Stable, recombinant expression of human insulin-like growth factor binding protein-1 (hIGFBP-1) in Chinese hamster ovary (CHO) cells

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Abstract

Stable expression of human insulin-like growth factor binding protein-1 (hIGFBP-1) at high levels has been achieved in Chinese hamster ovary (CHO) cells by co-transfection and subsequent co-amplification of expression vectors containing the hIGFBP-1 cDNA and a dihydrofolate reductase (DHFR) cDNA gene into DHFR-deficient cells. Stepwise selection of the DHFR⁺ transformants in increasing concentrations of methotrexate (MTX) generated cells which had high copy numbers of the hIGFBP-1 gene (around 100 copies in cells amplified in medium containing 100 nM MTX). Expression of hIGFBP-1 in mixed clones was found to increase with increasing copy number and an apparent correlation between intra- and extracellular levels of hIGFBP-1 produced by these cells was observed. It was further observed that continuous cultivation over eight months in medium supplemented with 100 nM MTX increased the production of hIGFBP-1 25 times. The productivity did not increase further after five more months cultivation in MTX containing medium. A subcloning of this cell line gave clones with an even higher productivity. Further amplification in 500 nM or 1 μ M MTX did not increase the hIGFBP-1 production.

Introduction

Insulin-like growth factor (IGF)-I and -II are peptides with mitogenic and metabolic properties. They circulate in plasma complexed to carriers or binding proteins (Baxter, 1994). Six distinct forms of IGFBPs have been structurally characterised and it has been shown, that the IGFs have affinity constants for four of these that are higher than for the type I IGF receptors (Clemmons, 1992). The IGFBPs have an important role in regulating the bioavailability of the IGFs in vivo serving to ensure that a stable reserve of potentially active growth factors is maintained in the circulation. (Baxter, 1994). IGF-1 is known to play a role in e.g. osteoporosis (Clark et al., 1995; Schacht, 1994), diabetes (Fisher et al., 1995; Kolaczynski and Caro, 1994) and obesity (Rasmussen et al., 1994).

Gene amplification as a means to overexpress transfected DNA sequences has been known for many years.

The dihydrofolate reductase (DHFR) gene is a well investigated example of such. DHFR is a cytosolic enzyme sensitive to the inhibitor methotrexate, MTX, which is a folate antagonist. Since MTX binds to and inhibits DHFR stoichiometrically, this amplification of the DHFR gene with a resultant increase in intracellular DHFR is responsible for the ability of the cells to grow in higher concentrations of MTX (Kaufman et al., 1985). Chinese hamster ovary cells, which are deficient in DHFR, can be transformed with a DHFR gene in combination with any particular gene of interest (Urlaub and Chasin, 1980). The DHFR⁺ transformants thus produced can be selected on the basis of their ability to grow in the absence of nucleosides and glycine. The gene sequences cotransfected with the DHFR gene are frequently integrated adjacent to the DHFR gene, and thus subsequent amplification of the DHFR gene results in coamplification of adjacent

DNA. DNA which has been coamplified in this manner is also coexpressed.

Other common amplification marker genes are e.g. glutamine synthetase (GS) (Sanders et al., 1987), adenosine deaminase (ADA) (Kaufman et al., 1986) and multiple drug resistance (mdr) (Kane et al., 1989). Some marker genes, e.g. GS, DHFR or ADA, are recommended for the use in combination with a cell line defective in the respective gene. Others, such as mdr, are dominantly acting and are not restricted to a specific cell line.

Human IGFBP-1 is a 26 kDa IGF binding protein with a total of 234 amino acids (Luthman et al., 1989). IGFBP-1 is the only one of the six binding proteins, that displays rapid dynamic regulation in vivo, with serum levels varying 10-fold or more in relation to meals. The N-terminal and C-terminal regions contain 18 conserved cysteines which are postulated to provide a framework for ligand binding (Lee et al., 1993). In vivo and in vitro studies indicate that insulin is the primary regulator of IGFBP-1 expression in human liver and that the primary effect of insulin is rapid inhibition of transcription (Lee et al., 1993). IGF-I administration causes complex changes in the property of circulating IGFBPs, so that a detailed knowledge of their regulation is essential if the therapeutic potential of IGF-1 is to be optimised. To understand the regulation of IGF bioactivity at the cellular level, it is necessary to study the IGFBPs. However, the availability of these proteins has been very low and we have therefore established a stable, amplified eukaryotic expression system for human IGFBP-1. We show that it was possible to increase the expression of hIGFBP-1 more than 25 times by using the DHFR system.

Materials and methods

hIGFBP-1 expression vector

The expression vector used is illustrated in Figure 1. The hIGFBP-1 expression vector contains the human IGFBP-1 cDNA under the control of the Chicken beta actin promoter. Competent *E. coli* was transformed with the vector and the plasmids were harvested using the protocol from Magic Minipreps (Promega, USA) and Qiagen maxiprep (Qiagen inc., USA). Diagnostic cuts ensured the correct composition of the vector.

Cell culture and transfection

DHFR-deficient CHO cells (DG44 N.Y., a generous gift from D.L. Chasin) were co-transfected with 10 mg of the expression vector and 1 mg of a dhfr-vector by using calcium phosphate-DNA coprecipitation method. Cells were cultured in selective medium (modified Hams F12 without nucleosides and glycine supplemented with 5% dialysed FBS (dFBS) (Hyclone, USA)) until colonies appeared. These colonies were pooled and transferred to another tissue culture flask for gene amplification. See Figure 2 for overview. The maximum number of cells obtained in a 25 cm² T-flask was determined by trypan blue exclusion after trypsinisation of the cells and resuspension in growth medium.

Gene amplification

For gene amplification, selective medium + 0.1 mg TPA l⁻¹ (TPA = phorbol 12-myristate 13-acetate) was added to the cells with stepwise increasing concentrations of MTX up to 100 nM MTX. The tumor promoter, TPA, has been shown to enhance the frequency of MTX resistance and DHFR gene amplification (Varshavsky, 1981). Colonies were pooled and transferred to a tissue culture flask and was denoted MIX100. When the cells were confluent, production medium (McCoy + 1% FBS + 10 mg ml⁻¹ Aprotinin (Sigma, USA)) was added to each tissue culture flask. The conditioned medium was harvested after 24 h. MIX100 was further amplified on 500 nM and 1 µM MTX.

Subcloning

Shortly after MIX100 was established, a subcloning was made in selective medium supplemented with 100 nM MTX. Appearing colonies were picked and tested at 100% confluence for hIGFBP-1 production in production medium. The most productive clones were tested repeatedly. Ten of these clones, denoted 'early subclones', were picked for further analysis. Subcloning of MIX100 was repeated after it had been kept in selective medium with 100 nM MTX for more than one year. Subclones were picked and analysed for hIGFBP-1 productivity.

BIACore analysis

Real-time biospecific interaction analysis (BIA) has been used to quantify the amount of hIGFBP-1 produced by the cells as the hIGFBP-1 concentration in

Figure 1. Schematic diagram of the hIGFBP-1 expression vector, showing the Chicken beta actin promotor, the 940 bp hIGFBP-1 gene and the SV40-3' splice site. See text for details.

the spent medium. The BIAcore™ system from Pharmacia Biosensor AB. IGF-I (Pharmacia, Sweden) was immobilised on Sensor Chip CM5. Human IGFBP-1 standards (Pharmacia, Sweden) and samples were injected followed by Mab anti-hIGFBP-1 (100 mg ml⁻¹, 6303 Medix Biochemica, Finland).

Southern blot analysis

DNA was extracted from exponentially growing cells by using the Qiagen Cell Culture DNA kit (Qiagen, USA). The 740 bp hIGFBP-1 fragment was isolated, transferred to an Eppendorf tube and labelled with ³²P by the use of random prime method and Amersham kit (Amersham, UK). A 1% horizontal agarose gel containing EtBr was loaded with Molecular Weight Marker VI (MW VI), 270 pg expression vector, 27 pg expres-

sion vector, 2.7 pg expression vector and 1 mg DNA extracted from the cells. The gel was electroforested. The DNA was transferred to GeneScreen filter paper (Du Pont, Boston USA) by the use of a vacuum blotter. Hybridisation was carried out in hybridisation buffer supplemented with ssDNA and the labelled hIGFBP-1 fragment. The filter was transferred to a plastic film and exposed to X-ray film (DuPont Gronex 4, Du Pont, France). For quantification, the bands in the autoradiograms were scanned, quantitated by determining the area under each peak and normalised to the plasmid band control, by the use of PhosphorImager™ and the computer program ImageQuant™ v3.3 (Molecular Dynamics inc., USA).

Figure 2. Schematic representation on the development of each cell line mentioned in this paper. See text for abbreviations.

Flow cytometry

Cells were fixed in 3% paraformaldehyde for 15 min at room temperature, washed with PBS twice, incubated in 0.01% Triton X-100 for 5 min at room temperature, washed twice with PBS and separated into two fractions. The cells were stained with Mab against hIGFBP-1 (6303, 5 mg ml⁻¹) or Mab HLA DR class II (M746, Dakopatts Denmark) as negative control for 45 min at room temperature, washed twice with PBS and incubated 45 min with 1 ml Fluorescein-conjugated F(ab')₂ fragment rabbit-anti-mouse (F313, Dakopatts, Denmark) in a total volume of 50 ml PBS and then analysed with flow cytometer (EPICS

753, Coulter Electronics, Florida) using a 488 nm laser beam. Fluorescein emission was detected using a 525 nm band pass filter. Immunobrite beads (Epics Division, Florida) were analysed as a standard at a concentration of 31 000, 115 000, 460 000 and 1 115 000 Fitec molecules/bead.

Results

Growth properties of the transfected cells

The CHO-DG44.*dhfr*⁻ which is the DHFR gene deficient cell line used for the transfection is an anchorage

Table 1. Stability of expression of hIGFBP-1 from S1 measured by the BIAcore. A confluent 25 cm² tissue culture flask containing 6 ml of production medium (McCoy + 1% FBS + 10 mg ml⁻¹ Aprotinin (Sigma, USA)) was harvested after 24 h. The BIAcore measurements were performed in triplicate. 'Days' indicates the time passed after the pressure of MTX has been removed from the S1 cells. S1 seems to be a stable producer of hIGFBP-1 in the absence of MTX since the productivity did not change for 4 months

Days	S1 mg IGFBP-1 l ⁻¹ 10 ⁻⁷ cells (± SD)
20	4.0±0.2
85	4.0±0.1
130	4.2±0.2

dependent cell line maintained in modified Hams F12 medium supplemented with 5% FBS. This cell line was cultured as a monolayer in tissue culture flasks before the transfection and the cells did not change their morphological appearance at any time after the transfection. However, the maximum number of cells in a 25 cm² tissue culture flask increased after transfection from 1.5·10⁵ cells per cm² to as much as 4·10⁵ cells per cm².

hIGFBP-1 gene copy number

Figure 3 shows a Southern blot analysis performed on DG44, MIX0, MIX20 and MIX100 in order to determine the number of hIGFBP-1 gene copies due to the amplification on MTX. No copies of the human IGFBP-1 gene were detected in DG44 cells, as expected (lane 5). MIX0 cells (lane 6) contained 5–10 copies of the hIGFBP-1 gene as well as MIX20 cells (lane 7). MIX100 cells (lane 8) contained as much as 100 copies of the hIGFBP-1 gene corresponding to a 10–20 fold increase with respect to gene copies compared to MIX0 cells.

Vector stability

Table 1 shows the hIGFBP-1 production from S1 cells (the highest producing early subclone) at different times. S1 was examined for stability of the amplified expression vector. The table shows that after a 4 month period of cultivation without selective pressure, S1 had not lost any of its hIGFBP-1 productivity measured by

Table 2. Productivity of hIGFBP-1 from MIX100 cells measured by the BIAcore. A confluent 25 cm² tissue culture flask containing 6 ml of production medium (McCoy + 1% FBS + 10 mg ml⁻¹ Aprotinin (Sigma, USA)) was harvested after 24 h. The BIAcore measurements were performed in triplicate. 'Days' indicates the time passed after the mixture of clones, MIX100, was established. The productivity of MIX100 cultured in the presence of 100 nM MTX increased considerably during the first 8 months. The productivity did not increase the following 5 months

Days	MIX100 mg IGFBP-1 l ⁻¹ 10 ⁻⁷ cells (± SD)
20	0.4±0.1
85	1.6±0.1
130	3.2±0.2
160	6.6±0.1
250	10.0±0.2
390	10.0±0.2

Table 3. Correlation between hIGFBP-1 gene copy number and relative productivity. The increase in gene copy number (10–20) times was not proportional to the increase in productivity (2 times). However, the productivity was increased considerably (25 times) when MIX100 was cultured for eight months in medium containing 100 nM MTX

Cell line	hIGFBP-1 gene copies	Relative productivity (24 h)	Productivity after 8 months (24 h)
MIX0	5–10	1	–
MIX20	5–10	1	–
MIX100	100	2	50

the BIAcore indicating that this cell line was a stable producer of hIGFBP-1. Meanwhile, MIX100 was cultured in the presence of 100 nM MTX in order to investigate whether the production of IGFBP-1 from this mixture of clones would increase with time in the presence of MTX. The productivity of MIX100 increased 25 times after an eight months cultivation period, as shown in Table 2.

hIGFBP-1 production

Table 3 shows a comparison between gene copy number and hIGFBP-1 productivity of the clone mixes. Amplification on 100 nM MTX resulted in a 2-fold increase in hIGFBP-1 productivity measured by the

Figure 3. Southern blot analysis. Lane 1 and 9: Molecular weight marker VI, lane 2, 3 and 4: 270 pg, 27 pg and 2.7 pg respectively of the hIGFBP-1 probe corresponding to 200, 20 and 2 IGFBP-1 gene copies, lane 5: DG44, lane 6: MIX0, lane 7: MIX20, lane 8: MIX100. For quantification, the bands in the autoradiograms were scanned, quantitated by determining the area under each peak and normalized to the plasmid band control. No copies of the hIGFBP-1 gene were detected in DG44 cells while MIX0 cells and MIX20 cells contained 5-10 copies of the hIGFBP-1 gene. MIX100 cells contained around 100 copies of the hIGFBP-1 gene.

BIACore compared to that of the non-amplified cell line. However, the productivity of MIX100 increased a further 25 times after a period of eight months under MTX pressure. Amplification in 500 nM MTX or 1 μ M MTX did not increase the specific productivity further. On the contrary, the productivity was decreased. A western blot analysis was used to determine the quality of the produced hIGFBP-1.

Figure 4 shows the results obtained from Flow Cytometry with DG44, MIX0, MIX100 and S1. An increased intracellular expression of hIGFBP-1 can be seen as a displacement of the curve to the right. The mean content of hIGFBP-1 molecules in MIX0 was used for normalisation of hIGFBP-1 concentration. MIX0 contained a broad spectrum of cells with varying levels of intracellular concentration of hIGFBP-1 spanning three orders of magnitude. Amplification in 100 nM MTX (MIX100) resulted in a 6-fold increase in content of hIGFBP-1 molecules per cell and with a peak displaced about one decade to the right. S1 con-

tained 35% more hIGFBP-1 molecules per cell than MIX100.

Discussion

The purpose of this work was to establish a stable CHO cell line producing high levels of hIGFBP-1 secreted into the medium. The high productivity has been achieved by using the DHFR gene amplification procedure, which has also been successfully used to obtain cell lines producing a.o. beta interferon (McCormick et al., 1984), human t-PA (Kaufman et al., 1985), Glucocorticoid receptors (Bellingham et al., 1992) and blood coagulation factors VII and XI (Kemball-Cook et al., 1994). Since a direct selection method for the amplification of the hIGFBP-1 gene was not available, a cotransfection method was used which may yield cells containing the hIGFBP-1 sequences linked to the DHFR sequences. Thus, selection for DHFR gene

Figure 4. Results obtained by flow cytometry showing the fluorescence from FITC molecules bound to Mab 6303 against hIGFBP-1 which again is bound to hIGFBP-1 in the cells. This is a way to measure the intracellular level of hIGFBP-1 as described in the text. Transfection lead to a very heterogeneous cell population (MIX0) where the intracellular level of hIGFBP-1 was spanning three orders of magnitude (logarithmic x-axis). Amplification in MTX (MIX100) lead to a displacement of the curve to the right indicating an increased intracellular level of hIGFBP-1. The difference in the intracellular level of hIGFBP-1 between MIX100 and the subclone S1 was about 35%. The peak of the curve (MIX100:s1) was not displaced to the right, but fewer cells were producing a lower amount of hIGFBP-1.

amplification by increasing MTX resistance provided for the coamplification of hIGFBP-1 sequences.

The productivity of MIX100 increased noteworthy during eight months of cultivation under selective pressure. The increasing productivity was not due to a continuous selection favouring cells with higher numbers of hIGFBP-1 gene copies, since the mean copy numbers for the whole cell culture did not increase with time. However, these results indicate that a selection of cells with an increased production and/or secretion rate may have occurred. We noticed, that these long-term amplified cells had an increased growth rate (results not shown) so that therefore it is likely that they have

outgrown the cells that just expressed enough DHFR to survive in the presence of MTX. This would lead to an increased IGFBP-1 production, since the IGFBP-1 genes and the DHFR genes are co-expressed. The long-term amplified MIX100 cells could be cultured serum-free in spinner flasks without any adaptation period at all which clearly indicates that these cells have been transformed in some way during the long amplification period.

When MIX20 cells were amplified on 100 nM MTX, the productivity measured by the BIAcore increased 2 fold, while the gene copy number increased as much as 10–20 fold. This phenomena might indicate that many gene copies have been integrated into sites where gene amplification is inefficient, as discussed by Kaufmann et al., 1985. The further amplification of MIX100 cells on 500 nM MTX (MIX500) and 1 μ M MTX (MIX1u) lead to a decrease in productivity even though the cells were more resistant to MTX. Gu et al. (1992) have presented similar results with CHO-DG44.*dhfr*⁻ transfected with a *dhfr* vector and a lac Z gene expression vector. The productivity declined when the cells were transferred from a medium containing 100 nM MTX to one containing 1 μ M MTX even though the intracellular concentration of DHFR increased. Similar results were reported by Page (1985). Interestingly, the intracellular level of hIGFBP-1 molecules in MIX500 was higher than in MIX100. The probable increased metabolic burden on MIX500 might have lead to a slower secretion rate. A similar phenomena was also observed when MIX100 was established from MIX20, where the intracellular level of hIGFBP-1 increased 6-fold while the level of hIGFBP-1 in the medium only increased 2-fold.

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