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High-Level Expression of Recombinant Human Interleukin-2 in Chinese Hamster Ovary Cells Using the Expression System Containing Transcription Terminator

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Abstract Many biological properties and the clinical potential of human interleukin-2 (hIL-2) draw much attention to its high-level expression in mammalian cells. Recombinant human IL-2 (rhIL-2) was expressed in Chinese hamster ovary (CHO) cells, using the recently developed expression system which confers position-independent expression. Stable CHO cell lines carrying several hundred amplified copies of the rhIL-2 gene were easily obtained and rhIL-2 was expressed at high levels after selection with increasing concentrations of methotrexate. Interestingly, the insertion of the transcription terminator of the human gastrin gene into the downstream region of the gene for rhIL-2 considerably increased rhIL-2 expression. Using the expression system with the transcription terminator, it was possible to get a CHO cell line expressing the rhIL-2 at a very high level, about 11.4 μg/10^6 cell/day, which is about 6 times higher than that previously reported. The biological activity of the rhIL-2 protein purified from the cell line was also confirmed by the cell proliferation assay.

Key words: IL-2, Chinese hamster ovary (CHO) cell, transcription terminator, expression

Interleukin-2 (IL-2), formerly referred to as T cell growth factor, is a lymphokine which is produced by lectin- or antigen-activated T cells. It has been reported that IL-2 has many biological activities such as induction of cytotoxic cells, activation of natural killer cells, enhancement of interferon-γ production by T cells, and stimulation of lymphokine-activated killer cells [9, 10, 13, 20, 21, 27]. IL-2 also has potential as a therapeutic agent in the treatment of cancers and defects in the immunological functions [3, 22]. In addition, it is also used as a tissue culture reagent due to its requirements in the survival of T-lymphocytes in culture. Natural human IL-2 (hIL-2) is a glycoprotein of 133 amino acids, whose molecular weight is heterogeneous, about 15–18 kDa, due to the variation in glycosylation on the threonine residue at position 3 [7]. hIL-2 has three cysteine residues, two of which form a disulfide bond that is required for biological activity. Several groups have cloned the cDNAs coding for hIL-2 and expressed it in many different systems such as *E. coli*, insect cells, and mammalian cells [8, 18, 31]. Recombinant hIL-2 (rhIL-2) produced in mammalian cells has the same glycosylation patterns as the natural form [23]. rhIL-2 produced in *E. coli* or insect cells is not glycosylated, but it has comparable biological activities to those of the natural hIL-2 or rhIL-2 produced in mammalian cells, suggesting that the post-translational modification by glycosylation may not contribute to the biological activities [23, 28]. However, glycosylation of hIL-2 has been suggested to play a role in clonal out-growth and long-term propagation of allo-activated human T cells [25]. In some cases, it was reported that glycosylated hIL-2 has higher specific activities than nonglycosylated hIL-2 [12, 18, 25].

Since hIL-2 can be used for many purposes, including as a therapeutic agent, the mass production of hIL-2 is very important for industry. Most recombinant glycoprotein drugs are produced using mammalian cells such as Chinese hamster ovary (CHO) cell [5, 16], even though bacterially produced recombinant proteins are biologically active, in order to reduce the probability of the side effects and possible problems in clinical trials. The post-translational modification of hIL-2 may not be critical for its biological activities, but it could affect its pharmacokinetic properties *in vivo*. Recently, a mammalian expression vector containing

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the human β-globin matrix attachment region has been developed, which may confer position-independent expression of foreign genes and thus enhance the expression level of recombinant proteins [16]. In the present study, the transcription terminator of the human gastrin gene was introduced into the expression vector containing the human β-globin matrix attachment region, and a CHO cell line secreting human rIL-2 was developed at a high level.

MATERIALS AND METHODS

Construction of Expression Plasmid Containing Human IL-2 cDNA

The hIL-2 expression plasmid, pMS, was constructed by subcloning hIL-2 cDNA into the expression vector pMSV [15]. hIL-2 cDNA was obtained by RT-PCR from mRNAs of human leukemic T-cell expressing hIL-2 [4, 30]. Oligonucleotide primers were designed to amplify the protein coding region for hIL-2 protein. The sense primer sequence was 5'-CTAGCTAGGCCACCATGTACAGGA-TGCAACTCCTG-3' and the antisense primer was 5'-GAAGATCTCAAGTCAGTGTTGAGAT-3'. Restriction enzyme sites (*Nhe*I and *Bgl*II) were engineered into the oligonucleotides, in order to facilitate cloning of cDNA into the vector pMSV. The Kozak sequence (GCCACC) was also added for proper start of translation in front of the translation initiator ATG [17]. The amplified hIL-2 cDNA was digested with *Nhe*I and *Bgl*II, and ligated to the vector digested with the same enzymes. The resulting expression plasmid construct was completely sequenced to confirm that it contains the same entire coding region of the hIL-2 protein previously reported [30]. The 74 bp terminator element of the human gastrin gene [14] was inserted into the downstream of the polyadenylation/clevage site of pMS plasmid, and the resulting plasmid was designated as pMSG.

Transfection and Selection

The dihydrofolate reductase (DHFR)-deficient CHO cells (DG44, provided by Dr. L. Chasin, Columbia University) were transfected by a liposome-mediated method. DG44 cells were seeded into a 6-well plate (2×10^5 cells/well), and cultured in α-MEM media (with nucleosides) supplemented with 10% cFBS (Gibco BRL, U.S.A.) for a day. Cells were rinsed with the same media prior to transfection. The rhIL-2 expression plasmid DNA, pMS or pMSG (10 μg), and DHFR expression plasmid (pDCH1P) (0.1 μg) [6] were co-transfected into the cells. After transfection, cells were grown in α-MEM media (with nucleosides) supplemented with 10% cFBS for two days and then transferred to α-MEM media (without nucleosides) supplemented with 10% dFBS. In order to amplify hIL-2 and DHFR expression units, the transfectants were grown in selective media containing increasing concentrations of methotrexate (MTX).

Western Blot Analysis

The transformants of CHO cells were seeded into a 48-well plate (1×10^5 cells/well), and cultured in 200 μl of α-MEM media (without nucleosides). After 48 h of incubation at 37°C, the culture supernatant was harvested and used for Western blot analysis. Culture supernant (7.5 μl) was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred onto a nitrocellulose membrane (Amersham Phamacia) and blocked with 5% skim milk. The rabbit anti-human IL-2 polyclonal antibody (Chemicon) and HRP-goat anti-rabbit IgG (Zymed) were used to detect IL-2 protein.

Southern and Northern Blot Analyses

Genomic DNA (15 μg) digested with *Cla*I and *Xho*I, and total RNA (15 μg) of CHO cell transformants, were used for Southern and Northern blot analyses, respectively. The digested DNA and total RNA were electrophoresed on a 0.8% agarose gel and a 1% formaldehyde-agarose gel, respectively. The blotting, prehybridization, and hybridization were done as described by Sambrook *et al.* [29]. The ^{32}P-labeled human IL-2 cDNA was synthesized by random priming method (Promega) and used as a probe.

Two-Dimensional Electrophoresis

Culture supernatant (10 ml) was concentrated using a Centricon (Amicon) to a volume of 500 μl and then dialyzed for 12 h at 4°C against 5 mM Tris-HCl (pH 7.5). The concentrated supernatant containing about 100 μg of protein was mixed with sample rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.2% Bio-lyte, 0.001% bromophenol blue) and the proteins were separated by the first-dimension electrophoresis using isoelectrofocusing. The second-dimension electrophoresis was done on a 12.5% Criterion precast gel (Bio-Rad). Gels were stained by silver nitrate, or subjected to Western blot analysis.

IL-2 Biological Assay

The activity of IL-2 was measured by proliferation assay using IL-2 dependent murine cytotoxic T-lymphocyte line CTLL-2 [12]. Serial dilutions of rIL-2 protein were incubated with CTLL-2 cells (about 5×10^3 cells) in a volume of 50 μl for 18 h, and followed by a 4 h pulse of 0.5 μCi of [^{3}H]thymidine. [^{3}H]thymidine uptake was determined by liquid scintillation counter. The units of IL-2 were calculated by determining the dilution factor required to give 50% of the maximal stimulation.

RESULTS AND DISCUSSION

Construction of the rhIL-2 Expression Plasmids

The recently developed expression vector, pMSV, was used for expression of the rhIL-2 cDNA. pMSV contains

the human β-globin matrix attachment region (MAR) and is known to confer position-independent expression of foreign genes in mammalian cells and is also known to make it much easier to obtain the stable cell line expressing a foreign gene at high level [15]. The rhIL-2 expression plasmid, pMS, was constructed by introducing rhIL-2 cDNA under the SV40 promoter of pMSV [15]. hIL-2 cDNA was cloned from mRNA of human leukemic T-cell by RT-PCR. Since AU-rich elements in the 3'-untranslated region of hIL-2 mRNA are known to cause rapid mRNA degradation *in vivo* [2], only the protein-coding region of hIL-2 cDNA was amplified and it was sucloned into the expression vector. To increase the translation efficiency, the sequence surrounding the initiator ATG was modified by the consensus sequence described by Kozak [17]. In addition, the plasmid pMSG was also constructed by inserting the transcription terminator of human gastrin gene [1, 14] into the expression plasmid to promote efficient transcription termination and thereby possibly enhancing rhIL-2 expression.

Selection of CHO Cells Expressing rhIL-2

Dihydrofolate reductase (DHFR)-deficient CHO cells (DG44) were co-transfected with the rhIL-2 expression plasmid (pMS or pMSG) and DHFR expression plasmid, pDCH1P [6]. The transfected cells were subsequently cultured in nucleotide-free medium to select the DHFR positive CHO cells. Then they were subcultured with a stepwise increase in the concentration of methotrexate (MTX) to amplify the DHFR gene together with rhIL-2 cDNA. The levels of rhIL-2 expression in cells adapted to different concentrations of MTX were analyzed by Western blot. Since the direct increase of MTX from 0 nM to 1 µM is impossible for the CHO cells, cells were first adapted to 10 nM, 50 nM, or 100 nM of MTX and then adapted to 1 µM of MTX. As shown in Fig. 1A, the adapted cells to 50 nM showed higher expression of rhIL-2 than the cells to 0 nM, 10 nM, or even to 100 nM, suggesting that the cells can be readily adapted to the direct increase of MTX concentration up to 50 nM, but not to 100 nM. However, regardless of MTX concentration at the intermediate adaptation step, all the cells finally adapted to 1 µM showed similar expression levels of rhIL-2 expression. In addition, further increase of MTX concentrations up to 40 µM did not increase the expression level (Fig. 1B). Interestingly, the expression levels of rhIL-2 in the cells transfected with pMSG were much higher than those in the cells transfected with pMS (Fig. 1A). This suggests that the transcription terminator of human gastrin gene enhances rhIL-2 expression. The transcription terminator of the human gastrin gene was also reported to enhance the expression in the transient system [14]. The enhancement of rhIL-2 expression by the transcription terminator could be achieved by increasing the concentration of free RNA polymerase II and the proper 3'-end processing of mRNA, and by preventing the promoter occlusion phenomena in the downstream genes during transcription of the repetitive genes [26].

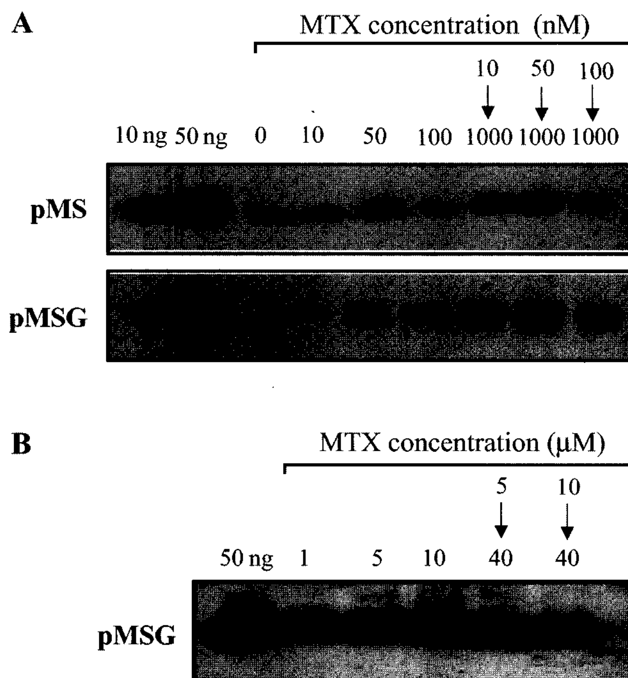

Fig. 1. rhIL-2 expression in CHO cells selected with MTX.
The CHO cells transfected with pMS (top panel in A) or pMSG (bottom panel in A, and B) were selected with increasing concentration of MTX as shown on the top of the panels. The culture supernatant (7.5 µl) was loaded on SDS-polyacrylamide gel and subjected to Western blot analysis using the antibody against human IL-2 (Chemicon). rhIL-2 purified from *E. coli* (Chemicon) was used as a control (lanes 1 and 2 in A, and lane 1 in B).

Southern and Northern Blot Analyses of the Transfected CHO Cells

The CHO cells transfected with pMSG were further characterized by Southern and Northern blot analyses. To determine copy numbers of rhIL-2 cDNA, chromosomal DNAs of CHO cells adapted at the different concentrations of MTX were analyzed by Southern blotting. As shown in Fig. 2A, copy numbers in the CHO cells could be approximately estimated by comparing the density of the DNA band (about 1 kb) hybridized to the rhIL-2 cDNA probe. The CHO cells adapted to higher selective pressure by MTX seemed to have more amplifications of rhIL-2 cDNA. The CHO cells adapted to 1 µM of MTX carried more than 300 copies of rhIL-2 cDNA per cell. Northern blot analysis of the cells showed that the expression level of rhIL-2 mRNA also increased as the concentration of MTX increased (Fig. 2B). Therefore, these results indicate that the levels of rhIL-2 production by the transfected cells correlated well with the amplified gene numbers as well as mRNA levels of rhIL-2.

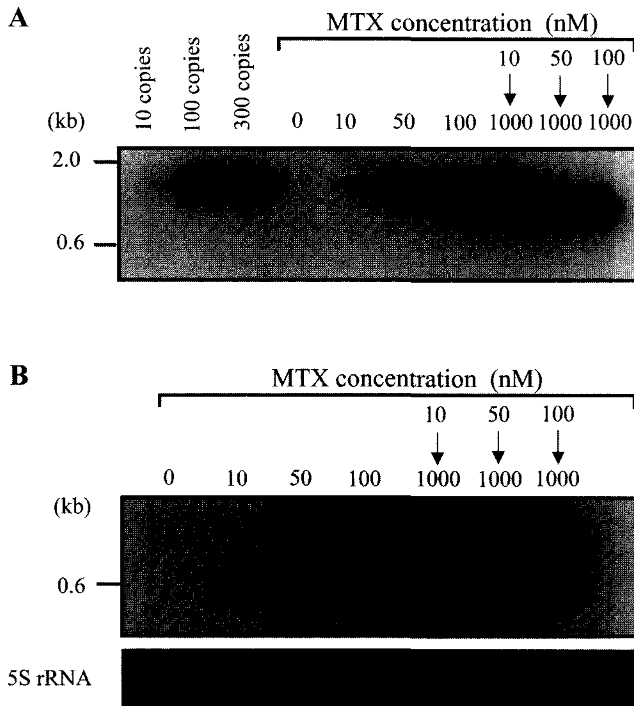

Fig. 2. Southern blot (A) and Northern blot (B) analyses of the CHO cells selected with MTX.
The P^{32}-labeled hIL-2 cDNA was used as a probe. The concentrations of MTX used for the selection are shown on the top of the panels. A. The genomic DNAs (15 μg) digested with *ClaI* and *XhoI* were loaded onto a 0.8% agarose gel and hybridized to the probe. As positive controls, the digested pMSG plasmid DNA corresponding to 10, 100, and 300 copies of the genomic DNA were loaded in lanes 1, 2, and 3, respectively. B. Total RNAs (15 μg) were loaded in each lane and hybridized to the probe. The hybridized rhIL-2 RNAs corresponding to about 600 bp can be seen. 5S rRNAs are shown as the control for sample loading.

Isolation of the CHO Cell Clones Expressing rhIL-2

To examine variation in the production level of rhIL-2 among the cells transfected with pMSG, 12 separate clones were isolated from the cells adapted to 1 μM and analyzed for their rhIL-2 production. As shown in Fig. 3, all the clones showed similar levels of rhIL-2 production. The level of rhIL-2 production determined by ELISA was about 11.4 μg/10^6 cell/day, which is about 6 times higher than those previously reported [24]. This similar level of

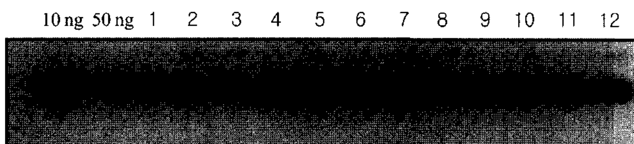

Fig. 3. Western blot analysis of the individual clones isolated from CHO cells adapted at 1 μM of MTX.
The culture supernatant (7.5 μl) of each clone was loaded on SDS-polyacrylamide gel and subjected to Western blot analysis using the antibody against hIL-2 (Chemicon). The rhIL-2 purified from *E. coli* (Chemicon) was used as a control (lanes 1 and 2).

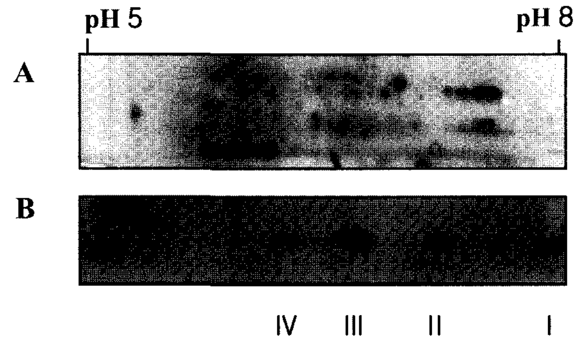

Fig. 4. Two-dimensional gel electrophoresis of the rhIL-2 expressed in CHO cells.
Total proteins (100 μg) in the culture supernatant were separated on an isoelectrofocusing strip, pH 5–8, and then transferred onto a precast gel (Bio-Rad). (A) Silver-stained gel. (B) Western blot analysis of 2-D gel. The four spots detected by Western blot analysis are marked by I, II, III, and IV.

high expression in all the clones is very surprising, since the conventional expression vector system has been shown to give very high variation and thus makes it very difficult to isolate highly expressing clones among the transfected cells. The MAR element of a pMSG vector system seems to allow the enrichment of high producers without significant variation in the expression level, probably by promoting the position-independent expression of the recombinant gene after the integration into the chromosome, which was also observed from the previous study [15].

Characterization of rhIL-2 Protein Produced in the CHO Cell Line

IL-2 is a heterogeneous glycoprotein with respect to size and charge, due to the variable sialyation. A non-glycosylated form of hIL-2 as well as two major forms of glycosylated hIL-2 were reported to be produced in mammalian cells [11, 19]. rhIL-2 protein produced in a CHO cell line was analyzed by two-dimensional gel electrophoresis. As shown in Fig. 4, the four spots (I, II, III, and IV) of rhIL-2 could be detected by Western blot analysis of the gel. Spots II and III were previously known to be two major glycosylated forms of hIL-2 with pI~7.6 and ~7.0, respectively. Spot IV was known to be a non-glycosylated form of hIL-2 with pI~8.0. However, Spot I has not been reported. Since Spot I has a lower pI value than Spot II, it seems to have additional sialic acid residue. Spot I could not be found previously, probably because of a low concentration of IL-2 *in vivo* or a different mammalian expression system. To determine the biological activity of rhIL-2 protein produced from CHO cell, rhIL-2 protein was purified from the culture supernatant using HPLC by the method described previously [8]. rhIL-2 was initially concentrated and then purified by cation-exchange chromatography and affinity chromatography. The purity of rhIL-2, as assessed by SDS-PAGE, was estimated to be

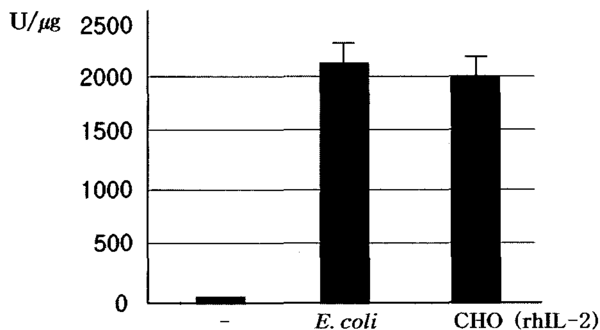

Fig. 5. The biological activity of rhIL-2 protein purified from CHO cells.

The biological activity was measured by the ability of proliferation of CTLL-2 cells using rhIL-2 protein purified from CHO cells. The units of IL-2 were calculated by determining the dilution factor required to give 50% of the maximal stimulation [12]. rhIL-2 protein from *E. coli* was used as a positive control. The standard deviations from the mean of three independent assays are indicated by error bars.

greater than 95% (data not shown). The biological activity of purified rhIL-2 was determined on a murine IL-2-dependent cytotoxic T cell line. It was found that it was biologically active as rhIL-2 produced from *E. coli* and its specific activity was about 2,000 U/µg (Fig. 5). rhIL-2 produced from CHO cells may not have an advantage over the one from *E. coli* in terms of functional activity *in vitro*. However, glycosylated rhIL-2 can be more stable than the non-glycosylated form *in vivo* like other protein drugs and may reduce side effects in clinical trials. The comparative studies on the pharmacokinetics would be important to determine which expression system is preferable for medical use.

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