Chinese hamster ovary cells produce sufficient recombinant insulin-like growth factor I to support growth in serum-free medium

Serum-free growth of IGF-I-producing CHO cells

S. M. N. Hunt¹,* S. C. O. Pak¹, M. W. Bridges¹, P. P. Gray¹ & M. J. Sleigh²†

¹ Department of Biotechnology, University of New South Wales, Kensington, NSW, Australia; ² CSIRO, Division of Biomolecular Engineering, North Ryde 2113, NSW, Australia

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Abstract

Insulin-like growth factor I has similar mitogenic effects to insulin, a growth factor required by most cells in culture, and it can replace insulin in serum-free formulations for some cells. Chinese Hamster Ovary cells grow well in serum-free medium with insulin and transferrin as the only exogenous growth factors. An alternative approach to addition of exogenous growth factors to serum-free medium is transfection of host cells with growth factor-encoding genes, permitting autocrine growth. Taking this approach, we constructed an IGF-I heterologous gene driven by the cytomegalovirus promoter, introduced it into Chinese Hamster Ovary cells and examined the growth characteristics of Insulin-like growth factor I-expressing clonal cells in the absence of the exogenous factor. The transfected cells secreted up to 500 ng/10⁶ cells/day of mature Insulin-like growth factor I into the conditioned medium and as a result they grew autonomously in serum-free medium containing transferrin as the only added growth factor. This growth-stimulating effect, observed under both small and large scale culture conditions, was maximal since no further improvement was observed in the presence of exogenous insulin.

Introduction

Insulin-like growth factor I (IGF-I) is a 70-amino acid basic peptide bound to specific carrier proteins in serum and other body fluids, where it can be present in great excess of up to 1 $\mu\text{g ml}^{-1}$. It plays an important role in the regulation of cell growth through its wide range of metabolic and mitogenic actions (reviewed in (Baxter, 1986; Froesch, 1985)).

The liver is the main source of serum IGF-I but many other tissues synthesize this growth factor. IGF-I elicits all the classical insulin effects on all the target tissues of insulin, and activates the same biological responses in cells. As an exogenous growth factor, it has been shown to exert potent growth promoting

stimuli for many different cultured cells *in vitro*. In contrast to the high levels present in serum, a few nanograms ml^{-1} can be effective.

Although *in vivo* observations suggest an endocrine modality of action, many cultured cells produce IGF-I and their proliferation is attributed to its autocrine and/or paracrine actions (Clemmons, 1984; Clemmons, 1983; Clemmons, 1981; Daughaday, 1989). This action is dependent on secretion of the protein and binding to specific, cell surface, type 1 IGF receptors (Dai, 1992a; Rechler, 1985).

With the increasing interest in producing recombinant proteins in cultured mammalian cells, attention has turned to measures to reduce cost and ensure consistency of such production methods. At laboratory scale, most mammalian cell culture systems employ serum. However, serum-free medium (SF) has been developed for many cell lines. Such media generally

* To whom correspondence should be addressed.

† Current address: Peptide Technology Ltd, Sydney 2099, Australia.

include growth factors such as insulin and transferrin, substituting for mitogenic factors in serum (Barnes, 1980).

Insulin and transferrin are the growth factors of choice for serum-free culture of Chinese Hamster Ovary (CHO) cells. However, it has been reported that IGF-I can replace the need for insulin (Ross, 1993). Although the mitogenic effects of insulin and IGF-I are similar, IGF-I may be a better candidate for achieving serum-free growth of CHO cells. The IGF-I effects occur at low concentrations compared to unphysiologically high levels of insulin required to support cell growth. In fact, the recommended levels for use as a growth factor in SF cell culture are $1\text{--}50\text{ ng ml}^{-1}$ for IGF-I (Froesch, 1985) compared with $1\text{--}10\text{ }\mu\text{g ml}^{-1}$ for insulin (Barnes, 1980). Human IGF-I has been shown to have growth promoting effects on several types of cells in culture, so presumably there is no significant differences in receptor specificity between the different cell types.

While growth of mammalian cells in serum-free culture is generally achieved by addition of exogenous growth factors, an alternative approach is transfection of host cells with growth factor-encoding genes, permitting autocrine cell growth. In considering this approach with CHO cells, additional factors favour the use of IGF-I over insulin. Levels of endogenous IGF-I of less than 10 ng ml^{-1} (in both naturally-expressing cells and transfected cells) have significant effects on the proliferation of these cells (Cascieri, 1988; Dai, 1992b). Furthermore, recombinant IGF-I has been found to be biologically equipotent to purified IGF-I (Ballard, 1987; McKinnon, 1991).

Cells transfected with the IGF-I gene secrete mature IGF-I that is correctly processed and biologically active into the conditioned medium (Ballotti, 1987; Bayne, 1988; Bayne, 1987; Bovenberg, 1990; Dai, 1992b; McKinnon, 1991). Levels of secreted IGF-I reported range from as little as $1\text{ ng ml}^{-1}\text{ day}^{-1}$ (Dai, 1992b), to 1500 ng ml^{-1} from similar cultures of CHO cells (McKinnon, 1991). These levels are comparable with amounts of other proteins produced in a variety of cell lines transfected with non-amplifiable genes (Bendig, 1988).

These results for IGF-I are in contrast with attempts to express human insulin in mammalian cells including CHO cells. In general, such cells fail to process proinsulin to the native form, and insulin-related products are detected only at low levels, of the order of less than $10\text{ ng}/10^6\text{ cells day}^{-1}$ (S.M.N. Hunt, Cytotechnology,

in press) and (Laub, 1983; Moore, 1983; Vollenweider, 1992; Vollenweider, 1993).

Although IGF-I has been successfully expressed in mammalian cells, the long term consequences of IGF-I endogenous expression have not been well defined. We investigated the effectiveness of endogenously-produced IGF-I in supporting serum-free growth of CHO cells by establishing a CHO cell line transfected with a heterologous gene containing cDNA encoding hIGF-I mRNA. The transfected cells secreted IGF-I into the conditioned medium and as a result the cells grew in serum-free medium with transferrin as the only added growth factor.

Materials and methods

Cell culture

CHO-K1 cells (ATCC No. CCL 61; obtained from the American Type Culture Collection, Rockville, MD, USA) were cultured in tissue culture dishes or T-flasks (Corning, Corning, NY) in a 1:1 mixture of DMEM:Ham's F-12 medium supplemented with 10% heat-inactivated foetal calf serum (FCS) in an incubator at 37°C , 5% CO_2 , 90% humidity. Serum-free medium consisted of a mixture of DMEM/Coon's modified F-12 (Gibco BRL) supplemented with $10\text{ }\mu\text{g ml}^{-1}$ each of human transferrin and bovine insulin (both from Sigma Chemical Co, St Louis, MO, USA) and with sodium selenite at $2 \times 10^{-8}\text{ M}$ except where otherwise noted. It is referred to as SF+ITS medium.

Larger-scale cultures were carried out in spinner flasks on microcarriers in medium + 2 g L^{-1} pluriol (BASF, Germany) + 10 mg L^{-1} cholesterol (Sigma USA).

Doubling time was used as a measure of cell growth. The cells were seeded at low density and cultured in plates or spinner flasks and the number of viable cells, determined on a hemacytometer at intervals during the growth period, was plotted against time in culture. The doubling time was calculated from the slope of the curve during the exponential growth period.

Gene cloning

A plasmid containing the IGF-I coding region driven by the human cytomegalovirus (CMV) promoter, and the gene for hygromycin resistance (Hyg B), was prepared (Fig. 1). The gene, designated pCMVIGF-I,

was constructed by modification of a pCEP4-derived plasmid, pCMVTf which contained the VP1 intron sequences and the SV40 polyadenylation signal from pSVL (Pharmacia), the CMV promoter, hygromycin resistance gene, and ampicillin resistance gene from pCEP4 (Invitrogen), and the coding sequence for human transferrin (S.M.N. Hunt, unpubl.).

Two IGF-I mRNAs are produced by alternative RNA processing from a single primary transcript (Rotwein, 1986). They encode the precursor peptides, IGF-IA (153 amino acid residues) and IGF-IB (195 amino acid residues). The IGF-IA coding sequence was isolated by polymerase chain reaction (PCR) on a Perkin-Elmer Cetus Instrument Thermal Cycler using specific oligonucleotide primers designed from published IGF-I sequences (Jansen, 1983), and a human liver cDNA library (in λ gt10, Clontech) as previously described (Friedman, 1988). Two oligonucleotides (IGFs1 and IGFa1) were prepared on a Model 391 PCR-Mate™ DNA Synthesizer and purified in a purification cartridge (Applied Biosystems). Oligonucleotide IGFs1 (cgagtctagaccacc**ATGCACACCATGTCCTCCTCG**) contained sequences homologous to the 5' end of IGF-I cDNA (shown in capital letters) around the methionine initiator at position -25 relative to the transcription start site (in bold). It also incorporated near-optimal translation initiation sequences (ACCATGC) to ensure optimal translation initiation of the gene (Kozak, 1986) and the *Xba* I endonuclease recognition site (underlined) to facilitate subsequent cloning of the PCR product. Oligonucleotide IGFa1 (GTGCAGAGCAAAGGATCCTGCG) was complementary to IGF-IA sequences 40 bp downstream of the TAG stop codon and included a *Bam* HI endonuclease recognition site (underlined).

The PCR-generated IGF-IA coding sequence was first cloned into the polylinker of pSVLN (Kelly, 1993) to generate pSVLNIGF-I and sequenced (Sequenase® Version 2.0 Kit, United States Biochemical, Cleveland, OH) to ensure that no PCR-generated mutations had been introduced. The IGF-IA coding sequence was then sub-cloned into the mammalian expression vector pCMVTf to generate pCMVIGF-I (Fig. 1).

Transfection

Plasmid DNA used for transfection of CHO cells was prepared by CsCl gradient centrifugation as previously described (Maniatis, 1982). For transient expres-

sion analyses, DNA was introduced by a liposome-mediated method. Transfectam® reagent (Promega Corporation; Madison, WI, USA) was used according to the manufacturer's recommendations. The optimal parameters determined for CHO cells were: Transfectam:DNA ratio of 2 μ l:1 μ g and a 4 h incubation time in the presence of the Transfectam/DNA mixture.

For stable expression analyses CHO cells were transfected with a total of 10 μ g of plasmid DNA by the calcium phosphate-mediated method (Chen, 1988). Forty-eight hours later Hyg B (Boehringer Mannheim, Germany) was added at 500 μ g ml⁻¹ of culture medium to select stable transfected cells. Stable colonies developed after 2 weeks and clonal lines were isolated by carefully picking cells from randomly selected colonies using a 'Pipetman' sterile disposable tip. After adequate amplification, aliquots of the cell clones were stored in liquid N₂ and the remaining cells used to verify expression of the transgene by Northern blot hybridization.

Northern blot analysis

Cytoplasmic RNA was isolated from CHO cells (Gough, 1988) and analyzed by Northern blot hybridization (Maniatis, 1982). A ³²P-labeled *Xba* I-*Bam* HI fragment from pSVLNIGF-I (Fig. 1) was used as an IGF-I-specific probe. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. The membranes were stripped and re-probed with the GAPDH-specific probe, ³²P-labeled pCHOGAP (a plasmid containing CHO-specific GAPDH cDNA fragment kindly provided by G. Brown, CSIRO). The hybridized membranes were screened after exposure to a PhosphorImager screen (Molecular Dynamics).

Western blot analysis

Conditioned medium was subjected to SDS-PAGE using a 4–20% gradient gel supplied by Novex, San Diego, CA (Laemmli, 1970). Human recombinant IGF-I (Boehringer Mannheim, Germany) was used as standard. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane (Schleicher and Schnell) using a semi-dry system and the IGF-I-specific bands were immunodetected using a 1:1000 dilution of a rabbit anti-human IGF-I primary antibody (abV ImmuneResponse, Inc., Derry, New Hampshire) and a 1:1000 dilution of an alkaline phosphatase-conjugated mouse anti-rabbit IgG immunoglobulin

Figure 1. Construction of the IGF-I-expressing plasmid pCMVIGF-I. An *Xba*I-*Bam*HI fragment of the PCR-isolated IGF-I coding sequence was ligated into the respective sites within the multiple cloning site of pSVLN to generate pSVLNIGF-I. An *Eco*RV-*Bam*HI fragment containing part of the VP1 intron and the IGF-I coding sequence was then isolated and ligated into the respective sites of pCMVtI in place of the VP1 intron + Transferrin sequence to generate pCMVIGF-I. The relevant restriction endonuclease recognition sites are shown.

secondary antibody (Boehringer Mannheim, Germany). The IGF-I-specific bands were visualised using NBT-BCIP (nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate) substrate system as previously described (Maniatis, 1982).

Results and discussion

Expression of CMVIGF-I fusion gene in CHO cells

The CMVIGF-I expression cassette was initially tested for IGF-I mRNA expression in a transient system. One μ g of the plasmid pCMVIGF-I was transiently transfected into CHO cells. Cytoplasmic RNA was isolated from the cells 48 h after transfection and analysed for IGF-I-specific mRNA by Northern blot. A transcript of approximately 1.0 kb, the expected size for the 450 bases of IGF-I mRNA together with 5' and 3' sequences from the expression vector, was detected in

the transfected cells (lane T) but not in the control (lane CHO), indicating correct expression of the IGF-I gene (Fig. 2).

CHO cell lines stably expressing the CMVIGF-I gene were generated by transfecting 10 μ g of pCMVIGF-I into CHO cells. Two separate transfections were done (#1 and #2). Six hygromycin-resistant clones were picked from #2 transfection (clones 2-1 to 2-6). Resistant cells from each transfection were also pooled into a bulk cell line (#1 and #2). Cytoplasmic RNA isolated from the 6 clonal lines and the bulk lines (#1 and #2) was analysed for IGF-I mRNA expression by Northern blot as above. IGF-I-specific transcripts were detected in both bulk lines and clonal line 2-5 (Fig. 2). Levels of GAPDH mRNA (internal control) are also shown. The transfected cells expressed IGF-I mRNA at levels comparable to that of the housekeeping gene GAPDH.

Figure 2. Analysis of transient and stable CMVIGF-I mRNA expression in transfected CHO cells. Ten μg of cytoplasmic RNA from CHO cells transiently transfected with pCMVIGF-I (lane T), stable bulk cell lines #1 and #2 and from clonal lines 2–1 to 2–6 (isolated from #2 bulk line) were analysed by Northern blot for IGF-I and GAPDH (internal control) mRNA expression. RNA from untransfected CHO cells was used as a control (CHO). IGF-I-specific transcripts were detected in the transiently transfected cells (T), the bulk lines #1 and #2 and clone 2–5 but not in the other 5 clones and untransfected CHO control.

Secretion of IGF-I by CHO/CMVIGF-I cells

Conditioned medium (24 h, SF+TS medium) from the #1 bulk cells and untransfected CHO cells (control) was analysed for the presence of IGF-I by Western blot analysis. An immunoreactive protein band migrating at about 7.6 kDa, the expected size for mature IGF-I, was detected in the standard and transfected line but not in the control (Fig. 3). No bands were detected between 14–15 kDa, the predicted size of the IGF-IA precursor.

Twenty-five cell clones were isolated from the bulk line #1 by limiting dilution (clones 1–1 to 1–25), and conditioned medium analysed as above. IGF-I-specific bands were detected in the standards and 14 of the 25 samples. The results (shown for clones 1–1 to 1–12 in Fig. 3) showed that 14 of the 25 clones tested were producing IGF-I at estimated levels greater than $100 \text{ ng}/10^6 \text{ cells day}^{-1}$ (based on the minimum levels detectable by western analysis, data not shown) with the highest producers (clones 1–2, 1–8, 1–9 and 1–11) secreting about $500 \text{ ng}/10^6 \text{ cells day}^{-1}$. Clones 1–2 and 1–9 were selected for serum-free growth characterization as described below. Cells from clone 2–5, which was found to express IGF-I-specific mRNA above, secreted IGF-I into the conditioned medium at a similar rate to cells from clones 1–2 and 1–9 (as determined by Western blot, data not shown).

We have shown that the transfected cells processed and secreted mature IGF-I at levels comparable to those previously reported for IGF-I expressed in CHO cells

(Bovenberg, 1990; McKinnon, 1991), and for other recombinant proteins produced in cells transfected with non-amplifiable genes (Bendig, 1988).

Small scale serum-free growth of IGF-I-producing CHO cells

The growth characteristics of cells from the CHO/CMVIGF-I clonal lines 2–5, 1–2 and 1–9 were analysed in SF+TS medium and medium +10% FCS. Cells (5×10^4) from each line were seeded in 100-mm plates and allowed to attach for 24 h in medium +10% FCS. The medium in half of the plates was then replaced by SF+TS medium and the cells cultured for a further 6 days. Viable cell counts were determined by trypan blue exclusion on days 2, 4 and 6. Duplicate plates were used for each time point and duplicate counts obtained from each plate. Two extra plates were set up as above with cells from clones 1–2 and 1–9 and the medium changed to SF+ITS 24 h later. A visual examination of the cells were made on days 2 and 4 for comparison with the same cells in the absence of insulin and the number of cells counted on day 6. Untransfected CHO cells cultured in SF+TS and SF+ITS medium were used as controls (cells counted on days 1, 2, 3 and 5 for SF+ITS medium).

The growth curves are shown for each cell line and treatment (Fig. 4). The doubling times (DT) for the transfected lines 2–5, 1–2 and 1–9 cultured in SF+TS medium were determined as $24.0 \pm 1.2 \text{ h}$, $22.3 \pm 0.3 \text{ h}$ and $25.8 \pm 0.3 \text{ h}$ respectively (average DT $\pm$

Figure 3. Western blot analysis of IGF-I secreted by transfected CHO cells. Conditioned media (24 h) from the stable bulk line #1 and control CHO cells (top panel) and clonal lines 1–1 to 1–12 (bottom panel) were analysed by Western blot. The same volume (16 µl) of IGF-I standard solutions (1 and 10 µg ml⁻¹) and conditioned medium was loaded in each lane. A mixture of pre-stained molecular weight markers was also loaded on the gel for location of the expected bands (data not shown). A band migrating at about 7.6 kDa, the expected size for mature IGF-I, was detected in the bulk line #1 and 9 of the 12 clones shown but not from the control cells.

SE). There was no additional stimulation of growth observed in the case of clones 1–2 and 1–9 when exogenous insulin was present in the medium, i.e. no obvious differences in confluency of cells were observed by visual examination of the plates on days 2 and 4 and the number of cells reached after 6 days in culture was not significantly different from cells grown in SF+TS (0.73×10^7 cells in SF+ITS vs 0.62×10^7 cells in SF+TS in the case of clone 1–2 and 0.34×10^7 in SF+ITS vs 0.27×10^7 in SF+TS for clone 1–9). The doubling times for the transfected cells in insulin-free medium were not significantly different from that of untransfected CHO cells in SF+ITS medium under similar conditions (23.6 ± 1.7 h). In the absence of insulin, the untransfected CHO cells remained viable and did double but at a much slower rate (DT greater than 200 h) indicating that either insulin or IGF-I was necessary for serum-free growth. These results indicate that the transfected cells were secreting sufficient biologically active IGF-I to maximally support their growth in insulin-free medium.

The serum-free growth characteristics of cells from clonal lines 1–10 and 1–14, which produced less than 100 ng/10⁶ cells/day of IGF-I, were examined as described above to test whether these levels could stimulate autocrine growth. Their DT of $43.1 \text{ h} \pm 9.9$ and $36.4 \text{ h} \pm 7.2$ respectively indicate that a production rate of less than 100 ng/10⁶ cells/day can stimulate autocrine growth in serum-free medium, although not maximally.

The transfected CHO cells produced about 10-fold more IGF-I than cells endogenously producing IGF-I and where an autocrine effect is observed (see (Dai, 1992a)). Such levels led to post-receptor desensitization to IGF-I in transfected mouse L cells over-expressing IGF-I (Cascieri, 1988). However, our transfected cells remained responsive despite prolonged exposure to IGF-I. Furthermore, they retained their wild-type morphology. Their growth characteristics in medium +10% FCS were similar to the parent cells (DT = 17.6 ± 0.3 h for line 2–5 and 16.3 ± 2.3 h for the parent line) and their growth characteristics in insulin-free serum-free medium were similar to those

Figure 4. Growth of CHO/CMVIGF-I cells in serum-free medium. Cells from stable clonal lines 2–5, 1–2 and 1–9 were cultured in different media as shown and the number of viable cells were determined by trypan blue exclusion after 48, 96 and 144 h of culture. The average number of cells are plotted on a $\log_{10}$ scale against time in culture. Untransfected CHO cells cultured in SF+TS and SF+ITS were used as controls and cells were counted after 24, 48, 72 and 120 h. The doubling times (DT) are shown in the graph legend as mean $\pm$ SE.

of the parent cells in the presence of insulin (or IGF-I). It has been speculated that intracellular accumulation of IGF-I, and not secreted IGF-I, is responsible for the observed desensitization of IGF-I-expressing cells to the effects of IGF-I in the medium (Dai, 1992a). We did not analyse IGF-I intracellular content but the fact that we did not detect any IGF-I precursor in the conditioned medium may indicate that processing was very efficient in the transfected CHO cells with no intracellular accumulation of IGF-I.

Large scale serum-free growth of IGF-I producing CHO cells

To obtain a better indication of the effect of endogenous IGF-I on the growth rate of the cells in insulin or IGF-I-free serum-free medium, the growth characteristics of cells from clone 2–5 were further examined in spinner flasks. In this system, there is no possibility of exogenous attachment or other factors influencing the growth rate, so any difference in growth rate can

Figure 5. Large-scale growth of CHO/CMVIGF-I cells in serum-free medium. Cells from the transfected clonal line 2–5 were cultured in spinners in either fresh SF+TS medium, 1:1 mixture of fresh:conditioned medium or SF+TS+100 ng ml⁻¹ insulin (SF+ITS). Untransfected CHO cells cultured in SF+ITS were used as a control. A sample was taken at 45, 69.5, 96 and 118 h and quadruplicate cell counts determined from each sample. The average number of cells ml⁻¹ of culture medium is plotted on a $\log_{10}$ scale against time in culture and the doubling times (DT) are shown in the graph legend as mean $\pm$ SE.

be more directly attributed to secreted IGF-I (M.W. Bridges, unpubl).

The cells were prepared by passaging once in SF+TS medium in a 150 cm² T-flask. The cells were then transferred to spinners at an inoculation density of 1×10^5 cells ml⁻¹. The medium was either 100 ml of fresh medium (SF+TS + 2 g L⁻¹ pluriol + 10 mg L⁻¹ cholesterol), 50 ml of fresh medium + 50 ml of conditioned medium (from the T-flasks above), or 100 ml of medium + 100 ng ml⁻¹ of insulin. The cells were grown for 5 days and the cell density measured daily (4 cell counts/sample). Untransfected CHO cells cultured in SF+ITS medium were used as a control. The growth curves are shown in Fig. 5. The doubling times (shown next to the graph legend) for the transfected line 2–5, cultured in SF+TS, SF+TS +50% conditioned medium or SF+ITS media, were 30.6 ± 5.8 h, 31.7 ± 4.8 h and 35.1 ± 4.9 h respectively. These growth rates are similar to that of untransfected CHO cells in SF+ITS medium under the above conditions (DT = 33.2 ± 4.3 h).

To compare the effects of endogenous IGF-I with those of exogenous IGF-I on the parent cell line in large scale cultures, we set up growth experiments in SF+TS

medium supplemented with IGF-I. Growth in spinner flasks on microcarriers was tested in SF+TS medium supplemented with either 100, 500 or 10,000 ng ml⁻¹ of IGF-I. The doubling times for these cultures were 30.5 ± 7.3 h and 34.5 ± 6.2 h with 500 and 10,000 ng ml⁻¹ of IGF-I respectively while 100 ng ml⁻¹ of IGF-I did not support growth. These growth rates are comparable to those of transfected cells in SF+TS medium (DT = 30.6 ± 5.8 h) and of CHO cells in SF+ITS (DT = 33.2 ± 4.3 h) as reported above.

The levels of IGF-I secreted by the clonal cells 2–5 (500 ng/10⁶ day⁻¹), which is presumably equivalent to a production rate of 21 ng/10⁶ cells h⁻¹, i.e. about 21 ng ml⁻¹ h⁻¹, was sufficient to optimally support cell growth from the start of the growth experiment in spinner flasks with no significant lag period since supplementing the medium with 50% pre-conditioned medium did not improve growth any further. In contrast, 100 ng ml⁻¹ of exogenous IGF-I, when added at the start of the experiment, did not support growth of the parent CHO cells in spinner flasks. These results are in agreement with previous experimental observations that continuous release of low amounts of IGF-I is a better mitogenic stimulus than temporary large concentrations (Blum, 1989). Small amounts of IGF-I added to baby hamster kidney fibroblast cells at hourly intervals (0.1 ng ml⁻¹ h⁻¹) stimulated DNA synthesis while 10–300 ng ml⁻¹ added at the beginning of the growth experiment did not have any significant effects. This could indicate that IGF-I is being lost either through breakdown in the culture medium or adsorption to the culture vessel thus resulting in a lower effective level. Presumably secretion of IGF-I from recombinant CHO cells endogenously expressing IGF-I would mimic the hourly additions described above. This observation further emphasizes the benefit of the IGF-I-expressing CHO cells for large scale cultures since, although the cost of exogenous IGF-I could be reduced by reducing the amounts used and increasing the frequency of the additions, this would not be a practical alternative.

Expression of IGF-I has been reported in mammalian cells (Bayne, 1987; Cascieri, 1988; Dai, 1992a; Dai, 1992b) including CHO cells (Bovenberg, 1990; McKinnon, 1991), but the long-term consequences of the IGF-I expression have not been well defined. In most reports the autocrine effect of IGF-I was measured at DNA synthesis level by thymidine incorporation. This is a widely used index of short term cell growth, examining effects on one aspect of cell growth over about 48 h. We describe here the longer term

(days vs hours) effects of endogenous IGF-I on the growth rate of transfected CHO cells as measured by doubling time, a growth index which encompasses all aspects of cell growth. DNA, RNA and protein synthesis, membrane assembly and mitosis must all be normal to obtain an adequate doubling time. Furthermore, we examined the effects of endogenous IGF-I under large scale as well as small scale culture conditions, the rate of growth in spinner cultures being an important parameter in cells which are to be scaled up for recombinant protein production (Friedman, 1989).

Although growth factors form an important component of serum, other factors (e.g. attachment factors) are also required for optimal serum-free growth. The serum-free growth rates we observed in the plate experiments for both transfected cells (in SF+TS medium) and untransfected cells (in SF+ITS medium) were slower than in the presence of serum (DT = 24.0 h and 23.6 h compared to 17.6 h and 16.3 h respectively). This attachment factor effect was also evident when comparing the growth rates (in SF+TS) of the transfected cells in spinners and culture plates (DT = 30.6 h and 24.0 h respectively). In spinners, there was no possible contamination with exogenous attachment and other factors and the growth rates, although slightly lower, can be more directly attributed to the endogenous IGF-I. Further fine tuning of effects other than a growth factor effect on serum-free growth is required. However, in the context of serum-free culture for recombinant protein production, advantages associated with serum-free growth, such as ease of product purification from the defined medium, could outweigh the disadvantage of the relatively slower growth. The final density reached by CHO cells was similar in both culture conditions (data not shown).

Conclusion

We have demonstrated that CHO cells expressing human IGF-I can grow in a transferrin-containing serum-free medium (a medium which does not support growth of the parent cell line) under both small and large-scale culture conditions and in a manner comparable to that of the parent cell line in serum-free medium supplemented with exogenous IGF-I or insulin. Secreted IGF-I is reported to be the major effector of IGF-I autocrine action (Dai, 1992a). We concluded, therefore, that the observed growth stimulation of the transfected cells was due to the autocrine effect of the endogenous IGF-I. Several clones were analysed

to exclude the possibility that the observed effect was due to some fortuitous alteration of the CHO genome by the transfection event. Our results also indicate that IGF-I can substitute for insulin to support serum-free growth (as previously reported (Ross, 1993)).

This study was undertaken as a first step towards generating a CHO cell line capable of autocrine growth in protein-free medium. We have also demonstrated similar results with Transferrin-expressing CHO cells and recently, we have generated a CHO cell line which expresses both IGF-I and Transferrin and is therefore capable of autocrine growth in protein-free medium, an important step towards improved production processes for recombinant protein in mammalian cells (S.C.O. Pak, Cytotechnology, In Press).

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- Address for correspondence:* S.M.N. Hunt, Department of Medical Oncology, Department of Medicine, Westmead Hospital, Westmead, NSW 2145, Australia.