRE and overexpressed recombinant human t-PA in the immobilized CHO cells. In the culture of immobilized cells culture, the cells containing HRE have higher productivity of recombinant protein than those without HRE on natural culture condition. These results provide the feasibility to use of HRE in the improvement of production of a useful protein in mammalian culture system.

Materials and Methods

Construction of t-PA expression vector containing HRE

Because the protein level from animal cells was very low, we used the strategies for gene amplification. *Dhfr* gene used be commonly, whose protein was essential for *de novo* pathway of nucleotide synthesis, and inhibited by methotrexate (MTX). *dhfr* gene from pSV-dhfr (ATCC 37146) was cloned into pCIneo expression vector(pCI/dhfr). t-PA gene from pETPFR (US patent 4,766,075, ATCC 40403), which contained t-PA cDNA from human melanoma cell, was subcloned 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). This t-PA gene was introduced into pCI/dhfr (pCI/dhfr/t-PA). We prepared 62 bps HRE fragment as described previously (3), which contained consensus sequence for binding of HIF-1, 5′-TACGTGCT-3′, as designated in Fig. 1A. 2 copies of HREs in neighboring reversely were introduced into t-PA and dhfr gene expression vector (pCI/dhfr/2HRE-t-PA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to some modified methods as described previously (3). Briefly, short double-stranded HRE oligonucleotide (W18: 5′-AG CTT GCC CTA CGT GCT GTC TCA G-3′) containing specific HIF-1 binding site were end-labeled using T4 polynucleotide kinase (Takara, Japan) in the presence of 10 μCi[γ-^{32}P] ATP and purified by spin column. Each binding reaction mixture contained 5 μg nuclear extract protein in 10 mM tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 5 % glycerol, 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$ and 0.1 μg of denatured salmon sperm DNA, and was incubated with 30 fmol of labeled duplex on room temperature for 30 min. To test competition experiments, 3 pmol (or 15 pmol) of unlabeled oligonucleotide competitor or mutant double-stranded HRE oligonucleotide (M18: 5-AG CTT GCC CTA AAA GCT GTC TCA G-3: underlined letter indicates mutated site.) were added 10 min before the addition of labeled duplex for 30 min. DNA-protein complexes were separated by electrophoresis on a 6 % nondenaturing polyacrylamide gel.

t-PA assay

The t-PA assay was performed according to some modified protocols as described previously (4). One hundred fifty micro-liters of growth media diluted with T-T buffer (100 mM Tri-HCl, pH 7.5, 0.02 % Triton X-100) were mixed with 10 ml of 1.67 mg/ ml of substrate (dissolved D-valkyl-L-leucyl-L-lysine-nitroanilide dihyrochloride (Fluka) in T-T buffer), 10 μl of 0.2 U/ml plasminogen (Calbio-Chem), and 1 μl of 1 mg/ml fibrinogen, and incubated for 24 hrs at 37 °C. The released concentration of *p*-nitroaniline (pNA) was determined by optical density at 405 nm with microplate reader (Bio-Rad).

Production and cryosection of cell-immobilized beads

The beads were prepared by the conventional Ba^{2+}-alginate methods. Beads were dehydrated with 30 % sucrose and then cryosected with 30 μm thickness at -20 °C using Cryo-microtom (HM505E, Cryostat, Meditech.). The cryosected sheets were stained with Methylene Blue/Basic Fuchsin solution. This solution stained nuclei and cytoplasm, blue and magenta, respectively.

Results and Discussion

Fig. 1 shows the sequences of HRE and the expresstion vector, pCI/dhfr/t-PA, pCI/dhfr/2HRE-t-PA. HRE contained consensus sequence for HIF-1 binding site, and followed other essential site for hypoxic induction. Two copies of HREs were introduced ahead of CMV promoter. *dhfr* gene was used for gene amplification depending the concentration of MTX.

To determine the induction of HIF-1α in CHO cells, EMSA was performed by incubation of W18 with nuclear extracts from CHO cell untreated or treated with $CoCl_2$ for 24 hrs. As shown in Fig. 2, HIF-1 DNA binding activity was strongly induced by treatment of 200 μM $CoCl_2$ to CHO cells (Fig. 2A lane 2) compared with untreated CHO cells as a control (lane 1). However, when the same experiment was done with M18, the HIF-1 DNA binding activity was not detected (data not shown). To assay competition reaction, when an excess amount of unlabeled W18 for competition was added to the reaction mixture, the retarded radioactive band was disappeared (Fig. 2, lane 4 and 5) whereas unlabeled M18 failed to compete

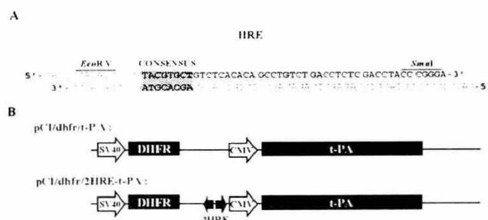

Fig. 1. Sequences of HRE and structures of plasmids. (A) 62 bps oligonucleotides of HRE contained consensus sequence, [illegible], for HIF-1 binding and essential site for hypoxic induction. **(B) Plasmids for t-PA production from CHO cells in culture of immobilization.** Two copies of HREs were neighboring inversely and positioned at ahead of CMV promoter. *Dhfr* genes used for gene amplification by inhibiting with MTX.

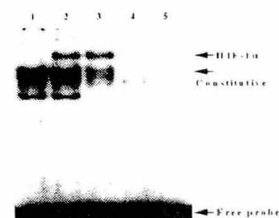

Fig. 2. DNA binding activity of HIF-1α induced by $CoCl_2$ in CHO cells. CHO (5 X 10[illegible]/60 mm dish) cells were split and followed incubation for 24 hrs. After treatment of 200 μM $CoCl_2$ for 24 hrs. Nuclear extracts (5 μg) were incubated with 32P-labeled W18 probe. Competition reaction was performed with unlabeled W18 and M18 oligonucleotides for 10 min at room temperature before adding of labeled probes. lane 1, untreated with $CoCl_2$; lane 2, treated with 200 μM $CoCl_2$; lane 3, treated with 200 μM $CoCl_2$ followed competition with 100 folds molar excess of M18; lane 4, treated with 200 μM $CoCl_2$ followed competition with 100 folds molar excess of W18; lane 5, treated with 200 μM $CoCl_2$ followed competition with 500 folds molar excess of W18.

showing specificity of the binding reaction (Fig. 2, lane 3).

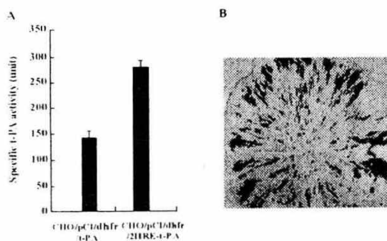

Fig. 3. Production of t-PA in immobilized cells and distribution of cells in bead. (A) CHO pCI dhfr t-PA, CHO pCI dhfr 2HRE-t-PA (2 x 10) immobilized in Ba -alginate (2 mL) macrobeads (3-4 mm in diameter) was complemented with fresh medium every day for 5 days. Ten of eighty beads were harvested and cultured with 2 ml fresh medium for another 2 days. The supernatant was assayed for t-PA activity. (B) The cell-immobilized beads cultured for 13 days were cryosected with 30 um thickness at -20 C using cryo-microtom. The sheets were stained with Methylene Blue Basic Fuchsin.

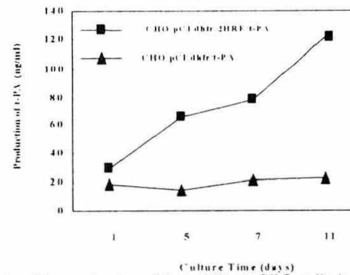

Fig. 4. t-PA production of immobilized CHO cells in repeated fed batch. t-PA expressing suspension adapted CHO cells (1 x 10 cells) were immobilized with 1.5ml alginate. The beads were exchanged with 8 ml fresh medium once every day and exchanged every two days from 17 day. Supernatant was harvested at pointed time and analysed with ELISA kits.

1 x 10^6 cells of CHO/pCI/dhfr/t-PA, or CHO/pCI/dhfr/2HRE-t-PA) was immobilized using Ba^{2+}-alginate methods. Fig. 3A shows production of t-PA from the immobilized cells for 2 day. Conditioned media were exchanged with 8 ml of fresh media every day(D=1 day ^{1}). Fig. 3B shows the photograph of the distribution of cells within beads. Almost cells were dispersed around surface of bead according with oxygen or nutrient gradient. On repeated fed batch culture, production of t-PA was 121 ng/ml/day and 22.8 ng/ml/day, CHO/pCI/dhfr/2HRE-t-PA and CHO/pCI/dhfr/t-PA cells, respectively. t-PA production of CHO/pCI/dhfr/2HRE-t-PA cells was higher above 5 fold than that of CHO/pCI/dhfr/t-PA cells at 11 day. At 20 day, production from CHO cells with HRE was 334.7 ng/m/day, while CHO cells without HRE was 20.1 ng/ml/day yet(data not shown). Maximum production of CHO cells with HRE was 2.7 mg/day.

These data indicates that HRE might effectively increase the protein production in immobilized cell culture. We reversed an adverse condition occurred during cell culture by introducing enhancer. We also propose that many of transcription factors induced from various stress be useful for protein production from animal cell culture

References

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