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Recombinant insulin-like growth factor-I (IGF-I) production in *Super*-CHO results in the expression of IGF-I receptor and IGF binding protein 3

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Abstract

Previously, we described the genetic construction *Super*-CHO, a cell line capable of autocrine growth under fully defined protein-free conditions. *Super*-CHO cells constitutively express insulin growth factor-I (IGF-I) and transferrin in sufficient amounts to support long-term, stable growth without the addition of exogenous growth factors, thus making it an ideal host for the production of recombinant biopharmaceuticals. although IGF-I has been successfully expressed in Chinese Hamster Ovary (CHO) cells, the long term effects of recombinant IGF-I expression have not been explored. In particular, the expression of the endogenous IGF-I receptor in response to IGF-I production has not been reported. We report here the transcriptional induction of the type I IGF receptor gene in *Super*-CHO. In addition, we examined the conditioned medium for the presence of IGF-I binding proteins. Ligand blot analysis reveals the presence of IGF binding proteins present in the medium conditioned by *Super*-CHO cells as well as CHO cells incubated in the presence of IGF-I. Furthermore, immunoaffinity reveals that *Super*-CHO expresses IGF binding protein-3 in response to IGF-I production. These results suggest the autocrine growth of *Super*-CHO involves a complex interaction of cell type specific factors which regulate its utility of IGF-I.

Introduction

The *Super*-CHO cell line expressing recombinant IGF-I and transferrin grows indefinitely in fully defined protein free medium. Growth rate and final cell densities are comparable to CHO-K1 cells growing in medium supplemented with insulin, transferrin and selenium. *Super*-CHO is an ideal host cell line for the production of recombinant biopharmaceutical products as it does not require foetal bovine serum (FBS) or other sources of mitogens, thus providing considerable regulatory and cost advantages. IGF-I and transferrin are secreted into protein free medium where they are presumed to bind to cellular surface receptors and sustain an autocrine fashion of cellular growth.

IGF-I is a member of a family of structurally related peptides that also includes insulin and IGF-II. Insulin-like growth factors (IGF-I and IGF-II), are found in a variety of cell types where they function in an autocrine/paracrine fashion to regulate cell growth (Baxter, 1986). IGFs are potent mitogens which exert their effect principally through binding to the type-I IGF receptor (Dufourny et al., 1997; LeRoith et al., 1995). IGF-I receptor binds IGF-I with high affinity and binds insulin with 500–1000 times lower affinity, explaining the well known role of insulin as a growth factor at high concentrations.

The interaction between the IGF and the IGF-I receptor is regulated by a family of IGF-binding proteins(Camacho-Hubner et al., 1992) of which six have been identified and cloned, designated IGFBP-1 to IGFBP-6 (Kelley et al., 1996). IGFBPs bind

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IGFs with very high affinities and confer upon the IGF regulatory system both functional and tissue specificity which influences their bioavailability and distribution. IGFBPs are produced by many cell types, which can modify them through differential post-translational modifications including phosphorylation, glycosylation, proteolysis and multimerization alter their biological activity (Coverley and Baxter, 1997; Koedam et al., 1997). It is believed that IGFBPs inhibit IGF-I activity by sequestering the peptide and consequently preventing interaction with its receptor. However, IGFBP-3 is known to both inhibit and potentiate IGF-I action depending on whether it is soluble or cell surface bound (Conover, 1992). The majority of IGFs in the adult circulation are tightly bound to IGFBP-3 in a 150 kDa ternary complex composed of 38–42 kDa IGFBP-3, IGF peptide and an 85 kDa acid-labile protein subunit (ALS)(Baxter and Martin, 1989; Hashimoto et al., 1997; Young Lee et al., 1996)

CHO cells do not normally produce IGF-I. Although IGF-I has been successfully expressed in mammalian cells, the long term consequences of recombinant IGF-I expression have not been well defined. We investigated the dynamics of IGF-I production in *Super*-CHO with respect to expression of the type I IGF-I receptor as well as to the implication of IGF binding proteins. As a consequence of IGF-I production in *Super*-CHO cells, we observed an induction of IGF-I receptor expression. In addition, it appears that IGFBP-3 is involved in the modulation of cell growth of *Super*-CHO. Therefore, the autocrine growth of *Super*-CHO involves a complex interaction of cellular proteins that regulate IGF-I utility resembling the dynamics of an IGF autocrine system. This report aims to extend characterisation of *Super*-CHO, a host cell line constructed for the production of biopharmaceutical products. In particular, we characterised *Super*-CHO with respect to its production of the growth factor, IGF-I.

Materials and methods

Cell lines and cell culture

The *Super*-CHO cell line was derived from the Chinese hamster ovary cell line CHO-K1 (ATCC CCL 61) cotransfected with plasmids expressing the human IGF-I under the control of the CMV promoter (pCMVIGF) and the human transferrin under the control of the SV40 early promoter (pSVLTf) (Pak. et al., 1996).

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Coons (1:1 mixture) medium referred to as protein-free medium (PFM). PFM was supplemented with either 10% foetal bovine serum (FBS) or IGF-I (Sigma) where indicated.

Cell labelling and immunoprecipitation

Cell monolayers in PFM were washed twice in PBS and incubated for 2 hr in labelling medium (methionine-free DMEM (GIBCO BRL). The medium was then replaced with fresh labelling medium containing 50 μCi mL^{-1}[^{35}S] methionine, 1175 Ci mmol^{-1} (ICN Pharmaceuticals, Inc. Irvine, CA). After labelling for 6 hr, the medium was removed, and cells were washed twice in PBS before solubilizing in RIPA buffer (150 mM NaCl, 1.0% NP-40, 1% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) containing protease inhibitors (100 μm leupeptin and 1 mM PMSF) for one hour, and then centrifuged at 14 000 rpm for 15 min. Immunoprecipitation was performed using 100 μL labelled lysate with 0.4 μg of M19 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rodent-specific anti IGFBP-3 antibody and protein G-Sepharose (Pharmacia Biotech Inc.). The immunoprecipitates were washed twice in RIPA and once in buffer consisting of 25 mM Tris-HCl, pH 7.4, 150 mM NaCl. Immunoprecipitates were solubilized in SDS-sample buffer containing 2-mercaptoethanol and denatured by heating at 95 °C for 5 min. Samples were electrophoresed on 4–20% acrylamide Tris-glycine gels (Novex, San Diego, CA). Gels were fixed in 10% (w/v) TCA and 2% (w/v) PEG-6000, and dried gels were exposed to a phosphoimaging screen (Bio-Rad, CS-imaging screen), and scanned using a Phosphoimager (Bio-Rad, model GS 363).

RNA extraction and northern blot analysis

Monolayer cultures of cells were grown in T150 flasks (Corning, New York) in PFM or PFM supplemented with 10% foetal bovine serum (GIBCO BRL). Cells were harvested at 70–80% confluence, using 0.2% EDTA in PBS. Total RNA was isolated from different cell lines by the acid-guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). For Northern analysis, total RNA (20 μg) was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and MOPS (a stock solution of 10X contained 0.2 M 3-(-N-morpholine) propanesulfonic acid, 5 mM sodium acetate, 5 mM EDTA, pH 7.0).

The RNA was transferred to Hybond-N nylon membrane (Amersham Life Science) by vacuum blotting with 20X SSC for 2–3 hr and RNA was bound to nylon membrane by UV cross-linking (Amersham Life Science). To confirm the integrity and the loading uniformity of the RNA on the membrane, methylene blue staining was employed (Herrin and Schmidt 1988). Complementary DNA (cDNA) for IGF-IR (a kind gift from Dr. R. Baserga, Jefferson Cancer Institute, Pennsylvania) and for IGF-I were used in this experiment. The probes were prepared using Gene Images Random Prime Labelling Module (Amersham, Life Science). Prehybridization of the nylon blots was carried out for at least 10–12 hr at 42 °C in a solution containing 50% deionised formamide, 5X Denhardt's solution (1X, 0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 1% SDS, 5X SSC and 200 μg mL^{-1} herring sperm DNA. After prehybridization, DNA probes, previously heated to 100 °C were applied to the blots. Hybridization was conducted overnight at 42 °C. The membranes were washed at room temperature in 1X SSC buffer containing 0.1% SDS(3×15 min) and (2×20 min) at 42 °C in 0.1% SSC-0.1% SDS. RNA detection was carried out using Gene Images CDP-star detection module (Amersham Life Science) according to the manufacturer's protocol.

Western blotting and chemiluminescent dot blot

Conditioned medium containing IGF-I and IGF-I complexes were assessed by Western blotting. In some cases, cross-linking of proteins was performed using disuccinimidyl suberate (DSS, Pierce, Rockford, IL) according to the manufacturer's protocol. Prior to electrophoresis, the samples were mixed with an equal volume of 2X Tris-glycine SDS sample buffer (Novex, San Diego, CA) and heated at 95 °C for 5 min. Samples were electrophoresed on 4–20% acrylamide Tris-glycine gels (Novex, San Diego, CA). Resolved proteins were electroblotted onto nitrocellulose membrane (Sartorius, Germany) using a semi-dry system at 2–3 mA cm^{-2} for 90 min. The transfer buffer consisted of 25 mM Tris-HCl (pH 8.5), 192 mM glycine and 20% (v/v) methanol. Following transfer, the blotted membranes were blocked by incubating for 1 hr at room temperature in a blocking solution composed of PBS containing 5% nonfat dry milk and 0.1% Tween-20. For the detection of IGF-I, the membranes were incubated with a rabbit anti-human IGF-I primary antibody (Peninsula Laboratories, Inc.), diluted 1:1000 and incubated for 2 hr at room temperature. The membranes were washed (4×10 min) with PBS/0.1% Tween-20 and then incubated with an alkaline phosphatase-conjugated mouse anti-rabbit conjugated IgG (Boehringer Mannheim) diluted 1:5000 in PBS containing 0.1% Tween-20. Membranes were washed and IGF-I and IGF-I complexes were visualised using Western Blue stabilised substrate for alkaline phosphatase (Promega Corp. Madison, WI). IGF binding protein-3 was detected using the antibody M-19 (Santa Cruz Biotechnology, Santa Cruz, CA), a rodent specific goat anti-IGFBP-3 followed by an alkaline phosphatase-conjugated rabbit anti-goat antibody (Pierce, Rockford, Il.).

A chemiluminescent dot blot assay was performed to detect IGFBP-3 in conditioned media as described by De Leon and Asmeron, 1997). Briefly, conditioned media were applied to ECL-hybond nitrocellulose (Amersham) membrane using a vacuum blotter (Hoeffer Scientific Instruments). Following adsorption, the membrane was dried and blocked in PBS containing 0.1% Tween-20 and BSA. Three successive 10 min washes in PBS containing 0.1% Tween-20 preceded incubation with primary antibody, M-19 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 in PBS containing 0.1% Tween-20. The membrane was washed three times in wash buffer before incubation in Supersignal reagent (Pierce, Rockford, Il.) followed by exposure to the Fluor-S MultiImager (Bio-Rad) system with maximum aperture without the 660 SP filter.

[^{125}I]IGF western ligand blot

This was performed according to the method described by Hossenlopp et al., 1986. Briefly, conditioned media were subjected to 12.5% SDS PAGE under nonreducing conditions followed by electrotransfer onto nitrocellulose (Sartorius, Germany). IGFBPs were detected by incubation with 100 000 cpm ^{125}I-IGF-I (ICN Pharmaceuticals, Inc. Irvine, CA) followed by autoradiography at –70 °C for 5 days using Kodak Autoradiographic film.

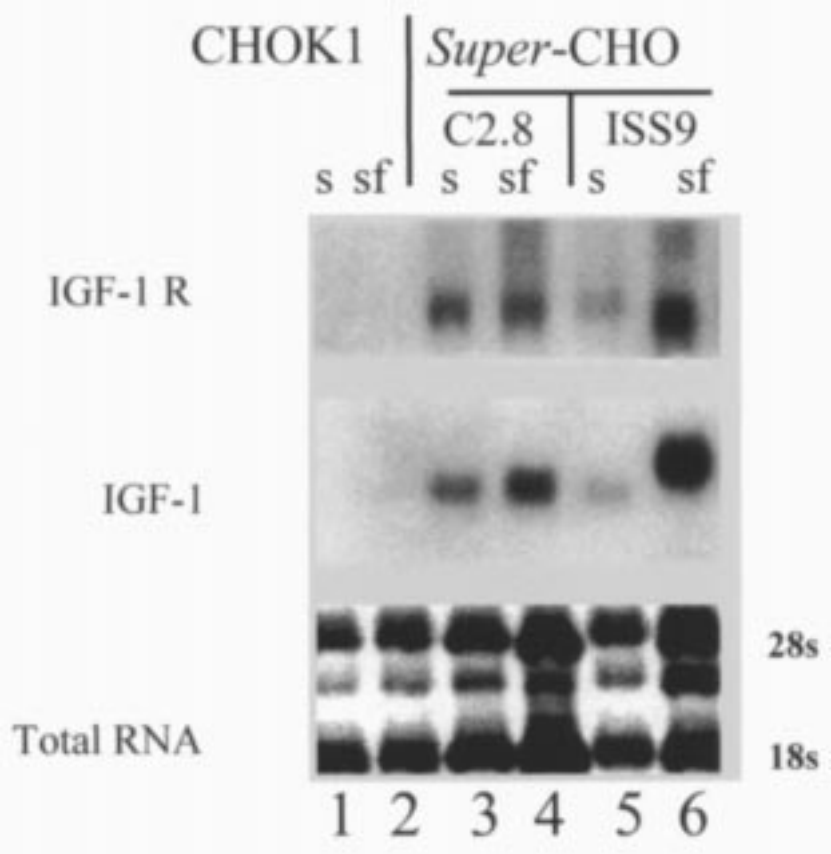

Figure 1. Northern blot analysis of IGF-I and IGF-I receptor. Expression of IGF type I receptor and IGF-I mRNA from *Super*-CHO clones C2.8 and ISS9 as well as the parental cell line, CHOK1 was analysed by Northern blot. IGF-I receptor-specific transcripts as well as transcripts of IGF-I were detected in *Super*-CHO clones but not in the control CHOK1 cells. Cells in serum-containing medium (s) or in serum-free medium (sf) were harvested for RNA isolation. Total RNA was visualised by methylene blue staining to confirm the integrity and loading uniformity of the RNA.

Results and discussion

IGF-I receptor expression

We examined the expression of the type I IGF receptor in the parent CHOK1 cell line compared to that of *Super*-CHO. We chose to investigate two clonal isolates of *Super*-CHO, named C2.8 and ISS9. Although both clones produce similar amounts of IGF-I (100 ng mL^{-1}) as assayed by RIA analysis (data not shown), they differ in their growth rates (doubling times are 24 and >35 hr, respectively). RNA was analysed for IGF-I and IGF-I receptor expression by Northern blot (Figure 1). RNA extractions were done using cells reaching confluence either in serum-containing (s) or serum-free medium (sf). IGF-I receptor-specific transcripts were detected in both clones of *Super*-CHO, C2.8 and ISS9 under serum and serum-free conditions, but not in the parent cell line, CHOK1 (top panel, Figure 1). Similarly, IGF-I-specific transcripts were detected in *Super*-CHO clones but not in CHOK1 (middle panel). Total RNA (bottom panel) with predominant 28S and 18S species is included to indicate comparable gel loading strategy.

RNA transcripts corresponding to IGF-I receptor in parental CHO cells were below our detection limits. Normally, CHO cells have between 5000 and 25 000 IGF-I receptors per cell (Kato et al., 1993; Steele-Perkins et al., 1988). The number of endogenous IGF-I receptors in CHO cells is known to fluctuate due to many factors. An upregulation of receptor by the respective ligand is not without precedent. Transcription of *Xenopus* thyroid hormone receptor genes is strongly up-regulated by their own ligand (Machuca et al., 1995). Expression of type I IGF receptors in keratinocytes were activated as a result of IGF-I exposure (Tavakkol et al., 1992; Ando et al., 1993).

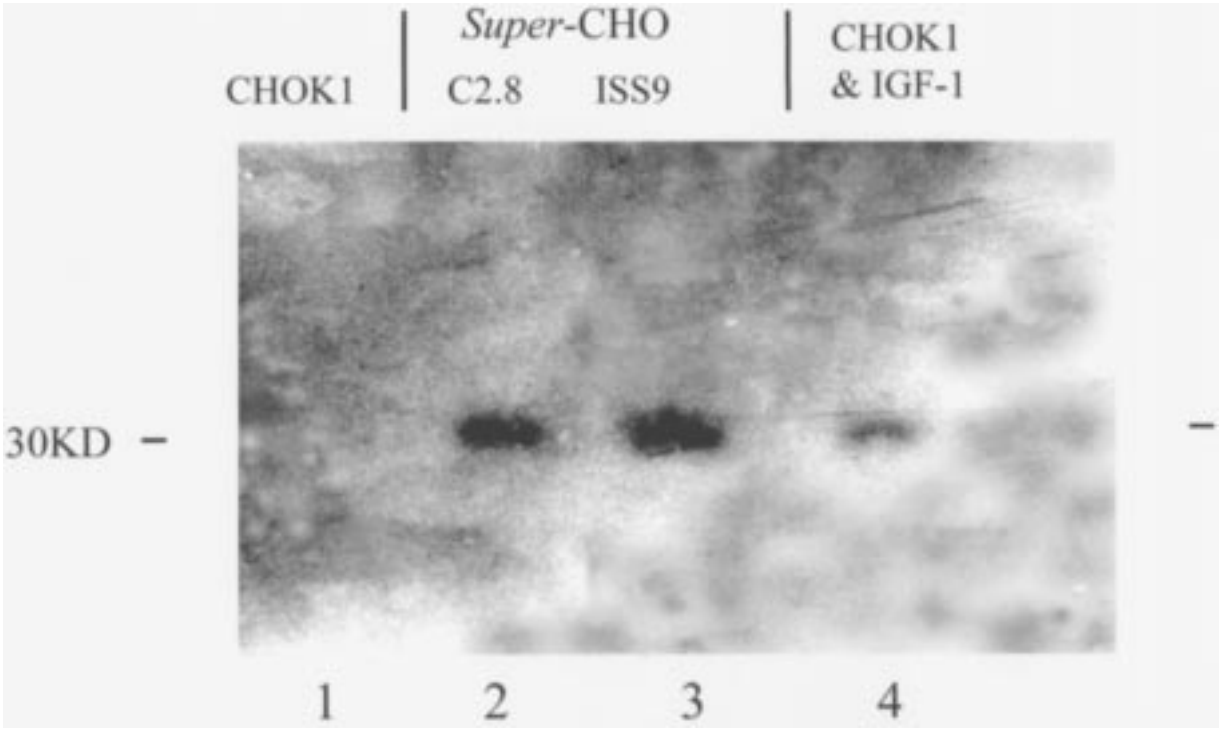

Figure 2. Western ligand blotting of IGF-I. Samples were prepared and processed for ligand blotting as described under Materials and Methods. Samples are media conditioned by CHOK1 cells (lane 1), in the presence of 50 ng mL^{-1} IGF-I (lane 4), and *Super*-CHO clones C2.8 and ISS9 (lanes 2 and 3). The ligand used was ^{125}I-IGF-I.

An up-regulation of IGF-I receptor expression was demonstrated in rat adult myocytes by IGF-I stimulation (Reiss et al., 1997). IGF-I binding is increased by platelet-derived growth factor (PDGF) treatment which activates the IGF-I receptor promoter (Baserga et al., 1997). In fact, an overexpression of the receptors for PDGF, epidermal growth factor (EGF) and IGF-I is observed when cells are grown in serum-free medium supplemented with their respective ligands (Rosenthal et al., 1991; Stewart et al., 1990). An upregulation of IGF-I receptor expression in *Super*-CHO may be due to the production and secretion of recombinant IGF-I in these cells.

Detection of IGF binding proteins in conditioned medium

The production of binding proteins in response to IGF-I was assessed by [^{125}I]IGF Western ligand blotting and shown in Figure 2 (Hossenlopp et al., 1986). Binding assays were performed on conditioned supernatents from confluent cell monolayers in PFM. Cells were seeded and grown overnight in serum-containing medium, washed twice with PFM, followed by a subsequent 48 hr incubation in PFM. A band of approximately 30 kDa is present in conditioned medium from *Super*-CHO, clones C2.8 and ISS9, but not in the that of the parent cell line, CHOK1. However, an IGF binding protein with similar migration is apparent in the conditioned medium of CHOK1 cells incubated in the presence of exogenously added IGF-I (50 ng mL^{-1}).

The production of binding proteins in response to IGF-I has been demonstrated in several studies (Conover, 1992; Donnelly and Holly, 1996), and it has been shown that expression and secretion of IGFBPs fluctuate under varying growth conditions (Birnbaum et al., 1994; Mouhieddine et al., 1994).

Western blot of IGF-I binding complexes in Super-CHO conditioned medium

Further evidence for the presence of IGF binding proteins was obtained in the conditioned medium of *Super*-CHO cells. Proteins present in PFM conditioned by *Super*-CHO cells were treated with the chemical cross linker, disuccinimidyl suberate (DSS) before separation on SDS-PAGE and transfer onto a

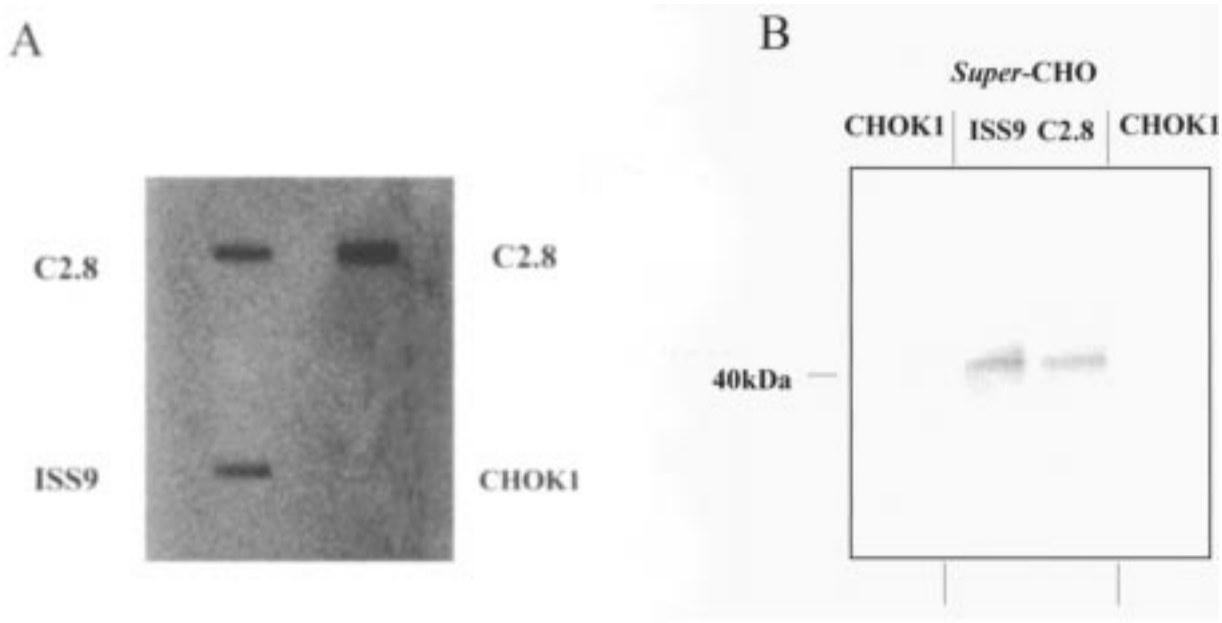

Figure 3. IGF-I and IGF-I binding proteins in conditioned media were detected using Western blot analysis. Cells were seeded in media containing 10% FBS to allow for attachment overnight, after which they were washed in PBS and incubated in PFM. Conditioned media was collected after 48 hr and incubated in the presence (+) or absence (–) of the cross-linker disuccinimidyl suberate (DSS). Following TCA precipitation, samples were electrophoresed, transferred to nitrocellulose and probed using antibody against IGF-I (see Section Material and Methods).

nitrocellulose membrane. The membrane was probed with anti-IGF-I antibody and IGF-I complexes were visualised as described in materials and methods and shown in Figure 3. IGF-I and complexes of IGF-I are present in the conditioned medium of *Super*-CHO, clone C2.8 in the presence of cross-linker (lane 1). A prominent IGF-I complex migrates at approximately 150 kDa and a doublet at 30–36 kDa . Minor complexes appear to migrate at 125 and 78 kDa. A 150 kDa complex consisting of IGF-I, IGFBP-3 and an acid-labile subunit (ALS) has been demonstrated in human plasma. This complex is considered as a reservoir of IGFs (Young Lee and Rechler, 1996; Ooi et al., 1997). IGFBP-3, a protein of approximately 35 kDa, has been demonstrated to form dimers in solution. Recently, it has been shown that the multimerization of IGFBP-3 may be responsible for generating distinctive conformations of the protein which in turn can regulate its ability to bind to the cell surface.(Koedam et al., 1997). IGFBP-3 is also subject to proteolysis, and it is possible that the 125- and 78 kDa protein complexes may be cross-linked dimers of IGFBP-3, IGF-I and their proteolytic products (Lalou and Binoux, 1993; Mohseni-Zadeh and Binoux, 1997). In the absence of cross-linker (lane 2), IGF-I is visualised as a complex migrating at 36 and 150 kDa, suggesting a strong association with these proteins. The absence of a 30 kDa complex may represent a weak association with IGF-I. The 150 kDa complex appears less intense in the absence of cross-linker, probably due to disassociation during electrophoresis. The IGF-I binding protein profile of clone ISS9 differs from that of clone C2.8 both in the presence and in the absence of cross-linker (lanes 3 and 4). Cross-linking proteins in the conditioned medium of *Super*-CHO clone ISS9 results in the appearance of the 150 kDa IGF-I complex while the 30- or 36 kDa complex is not visible (lane3). This suggests that under normal circumstances, IGF-I is bound tightly in the 150 kDa complex, but disassociates during electrophoresis if not chemically cross-linked (lane 4). The difference in profiles of clones C2.8 and ISS9 may help to explain the differences in growth capabilities of both *Super*-CHO clones. Although similar quantities of IGF-I are produced in both C2.8 and ISS9, their growth may depend on the nature of the interaction of IGF-I and IGF binding proteins, regulating the utility of IGF-I by the cells. IGF-I and/or IGF-I complexes are not present in PFM conditioned by CHO-K1 cells in the presence (lane 5) or in the absence (data not shown) of

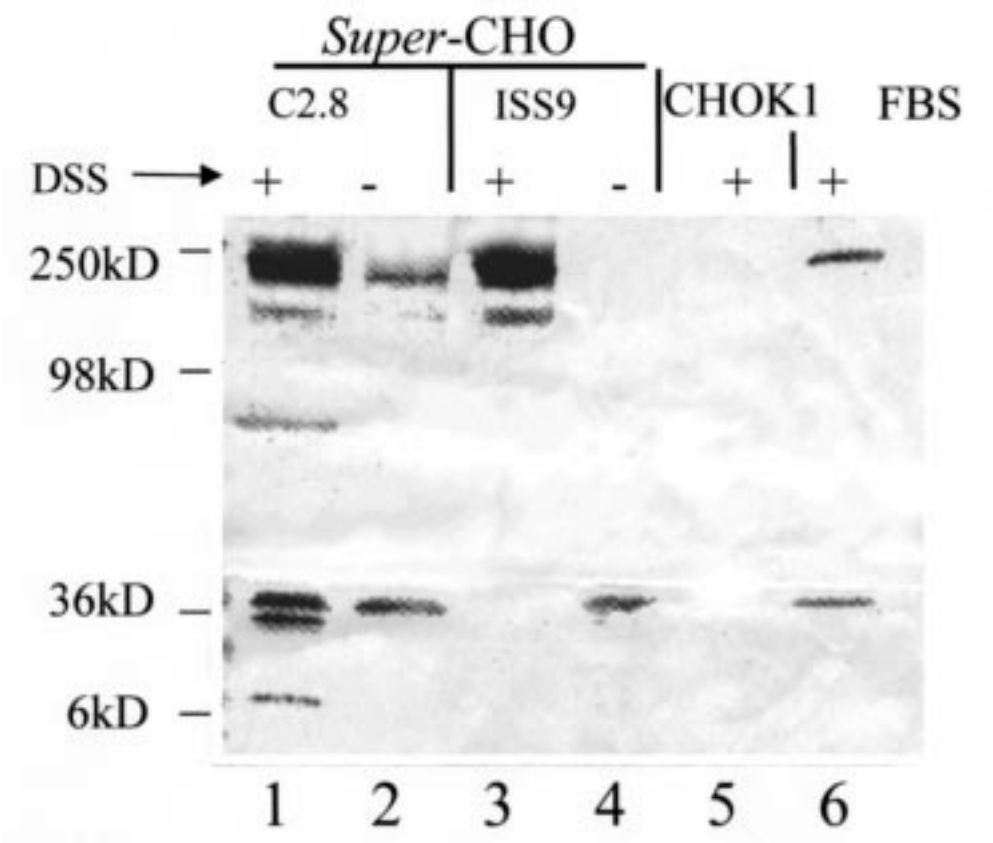

Figure 4. Detection of IGFBP-3 in conditioned media and in cellular lysates. A) IGFBP-3 dot blot. Conditioned media was obtained from cells maintained in PFM for 48 hr and applied to a nitrocellulose membrane using a vacuum blotting apparatus (see Materials and Methods). IGFBP-3 was detected using the rodent-specific anti-IGFBP-3 antibody, M-19 (Santa Cruz Biotechnology). B) Immunoprecipitation of ^{35}S Methionine-labelled cellular lysates using the M-19 antibody. Estimation of molecular weight was determined by co-migration of See Blue size markers (Novex, San Diego, CA).

cross-linker. Unconditioned medium containing 10% FBS (lane 6) includes IGF-I complexes and are visible when cross-linked to the molecule.

Detection of IGFBP-3

The M-19 antibody (Santa Cruz Biotechnology) which is specific for rodent IGFBP-3 was used to detect IGFBP-3 both in conditioned media and cellular lysate of *Super*-CHO and CHOK1 (Figure 4). IGFBP-3 can be detected in the conditioned media from *Super*-CHO clones C2.8 and ISS9, but not from that of CHOK1 cells (Figure 4A). In addition, a protein of approximately 40 kDa corresponding to CHO IGFBP-3 was detected in ^{35}S-methionine labelled cellular lysates of *Super*-CHO clones, but not from that of CHOK1 when immunoprecipated using the same antibody (Figure 4B). Cell surface associated IGFBP-3 has been demonstrated to either inhibit or stimulate IGF action depending on the cell type (Conover et al., 1996). Our results suggests that *Super*-CHO expresses its own IGFBP-3, but it remains to be determined whether IGFBP-3 is membrane bound and/or intracellular.

Conclusion

The engineered *Super*-CHO cell line is capable of stable growth under fully defined protein-free conditions making it an ideal host for the production of biopharmaceutical products. The constitutive expression of IGF-I and transferrin eliminates the need for exogenous growth factors in the culture medium. The growth of *Super*-CHO has been shown to be due to the autocrine effect of the endogenous IGF-I (Pak. et al., 1996). We detected IGF-I receptor-specific RNA

transcripts in two different clonal isolates of *Super*-CHO which differ in their growth rates whereas RNA transcripts corresponding to IGF-I receptor in parental CHO cells were below our limits of detection.

Although the biological effects of IGFs are mediated by cell surface receptors, their bioavailability is regulated by a family of at least six homologous proteins that have co-evolved with the IGFs called IGF binding proteins (IGFBPs). The IGFBPs modulate the actions of IGF in endocrine, paracrine and autocrine settings. They influence IGF bioavailability and can either inhibit or potentiate IGF action. Inhibition is achieved by sequestration of IGFs from their receptors (Angervo et al., 1991; Cohen. et al., 1993) and the mechanism of potentiation involves a cellular association by IGFBPs bound to IGFBP receptors or otherwise (Conover, 1992). Potentiation of IGFs can also be achieved by the proteolysis of IGFBPs into fragments with lower affinity for IGFs, thereby increasing free IGF bioavailability (Cohen et al., 1994).

Since IGFBPs play an important role in directly regulating cell growth we investigated the IGF autocrine system of *Super*-CHO to determine the implication of IGFBPs. We report that *Super*-CHO cells synthesise IGFBP-3 in response to IGF-I production. We noted an apparent difference in the size of soluble IGFBP (about 30 kDa) in conditioned media and that of endogenous IGFBP-3 (40–45 kDa). Proteolysis of IGFBP-3 may be occurring extracellularly since it is known that specific IGFBP proteases can release IGFs from reservoir complexes. The activation of these proteases might be mediated by binding to IGF (Mohseni-Zadeh and Binoux, 1997; Zumbrunn and Trueb, 1996). Proteolytic enzymes specific to the host CHO cell may be operative in the dynamics of IGF-I regulated growth. In conclusion, the IGF-I-mediated growth characteristics of *Super*-CHO is governed by a complex interaction of host cell specific factors which regulate its utility of the growth factor and are coordinated by altering the expression of IGFBPs, IGF-I receptor and perhaps other relevant genes.

References

Ando Y and Jensen PJ (1993) Epidermal Growth Factor and Insulin-Like Growth Factor I Enhance Keratinocyte Migration. J Invest Dermatol 100, 633–639.

Angervo M, Koistinen R, Suikkari AM and Seppala M (1991) Insulin-like Growth Factor Binding Protein Inhibits the DNA Amplification induced by Insulin-like Growth Factor-I in Human Granulosaluteal cells. Hum Reprod 6, 770–773.

Baserga R, Hongo A, Rubini M, Prisco M and Valentinis B (1997) The IGF-I Receptor in Cell Growth, Transformation and Apoptosis. Biochimica et Biophysica Acta 1332, F105–F126.

Baxter RC (1986) The Somatomedins: Insulin-like Growth Factors. Adv Clin Chem 25, 49–115.

Baxter RC and Martin, JL (1989) High Molecular Weight Insulin-like Growth Factor Binding Protein Complex. J Biol Chem 264, 11843–11848.

Birnbaum RS and Wiren KM (1994) Changes in Insulin-Like Growth Factor-Binding Protein Expression and Secretion during the Proliferation, Differentiation, and Mineralization of Primary Cultures of Rat Osteoblasts. Endocrinology 135, 223–230.

Camacho-Hubner C, Busby WH, Mccusker RH, Wright G and Clemmons DR (1992) Identification of the Forms of Insulin-like Growth Factor-binding Proteins Produced by Human Fibroblasts and the Mechanisms that Regulate their Secretion. J Biol Chem 267, 11949–11956.

Chomczynski P and Sacchi W (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 165–169.

Cohen P, Lamson G, Okajima T and Rosenfeld RG (1993) Transfection of the Human Insulin-Like Growth Factor Binding Protein-3 gene into Balb/c Fibroblasts Inbhibits Cellular Growth. Mol Endcrinol 7, 380–386.

Cohen P, Peehl DMBB, Liu FHRL and Rosenfeld RG (1994) Insulin-like Growth Factor Axis Abnormalities in Prostatic Stromal Cells from Patients with Benign Prostatic Hyperplasia. J Clin Endocrinol Metabol 79, 1410–1414.

Conover CA (1992) Potentiation of Insulin-Like Growth Factor (IGF) Action by IGF-Binding Protein-3: Studies of Underlying Mechanism. Endocrinology 130(6): 3191–3199.

Conover CA, Clarkson JT and Bale LK (1996) Factors Regulating Insulin-Like Growth Factor-Binding Protein 3 Binding, Processing, and Potentiation of Insulin-Like Growth Factor Action. Endocrinology 137, 2286–2292.

Coverley JA and Baxter RC (1997) Phosphorylation of Insulin-Like Growth Factor Binding Proteins. Mol and Cellular Endocrinology 128, 1–5.

De Leon DD and Asmerom Y (1997) Quantification of Insulin-Like Growth Factor I (IGF-I) without Interference by IGF Binding Proteins. Endocrinology 138, 2199–2202.

Donnelly MJ and Holly JMP (1996) The Role of IGFBP-3 in the Regulatlion of IGFBP-4 Proteolysis. J Endocrinology 149, R1–R7.

Dufourny B, Alblas J, van Teeffelen HAAM, Van Schaik FMA, Van der Burg B, Steenbergh PH and Sussenbach JS (1997) Mitogenic Signaling of Insulin-like Growth Factor I in MCF-7 Human Breast Cancer Cells Requires Phosphatidylinositol 3-Kinase and is Independent of Mitogen-activated Protein Kinase. J Biol Chem 272, 31163–31171.

Hashimoto R, Ono M, Fujiwara H, Higashihashi N, Yoshida M, Enjoh-Kimura T and Sakano K (1997) Finding Sites and Binding Properties of Binary and Ternary Complexes of Insulin-like Growth Factor-II (IGF-II), IGF-binding Protein-3, and Acid-labile Subunit. J Biol Chem 272, 27936–27942.

Herrin DL and Schmidt GW (1988) Rapid, Reversible Staining of Northern Blots Prior to Hybridization. Biotechniques 6, 196–200.

Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, and Binoux M (1986) Analysis of Serum Insulin-like Growth Factor Binding Proteins Using Western Blotting: Use of the Method for Titration of the Binding Proteins and Competitive Binding Studies. Anal Biochem 154, 138–143.

Kato H, Faria TN, Stannard B, Levy-Toledano R, Taylor SI, Roberts CTJ and LeRoith D (1993) Paradoxical Biological Effects of Overexpressed Insulin-like Growth Factor-I Receptors in Chinese Hamster Ovary Cells. J Cell Physiol 156, 145–152.

Kelley KM, Youngman OH, Gargosky SE and Gucev ZS (1996) Insulin-like Growth Factor-binding Proteins (IGFBPs) and their Regulatory Dynamics. Int J Biochem Cell Biol 28, 619–637.

Koedam JA, Hoogerbrugge CM and Van Buul-Offers SC (1997) Insulin-like Growth Factor Binding Proteins-3 and -5 Form Sodium Dodecyl Sulfate-Stable Multimers. Biochem Biophys Res Comm 240, 707–714.

Lalou C and Binoux M (1993) Evidence that Limited Proteolysis of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) occurs in the normal state outside of the bloodstream. Regul Pept 48, 179–188.

Lee CY and Rechler MM (1995) A Major Portion of the 150-kilodalton Insulin-Like Growth Factor Binding-Protein (IGFBP) Complex in Adult Rat Serum Contains Unoccupied, Proteolytically Nicked IGFPB-3 that Bind IGF-II Preferentially. Endocrinology 136, 668–678.

LeRoith D, Werner H, Beitner-Johnson D and Roberts Jr, CT (1995) Molecular and Cellular Aspects of the Insulin-like Growth Factor I Receptor. Endocrine Reviews 16, 143–163.

Machuca I, Esslemont G, Fairclough Lynne and Tata JR (1995) Analysis of Structure and Expression of the *Xenopus* Thyroid Hormone Receptor-B Gene to Explain its Autoinduction. Mol Endcrinol 9, 96–107.

Mohseni-Zadeh S and Binoux M (1997) Insulin-like Growth Factor (IGF) Binding Protein-3 Interacts with the Type I Receptor, Reducing the Affinity of the Receptor for its Ligand: an Alternative Mechanism in the Regulation of IGF Action. Endocrinology 138, 5645–5648.

Mouhieddine OB, Cazals V, Maiatre B, Le Bouc Y, Chadelat K and Clement A (1994) Insulin-Like Growth Factor-II (IGF-II) Type 2 IGF Receptor, and IGF-Binding Protein-2 Gene Expression in Rat Lung Alveolar Epithelial Cells: Relation to Proliferation. Endocrinology 135, 83–91.

Ooi GT, Cohen FJ, Tseng LYH, Rechler MM and Boisclair YR (1997) Growth Hormone Stimulates Transcription of the Gene Encoding the Acid-Labile Subunit (ALS) of the Circulating Insulin-Like Growth Factor-Binding Protein Complex and ALS Promoter Activity in Rat Liver. Mol Endcrinol 11, 997–1007.

Pak SCO, Hunt SMN, Bridges MW, Sleigh MJ and Gray PP (1996) Super-CHO – A cell line capable of autocrine growth under fully defined protein-free conditions. Cytotech 22, 139–146.

Reiss K, Cheng W, Pierzchalski P, Kodali S, Li B, Wang S, Liu Y and Anversa P (1997) Insulin-like Growth Factor-I Receptor and its Ligand Regulate the Reentry of Adult Ventricular Myocytes in the Cell Cycle. Exp Cell Res 235, 198–209.

Rosenthal SM, Brown EJ, Brunetti A and Goldfine ID (1991) Fibroblast Growth Factor Inhibits Insulin-Like Growth Factor-II (IGF-II) Gene Expression and Increases IGF-I Receptor Abundance in BC3H-1 Muscle Cells. Mol Endcrinol 5(678): 684.

Steele-Perkins G, Turner J, Edman JC, Hari J, Pierce SB, Stover C, Rutter WJ and Roth RA (1988) Expression and Characterization of a functional Human Insulin-like Growth Factor I receptor. J Biol Chem 263, 11486–11492.

Stewart AJ, Johnson MD, May FEB and Westley BR (1990) Role of Insulin-Like Growth Factors and the Type I Insulin-Like Growth Factor Receptor in the Estrogen-stimulated Proliferation of Human Breast Cancer Cells. J.Biol.Chem 265, 21172-21178.

Tavakkol A, Elder JT, Griffiths CEM, Cooper KD, Talwar H, Fisher GJ, Keane KM, Foltin SK and Voorhees JJ (1992) Expression of Growth Hormone Receptor, Insulin-Like Growth Factor I (IGF-I) and IGF-I Receptor mRNA and Proteins in Human Skin. J Invest Dermatol 99, 343–349.

Young Lee C and Rechler MM (1996) Proteolysis of Insulin-Like Growth Factor (IGF)-Binding Protein-3 (IGFBP-3) in 150-Kilodalton IGFBP Complexes by a Cation-Dependent Protease Activity in Adult Rat Serum Promotes the Release of Bound IGF-I. Endocrinology 137, 2051–2058.

Zumbrunn J and Trueb B (1996) Primary Structure of a Putative Serine Protease Specific for IGF-Binding Proteins. FEBS Letters 398, 187–192.