

## High Productivity of t-PA in CHO Cells Using Hypoxia Response Element

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**Abstract** The dissolved oxygen level of any cell culture environment has a critical effect on cellular metabolism. Specifically, hypoxia condition decreases cell viability and recombinant protein productivity. In this work, to develop CHO cells producing recombinant protein with high productivity, mammalian expression vectors containing a human tissue-type plasminogen activator (t-PA) gene with hypoxia response element (HRE) were constructed and stably transfected into CHO cells. CHO/2HRE-t-PA cells produced 2-folds higher recombinant t-PA production than CHO/t-PA cells in a Ba<sup>2+</sup>-alginate immobilized culture, and 16.8-folds in a repeated batch culture. In a non-aerated batch culture of suspension-adapted cells, t-PA productivity of CHO/2HRE/t-PA cells was 4.2-folds higher than that of CHO/t-PA cells. Our results indicate that HRE is a useful tool for the enhancement of protein productivity in mammalian cell cultures.

**Key words:** Chinese hamster ovary cell, hypoxia, hypoxia inducible factor-1 $\alpha$ , hypoxia response element, immobilized cells, tissue-type plasminogen activator

To produce recombinant proteins in a bioreactor, high-density cell culture and immobilization of cells in capsules or beads have been commonly used [1, 13–15, 18, 35, 36]. Immobilization of cells offers several advantages over conventional suspension culture methods: the achievement of large cell densities, the protection of mammalian cells from shear forces, and the simplification of product purification. However, cell growth and protein production were often limited by the supply of nutrients, serum,

growth factors, and accumulation of toxic wastes due to low mass transfer rates. Among the limitations, the amount of dissolved oxygen in beads often determines the productivity of immobilized cells [8, 22, 28]. Limitation of dissolved oxygen in beads depends on several factors, such as the oxygen supply, the cellular uptake kinetics, and the dense matrix created by the cells and by the polymeric network of the gel. Although diffusivity of oxygen depends on the components of the beads, oxygen is able to diffuse to only a shallow depth [22]. It was also reported that the oxygen consumption rate of entrapped cells is higher than that of free cells [33]. High-density cell culture systems were used in large-scale production of biopharmaceuticals because of their high productivity and efficient consumption of substrates [1]. In these systems, the larger the cell density, the more difficult is the oxygen supply [8].

Oxygen plays a critical biological role as the terminal electron acceptor in mitochondria of cells. Most cells have developed ways to sense alterations in oxygen and acquired the ability to conditionally modulate the expression of genes involved in adaptive physiological responses to hypoxia [21, 31, 34]. The expression of hypoxia inducible genes is controlled by binding of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) to hypoxia response element (HRE), which is in the regulatory region of the genes. HIF-1 $\alpha$ , a subunit of the HIF-1 complex, is induced either under hypoxia condition or by the treatment of hypoxia-mimicking cobaltous ion or desferrioxamine [2, 25, 26, 34]. An HRE was found in the 3'-flanking region of the erythropoietin (*epo*) gene [26]. The HRE regulates the expression of the *epo* gene under hypoxia condition, which maintains the oxygen homeostasis by stimulating the generation of red blood cells. This HRE is composed of functionally tripartite sites, with the first two sites essential for hypoxia inductivity and a third site

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functioning to amplify the induction signal [2, 12, 26]. Recently, a high productivity of recombinant proteins even under low oxygen concentration was studied using the mechanism underlying the hypoxia activation of gene transcription [17, 19, 27].

In this study, to develop a CHO cell line producing human tissue-type plasminogen activator through immobilization or high-density cell culture, we designed mammalian expression vectors containing a diverse copy number of HRE and tried to overexpress a human tissue-type plasminogen activator. In immobilized and non-aerated batch culture, the cells containing HRE showed higher t-PA productivity. Hence, these results provide a feasible application of HRE in the production of recombinant proteins in mammalian cell culture systems.

## MATERIALS AND METHODS

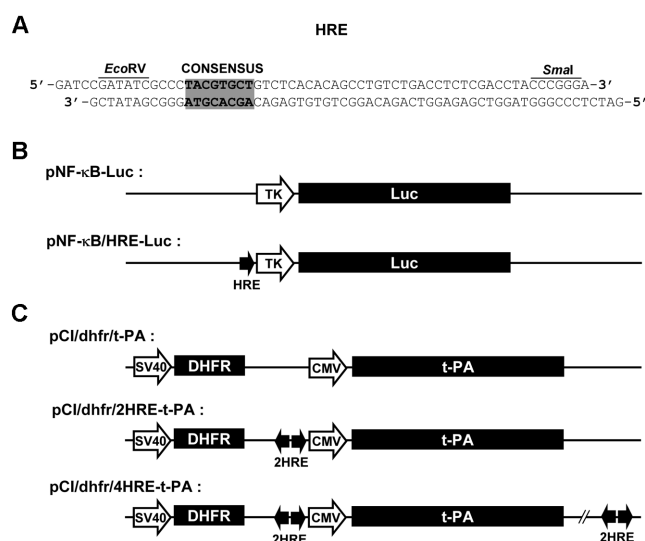
### Cell Culture and Stable Cell Line

Dihydrofolate reductase (*dhfr*)-deficient CHO (CHO *dhfr*<sup>-</sup>, CRL-9096, ATCC) cells were maintained in Iscove's modified Dulbecco's medium (IMDM, Gibco-BRL) containing 4 mM L-glutamine, 3.024 g/l sodium bicarbonate, 0.1 mM hypoxanthine, 0.016 mM thymidine, 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. The plasmid containing *t-PA* controlled with or without HRE was transfected into CHO *dhfr*<sup>-</sup> cells using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Two days after transfection, resistant cells were selected with 600 µg/ml of G418 (Gibco-BRL) for two weeks and then adapted to growth medium.

To amplify the recombinant gene, cells were cultured in MEM-α (Gibco-BRL) without deoxyribo- and ribonucleotides, and then adapted in the media with 0.02, 0.1, 0.5, 1, 5, and 10 µM methotrexate (MTX) (Sigma) [6, 7], as an inhibitor of DHFR protein in the *de novo* pathway. In immobilized and non-aerated high-density batch culture, suspended cells were adapted to CHO-s-SFMII DPMII (Gibco-BRL), a serum-free medium.

### Construction of t-PA Expression Vector Containing HRE

To clone HRE, a 62-bp [11, 12] synthetic HRE duplex was designed to include a consensus sequence for the binding of HIF-1, 5'-TACGTGCT-3', and cohesive sequences at both ends, 5'-GATC-3', identified to restricted sequences with BglII or BamHI and two restriction enzyme sites, EcoRV and SmaI (Fig. 1A). Each strand of HRE obtained from Bioneer Inc. (Korea) was boiled and annealed from 95°C to 25°C for 3 h. After precipitation with ethanol, the HRE fragment could be introduced into the BglII or BamHI site.



**Fig. 1.** Sequences of HRE in the 3' flanking region of the *hEPO* gene and structures of plasmids.

**A.** The 62-bp oligonucleotide of HRE contained a consensus sequence, -TACGTGCT-, for HIF-1α binding. Also, it was appended with synthetic restriction enzyme sites, EcoRV and SmaI. The same cohesive sequences at both ends were protruded as the same as BamHI or BglII. **B.** Plasmids for t-PA production from CHO cells in immobilization and non-aerated batch culture. Two copies of HREs were neighboring inversely and positioned ahead of a CMV promoter and in polyadenylation signal. The *dhfr* gene was used for gene amplification.

To introduce 2 copies of HRE into the *t-PA* gene expression vector, a HRE fragment shown in Fig. 1A was cloned at unique BamHI of pSV-dhfr (ATCC 37146) and at BglII restriction enzyme site of pCIneo (Promega). The resulting recombinant plasmids were designated as pSV-dhfr/HRE and pCIneo/HRE, respectively. The blunted dhfr-HRE fragment, a PvuII/EcoRV fragment of the pSV-dhfr/HRE vector, was then subcloned into pCIneo/HRE digested with EcoRV. The resulting plasmid was designated as pCI/dhfr/2HRE, in which each HRE was neighboring reversely. To make a vector containing four copies of HRE, two copies of HRE fragment eluted from pCI/dhfr/2HRE digested with SmaI were then subcloned into a HpaI site within the SV40 late polyadenylation signal of pCI/dhfr/2HRE (because HRE in the *epo* gene was in the 3' flanking region). The vector containing four copies of HRE was designated as pCI/dhfr/4HRE. To construct a control vector, a blunted *dhfr* gene expression cassette from pSV-dhfr digested with BamHI/Klenow/PvuII was ligated one by one into the blunted pCIneo digested with BglII/Klenow in order. This vector was called pCI/dhfr.

To clone the *t-PA* gene, an approx. 1.7-kbp fragment of *t-PA* cDNA of human melanoma cell was obtained from pETPFR (US patent 4,766,075, ATCC 40403) 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 the *t-PA* gene digested with *SpeI/XhoI* was ligated into pCI/dhfr, pCI/dhfr/2HRE, and pCI/dhfr/4HRE digested with *XbaI/XhoI* (Fig. 1B). The resulting recombinant plasmids were designated as pCI/dhfr/t-PA, pCI/dhfr/2HRE-t-PA, and pCI/dhfr/4HRE-t-PA and the transfectant cells were named CHO/t-PA, CHO/2HRE-t-PA, and CHO/4HRE-t-PA, respectively.

### Electrophoretic Mobility Shift Assay (EMSA)

In order to detect induction of HIF-1 $\alpha$  with the treatment of CoCl<sub>2</sub>, nuclear extracts were prepared according to the methods as described previously [26]. Briefly, a short double-stranded HRE oligonucleotide (W18: 5'-AG CTT GCC CTA CGT GCT GTC TCA G-3') containing specific HIF-1 $\alpha$  binding site was end-labeled using T4 polynucleotide kinase (Takara, Japan) in the presence of 10  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] ATP and purified by spin column. Each binding reaction mixture contained 5  $\mu$ g of nuclear extract proteins 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<sub>2</sub>, and 0.1  $\mu$ g of denatured salmon sperm DNA, and was incubated at room temperature for 30 min. To perform competition experiments, 3 pmol (100 $\times$ ) or 15 pmol (500 $\times$ ) of unlabeled W18 oligonucleotide or 3 pmol (100 $\times$ ) of unlabeled M18 (5'-AG CTT GCC CTA AAA GCT GTC TCA G-3': underlined letters indicate mutated site) were reacted for 10 min before the addition of 30 fmol of labeled duplex for 30 min. DNA-protein complexes were separated by electrophoresis on 6% nondenaturing polyacrylamide gel.

### Determination of t-PA mRNA Expression in a Hypoxia-Mimic Condition

Total RNA was extracted by using RNA sol (Gibco-BRL) at the indicated time points after exposure to hypoxia-mimic conditions for 24 h, and the first cDNA was prepared by reverse transcriptase with 1  $\mu$ g of total RNA using a random primer kit (Perkin-Elmer). PCR was performed with specific primers located within the *t-PA* gene (P1; 5'-GAC GCT GTG AAG CAA TCA TG-3' in exon 2, P2; 5'-GAG GCT GGC TGT ACT GTC-3' in exon 10) with the thermal cycle profile; 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. In order to normalize the level of expression, co-amplification of the  $\beta$ -actin gene was performed with the appropriate primers oligo 1 (5'-GAT GAC GAT ATC GCT GCG CT-3') and oligo 2 (5'-GAT CAT TGC CGA ATA GTG ATG GAC CT-3'). The PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized with a UV illuminator.

### t-PA Activity Analysis

The t-PA activity analysis was performed according to the protocols described previously [6, 7]. The conditioned medium was centrifuged to remove cell debris, and 150  $\mu$ l

of growth media diluted (1:1 volume ratio) with T-T buffer (100 mM Tris-HCl, pH 7.5, 0.1% Tween 80, 0.15 M NaCl) were mixed with 10  $\mu$ l of 1.67 mg/ml of substrate [D-vakyl-L-leucyl-L-lysine-nitroanilide dihydrochloride (Fluka)] in T-T buffer, 10  $\mu$ l of 0.2 U/ml plasminogen (Calbio-Chem), and 1  $\mu$ l of 1 mg/ml fibrinogen, and then incubated for 24 h at 37°C. The quantity of released *p*-nitroanilide was converted to t-PA production; that is, 400 IU equal to 1  $\mu$ g of t-PA (Sigma). To determine t-PA production from CHO cells, the supernatants were assayed with an ELISA kit (Biopool, U.S.A.) according to the manufacturer's instructions.

### Gel Electrophoresis and Immunoblotting of t-PA

t-PA-producing cells at a concentration of  $5 \times 10^6$  cells/8 ml MEM- $\alpha$  were incubated in a 100-mm dish for 24 h and then harvested 24 h after the treatment of various concentrations of CoCl<sub>2</sub>. The cells were washed 3 times with ice-cold PBS. The pellets were lysed with a lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgSO<sub>4</sub>, 0.5 mM EGTA, 0.1% 2-mercaptoethanol, 10  $\mu$ g/ml leupeptin and aprotinin, and 1 mM PMSF) containing 1% Nonidet P-40 and placed on ice for 1 h. Cellular debris was removed by centrifugation in a microcentrifuge at 13,000 rpm for 20 min. The supernatants were prepared for SDS-PAGE by the addition of 5 $\times$ SDS sample buffer and boiled for 10 min. Samples (20  $\mu$ g protein) were resolved on 7.5% acrylamide gels at 150 V for 1.5 h and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, pore size=0.2  $\mu$ m). On transfer to the PVDF membrane, immunoblotting was performed using rabbit anti-human t-PA antibody (ICN, 1/250 dilution). After rocking overnight at 4°C, the membrane was washed 3 times with TBS containing 0.1% tween-20 (TTBS), and HRP-conjugated goat anti-rabbit IgG (Santa Cruz, 1/2,000) was added. The antigen-antibody complexes were visualized using chemiluminescence (Luminol reagents, Santa Cruz).

### Immobilized Cell Culture

The cell-immobilized beads were prepared by the conventional Ba<sup>2+</sup>-alginate method [10] with some modifications. Since steam sterilization causes hydrolysis of alginate, filter sterilization was used for alginate preparation. The solution composed of both 0.15 M glucose and 1/2 diluted IMDM was mixed with 2% alginate solution containing suspension-adapted t-PA expressing CHO cells [3]. After gentle mixing, these viscous solutions were dropped into a gently stirred 0.12 M BaCl<sub>2</sub> solution through a needle. After hardening in new 0.12 M BaCl<sub>2</sub> solution for 40 min, the beads were washed 3 times and suspended with CHO-s-SFM II-DPM II (Gibco-BRL).

### Bead Staining with Methylene Blue/Basic Fuchsin

The cell-immobilized beads were washed and fixed with 4% formaldehyde for 1 h. Then, the beads dehydrated with

30% sucrose in CHO-s-SFM II DPM were cryosected with 30- $\mu$ m thickness at  $-20^{\circ}\text{C}$  using Cryo-microtome (HM505E, Cryostat, Mediatech.). The cryosected sheets were stained with methylene blue/basic fuchsin solution [5]. The solution stained nuclei and cytoplasm into blue and magenta, respectively.

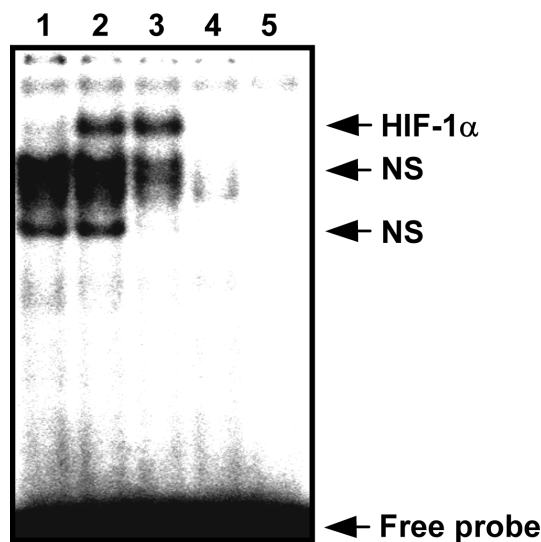
### Non-Aerated High-Density Culture

Suspension-adapted t-PA-expressing CHO cells ( $8 \times 10^6$ ) were cultured in tightly capped 15-ml tubes with 4 ml of CHO-s-SFM II-DPM II (Gibco-BRL) for 2 days in a  $37^{\circ}\text{C}$   $\text{CO}_2$  incubator. There was no exchange of any gas and all the tubes were set vertically. The culture was then centrifuged, and the supernatant was used for t-PA assay. The t-PA assay was performed as described above.

## RESULTS

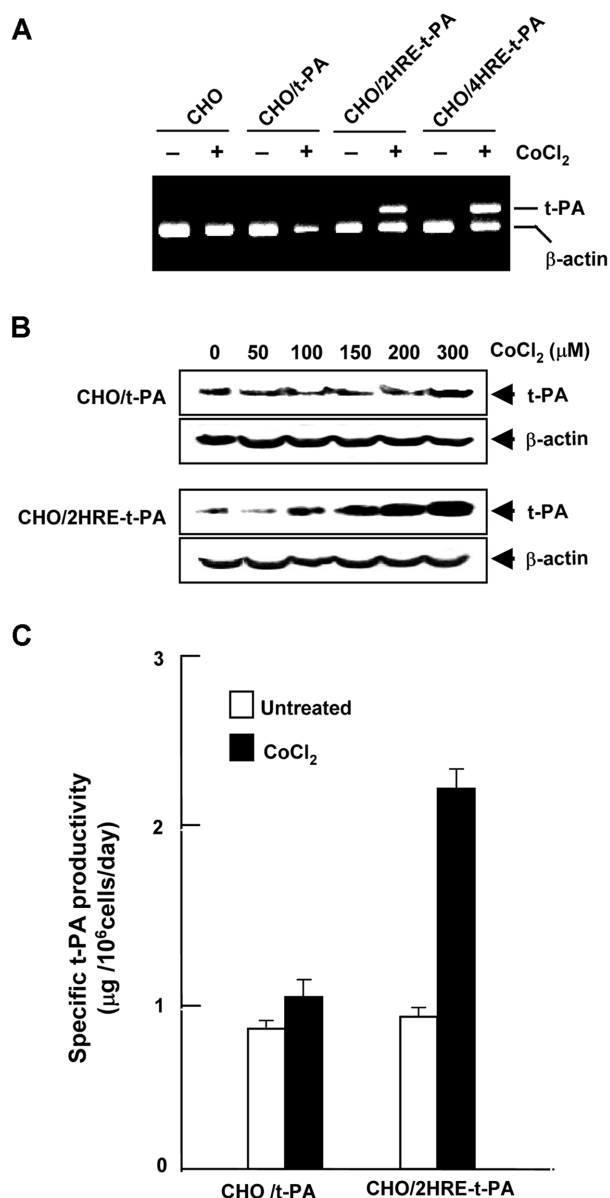
### DNA Binding Activity of HIF-1 $\alpha$ by $\text{CoCl}_2$

To determine the induction of HIF-1 $\alpha$  in CHO cells, an EMSA was performed by incubating W18 with nuclear extracts from CHO cells untreated or treated with  $\text{CoCl}_2$  for 24 h. As shown in Fig. 2A, HIF-1 $\alpha$  DNA binding activity was strongly induced by the treatment of CHO



**Fig. 2.** DNA binding activity of HIF-1 $\alpha$  induced by  $\text{CoCl}_2$  in CHO cells.

CHO ( $5 \times 10^5/60$  mm dish) cells were split, followed by incubation for 24 h. After treatment with 200  $\mu\text{M}$   $\text{CoCl}_2$  for 24 h, nuclear extracts (5  $\mu\text{g}$ ) were incubated with a  $^{32}\text{P}$ -labeled W18 probe. Competition reaction was performed with unlabeled W18 and M18 oligonucleotides for 10 min at room temperature before adding labeled probes. Lane 1: untreated with  $\text{CoCl}_2$ ; lane 2: treated with 200  $\mu\text{M}$   $\text{CoCl}_2$ ; lane 3: treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  followed by competition with 100-fold molar excess of M18; lane 4: treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  followed by competition with 100-fold molar excess of W18; lane 5: treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  followed by competition with 500-fold molar excess of W18.



**Fig. 3.** Expression level of t-PA induced by  $\text{CoCl}_2$  treatment, revealed by RT-PCR and Western blot.

CHO and t-PA-expressing CHO cell lines were treated with (+) or without (–) 200  $\mu\text{M}$   $\text{CoCl}_2$  for 24 h. Total RNAs were harvested according to the manufacturer's instructions. RT-PCR was performed as described in Materials and Methods.  $\beta$ -Actin probes were used as control. CHO: lanes 1, 2; CHO/t-PA: lanes 3, 4; CHO/2HRE-t-PA: lanes 5, 6; CHO/4HRE-t-PA: lanes 7, 8. **B.** Western blot analysis of t-PA protein level in CHO/t-PA cells (upper), and in CHO/2HRE-t-PA cells (lower). Cell lysates were prepared from CHO cells cultured with 0, 50, 100, 150, 200, and 300  $\mu\text{M}$   $\text{CoCl}_2$  (lanes 1–6) for 24 h. Cell lysates (20  $\mu\text{g}$ ) were fractionated by SDS-PAGE and incubated with a 1:250 dilution of purified antibodies raised against recombinant melanoma t-PA (top).  $\beta$ -Actin was blotted as control (bottom). **C.** Production of t-PA by treatment of  $\text{CoCl}_2$  on t-PA-expressing CHO cells. After 24 h of incubation, CHO cells with or without HRE were treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  for another 24 h. Cell cultured medium was harvested, and production of t-PA was assayed with chromogenic substrates. One unit was defined as the concentration of enzyme to hydrolyze 1 pmol of chromogenic substrate, *p*-Val-Leu-Lys-pNA dihydrochloride.

cells with 200  $\mu\text{M}$   $\text{CoCl}_2$  (Fig. 2, lane 2), compared with untreated cells. When an excess amount of unlabeled W18 for competition was added to the reaction mixture, the retarded radioactive band gradually disappeared as the concentration of W18 was higher (Fig. 2, lanes 4 and 5), whereas unlabeled M18 failed to compete. This showed the specificity of the binding reaction (Fig. 2, lane 3). Nonspecific DNA binding activities disappeared in the competition reaction. This was attributed to reaction with unlabeled W18 with higher binding activity and M18 with lower binding activity. These results indicate that the CHO cells have a physiological response to defend cell damage in the hypoxia-mimic condition by inducing HIF-1 $\alpha$  transcription factor.

### HRE-Dependent Expression of Recombinant t-PA

To show the validity of the use of HRE for the production of recombinant t-PA in high-density culture and immobilized cell culture, we established the recombinant CHO cells through the introduction of recombinant HRE-dependent t-PA expression plasmids, shown in Fig. 1B. In order to determine the t-PA expression regulated by hypoxia, the t-PA-expressing cells were treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  for 24 h, and then RT-PCR was performed with 1  $\mu\text{g}$  of total RNA using t-PA specific primers as described in Materials and Methods (Fig. 3A). When not treated with  $\text{CoCl}_2$ , the 938-bp PCR product was not detected in all CHO/t-PA, CHO/2HRE-t-PA and CHO/4HRE-t-PA cells. When treated with 200  $\mu\text{M}$   $\text{CoCl}_2$  for 24 h, however, PCR products were intensively detected only in CHO/2HRE-t-PA and CHO/4HRE-t-PA. CHO/4HRE-t-PA cells showed slightly more

PCR product than CHO/2HRE-t-PA. The number of HRE was attributed to the transactivity of HIF-1 $\alpha$  in hypoxia-mimic condition. However, two copies of HRE within the SV40 late polyadenylation signal did not mathematically increase the transactivity of HIF-1 $\alpha$ .

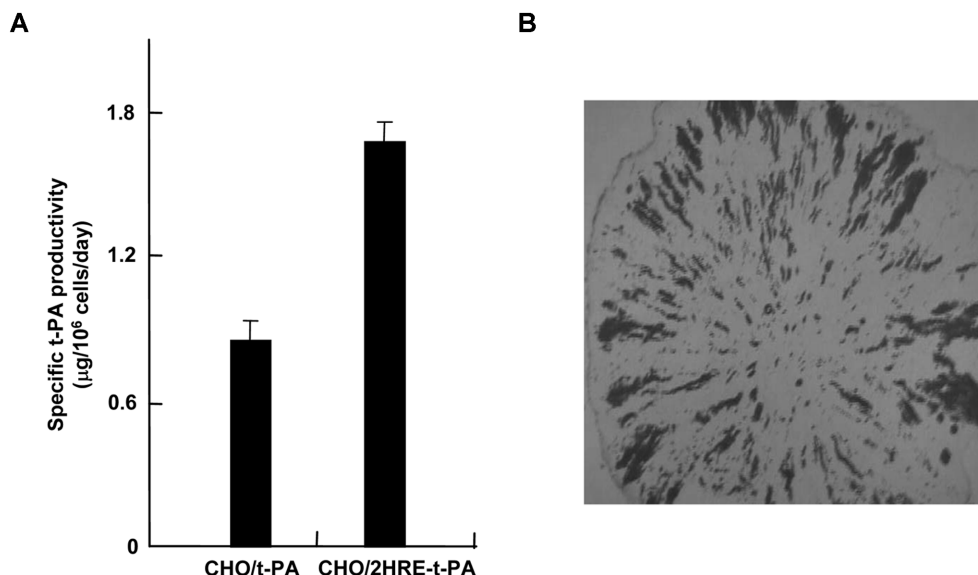
In order to further confirm that the enhancement of t-PA production was regulated by HRE, CHO cells harboring the t-PA gene with or without HRE were treated with various concentrations of  $\text{CoCl}_2$ . Then, the expression level of t-PA was determined by immunoblotting using polyclonal antibody against t-PA. As shown in Fig. 3B, the expression levels of CHO/2HRE-t-PA cells were proportionally increased with  $\text{CoCl}_2$  concentration, whereas those were not changed in CHO/t-PA cells.

Figure 3C shows the specific t-PA productivity of CHO cells harboring the t-PA gene with or without HRE. CHO/2HRE-t-PA cells showed 2-folds higher t-PA productivity than that of CHO/t-PA cells, which coincided with the results of RT-PCR and immunoblotting experiments described above. When not treated with  $\text{CoCl}_2$ , both cell lines maintained a similar level of t-PA activity.

### Production of t-PA in Immobilized Cell Culture

High-density cell culture and immobilized cell culture have been used to produce recombinant protein in large production. Therefore, in this work to inspect hypoxia induction in macrobeads without  $\text{CoCl}_2$ , we immobilized CHO cells with  $\text{Ba}^{2+}$ -alginate.

$\text{Ba}^{2+}$ -alginate macrobead was prepared with 3.5 mm diameter. After immobilization, CHO/t-PA or CHO/2HRE-



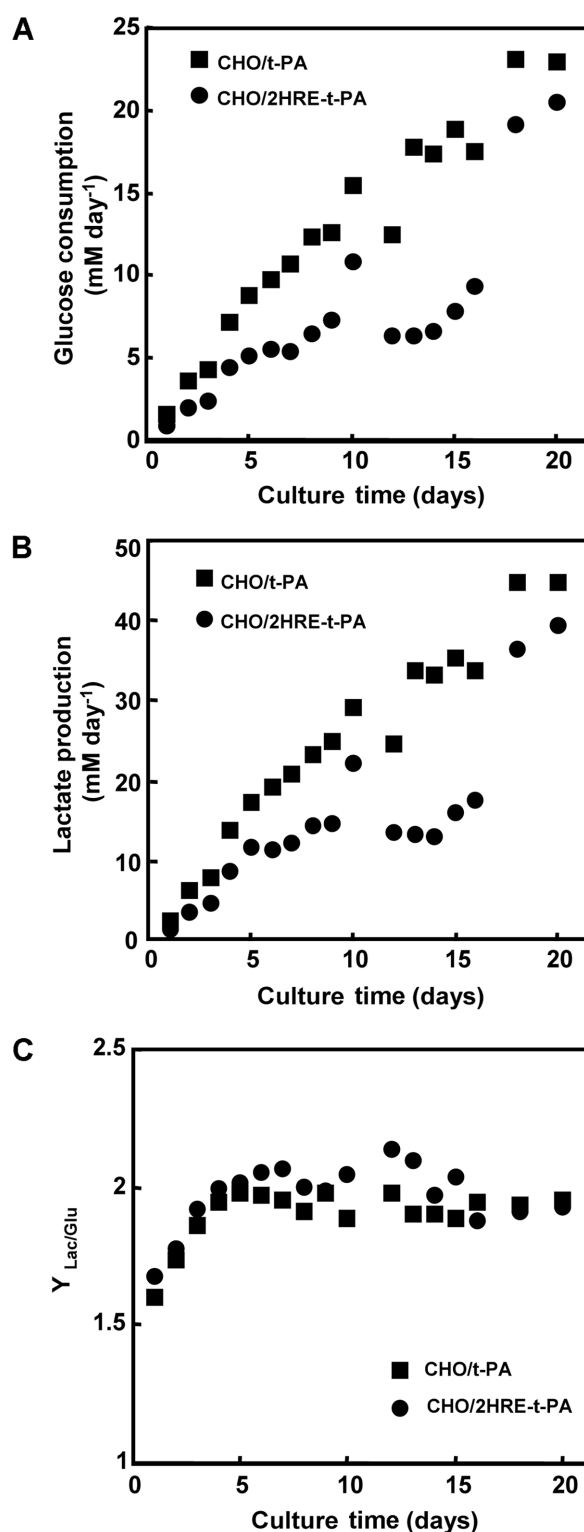
**Fig. 4.** Production of t-PA in immobilized cells and distribution of cells in bead.

**A.** CHO/t-PA and CHO/2HRE-t-PA ( $2 \times 10^7$ ) cells immobilized in  $\text{Ba}^{2+}$ -alginate (2 ml) macrobeads (3–4 mm in diameter) were refreshed daily with CHO-s-SFMII-DPMII medium for 5 days. Ten of eighty beads were harvested and cultured with 2 ml of fresh medium for another 2 days. The supernatants were assayed for t-PA production. **B.** The cell-immobilized beads cultured for 13 days were cryosected with 30 mm thickness at  $-20^\circ\text{C}$  using Cryo-microtome. The sheets were stained with methylene blue/basic fuchsin.

t-PA ( $2 \times 10^7$  cells/8 ml CHO-s-SFMII-DPMII) was cultured for 5 days for maximum cell viability with medium daily refreshed. Then, ten of eighty beads were harvested and cultured with 2 ml of fresh medium for another 2 days to assay t-PA production. As shown in Fig. 4A, CHO/2HRE-t-PA cells produced 1.95-folds higher t-PA than CHO/t-PA cells, indicating that an oxygen gradient occurred and the transactivity of HIF-1 $\alpha$  existed within the beads. Riley *et al.* [22] reported that oxygen penetrates to a maximum depth of about 0.4 mm under typical culture conditions. In our experiments, the beads had a diameter of 3.5 mm, and the denser cell densities on outer beads surfaces might inhibit some oxygen penetration. Accordingly, the interior cells inside a 0.4-mm depth receive an insufficient supply of oxygen. Although we could not suggest that the data indicate the minimal depth for hypoxia to occur, it is quite possible that there exists an oxygen gradient in the interior bead.

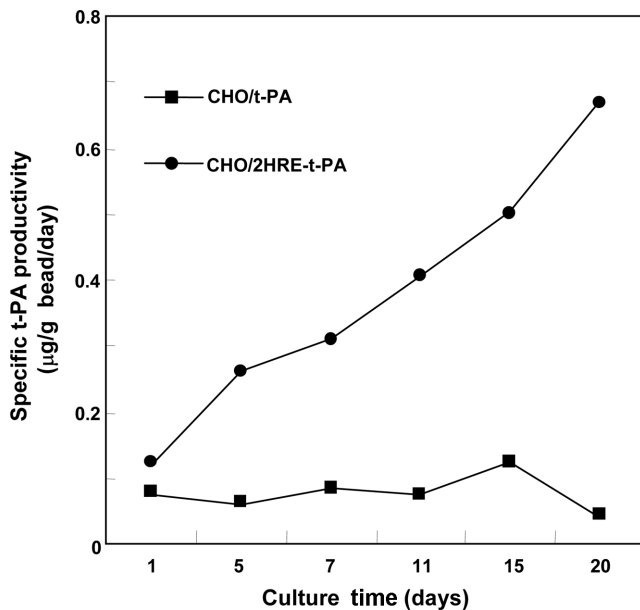
Figure 4B shows the cell distribution within the beads cultivated with daily refreshed medium for 13 days. Viable cells are shown as dark blue, and a number of viable cells decreased from the surface of beads to the core. Most viable cells were concentrated near the bead surface. This result indicates that the cells near the center experienced hypoxia and died.

Figure 5 shows the profile of immobilized cells cultivated for 20 days. Both CHO/2HRE-t-PA and CHO/t-PA ( $1 \times 10^6$ ) cells were immobilized in 2 ml of alginate solution, which resulted in producing about 60 beads. After 2 days of cultivation, the beads were exchanged with 8 ml of fresh medium everyday ( $D=1/\text{day}$ ) at the beginning of culture and every two days ( $D=0.5/\text{day}$ ) from day 17. Figure 5 shows the metabolic profiles of glucose and lactate. Consumption of glucose in CHO/t-PA cells constantly increased at the beginning of cultivation and then maintained the first static state of 17.8 mM/day from 13 days to 17 days, of which consumption level was 77% of medium added. When sampled at every two days with exchange of 8 ml every two days ( $D=0.5/\text{day}$ ), glucose was found to be totally deprived. On the other hand, in CHO/2HRE-t-PA cells, glucose consumption was maintained only at 6.7 mM/day glucose from 5 days until the end of cultivation of  $D=1/\text{day}$ . After 17 days, with  $D=0.5/\text{day}$ , glucose consumption increased to 86% of medium added. CHO/t-PA cells consumed about 1.7 times more total glucose than CHO/2HRE-t-PA cells throughout the cultivation period. The profile of lactate production is shown in Fig. 5B. Lactate production was accompanied by a corresponding consumption of glucose in both cell lines. Both cell lines showed different cell physiology. CHO/2HRE/t-PA cells consumed less glucose and produced less lactate than CHO/t-PA cells. Cell growth of CHO/2HRE-t-PA was slower than that of CHO/t-PA (data not shown). Figure 5C shows the yield of lactate from glucose. This yield was used as an index for the level of aerobic or anaerobic



**Fig. 5.** Profiles of glucose consumption (A), lactate production (B), and lactate yield from glucose (C) in repeated batch culture. t-PA-expressing suspension-adapted CHO cells ( $1 \times 10^6$ ) were immobilized with 1.5 ml of alginate solution.

The beads were exchanged with 8 ml of fresh medium every day until 17 days and exchanged every two days from day 17. Supernatants were harvested at indicated times and assayed with YSI analyzer.

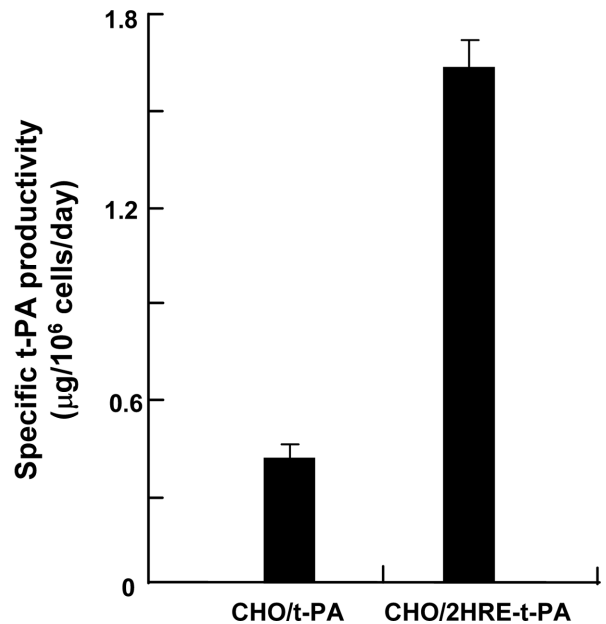


**Fig. 6.** t-PA production of immobilized CHO cells in repeated batch culture. t-PA-expressing suspension-adapted CHO cells ( $1 \times 10^6$ ) were immobilized with 2 ml of alginate solution. The beads were exchanged with 8 ml of fresh medium every day until 17 days and exchanged every two days from day 17. Supernatants were harvested at indicated times and analyzed by ELISA.

respiration [8]. The ratio of the two cell lines increased from the beginning of the culture until 5 days and was maintained thereafter at maximum theoretical yield, 2 mol of lactate per 1 mol of glucose, until the end of the culture. This result indicates that most cells respired through an anaerobic pathway after 5 days. Compared with CHO/t-PA, the yield of lactate in CHO/2HRE-t-PA cells was higher. Semenza *et al.* [24, 25] and Wang *et al.* [32] reported that hypoxic cells required more energy metabolism through glycolysis enzymes. Several enzymes related to glucose consumption and lactate production have a site for HIF-1 $\alpha$  binding activity. Figure 6 shows the production of t-PA in both cell lines. CHO/2HRE-t-PA cells increased the t-PA production from initial cultivation, and the t-PA productivity was 0.675  $\mu\text{g/g}$  bead/day at 20 days, while the productivity of CHO/t-PA cells was 0.04  $\mu\text{g}/10^6$  cells/day. Therefore, the t-PA productivity was increased 16.8 times with the introduction of HRE.

#### Production of t-PA in Non-Aerated High-Density Culture

In order to demonstrate the induction of hypoxia in high-density cell culture, which is one of the most common methods for large-scale production of proteins, suspension-adapted t-PA-expressing CHO cells ( $8 \times 10^6$ ) were cultured in 15-ml tightly capped tubes with 4 ml of CHO-s-SFM II-DPM II (Gibco BRL) medium for 2 days. There was no air entrance and exit, except oxygen dissolved in medium and oxygen in the tube before capping. Furthermore, because



**Fig. 7.** Production of t-PA in non-aerated high-density culture. CHO/t-PA or CHO/2HRE-t-PA ( $8 \times 10^6$ ) suspension-adapted CHO cells were cultured in 15-ml tubes tightly capped with 4 ml of CHO-s-SFM II-DPM II (Gibco-BRL) for 2 days at 37°C in a CO<sub>2</sub> incubator. Then, the cultured medium was harvested and centrifuged. Production of t-PA was assayed with chromogenic substrates.

solubility of oxygen in the medium was low, the oxygen in the tube was not able to diffuse into cells precipitated gravitationally. Hence, cells experience oxygen limitation after a certain period of time, and hypoxia condition leads to apoptosis. As shown in Fig. 7, CHO/2HRE-t-PA produced 4.2-folds higher t-PA than CHO/t-PA cells.

#### DISCUSSION

Mammalian cells are very sensitive to environmental changes during cell culture, such as nutrients, serum, pH, temperature, osmolality, shear, oxygen, and toxic metabolites. Therefore, to enhance productivity, recombinant techniques have been exploited for cell survival and modification of cell physiology [17, 19, 30].

In this study, we constructed plasmids containing the t-PA gene modulated by 2 or 4 copies of HRE. RT-PCR and immunoblotting indicated that CHO cells with HRE showed high protein productivity when treated with CoCl<sub>2</sub>. Although we did not show direct evidence of HIF-1 $\alpha$  induction within beads and high-density culture, natural hypoxia induction during cell immobilization and non-aerated high-density cultivation enhanced the productivity 16.8 times and 4.2 times in the cells introduced with HRE-regulating t-PA gene, respectively. With a long period of culture, HIF-1 $\alpha$  accumulated in the cells within the interior beads. It is quite likely that some of the HIF-1 $\alpha$  increased

transcription levels of the *t-PA* gene. It could also be possible that something other than HIF-1 $\alpha$  induced cell apoptosis [4], and cell disruption, which increased t-PA productivity.

HIF-1 $\alpha$  is able to induce the expression of HRE containing target genes within their transcriptional promoters. These target genes promote cell survival by delivery of O<sub>2</sub> to oxygen-deprived tissues (e.g., erythropoietin), promoting the formation of new vasculature (e.g., vascular endothelial growth factor), increasing glucose transport (e.g., glucose transporter-1), and increasing glycolysis flux (e.g., lactate dehydrogenase). However, when cells are exposed to chronic or extreme hypoxia, or the protective adaptive mechanism initiated by HIF-1 $\alpha$  is not sufficient, apoptosis results [23]. Surprisingly, HIF-1 $\alpha$  has been proved to play a role in hypoxia-mediated apoptosis [4]. Embryonic stem cells in which the gene encoding HIF-1 $\alpha$  has been disrupted do not undergo apoptosis in response to hypoxia.

Masuda *et al.* [17] proposed the use of HRE and a promoter of the *lactate dehydrogenase A* gene, a hypoxia-inducible gene, instead of a CMV promoter, and they could increase EPO production 6–10-folds in a 2% oxygen condition, compared with that in a 21% oxygen condition. Goldberg *et al.* [9] reported that EPO mRNA stability in Hep 3B cells was altered by changes of oxygen tension. This would amplify the effect of hypoxia or hypoxia-mimic treatment on *epo* gene transcription and lead to a greater fold induction of EPO mRNA.

Although the productivity of t-PA was increased by introducing HRE in our studies, many cells under hypoxic condition died from apoptosis. Hence, it is necessary to supersede apoptosis in a hypoxic condition. It has been reported that the cell survival in response to various stresses including hypoxia was enhanced by overexpressing the *bcl-2* family member [16, 30]. Also, it is necessary to study for large-scale production fed-batch culture, perfusion culture using CHO cell with HRE and the anti-apoptotic family.

Based on the present results, HRE appears to be a very useful element for overproduction of recombinant proteins in CHO cells under different culture systems.

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