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Long-term stable production of monocyte-colony inhibition factor (M-CIF) from CHO microcarrier perfusion cultures

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Abstract

Monocyte-colony inhibition factor (M-CIF) was produced in microcarrier perfusion cultures from engineered Chinese hamster ovary (CHO) cells. Three and fifteen liter microcarrier perfusion bioreactors equipped with internal spin filters were operated for over two months. Approximately 60 L and 300 L of culture filtrate were harvested from the 3L and 15L microcarrier perfusion bioreactors respectively. During the perfusion operation, cell density reached $2–6 \times 10^6$ cells/ml. Importantly, stable expression of M-CIF from the CHO cells under non-selection condition was maintained at a level of 4–10 mg/L. Specific productivity was maintained at 1.8–3.4 mg/billion cells/day. The ability of the recombinant CHO cells to migrate from microcarrier to microcarrier under our proprietary HGS-CHO-3 medium greatly facilitated microcarrier culture scale-up and microcarrier replenishment. Future directions for microcarrier perfusion system scale-up and process development are highlighted.

Introduction

An increasing number of proteins with therapeutic potentials have been produced in eukaryotic expression systems due to high fidelity of correct post-translational modifications (Cartwright, 1994). Slow growth and low volumetric productivity have promoted the use of internal and external cell retention devices to achieve high cell density and volumetric productivity. Various cell retention devices have been used, such as spin filters (Himmelfarb *et al.*, 1969; Griffiths, 1988; Avgerinos *et al.*, 1990; Emery *et al.*, 1995), hollow fibers (Omasa *et al.*, 1995), gravity settlers (Hansen *et al.*, 1993; Searles *et al.*, 1994), centrifugation devices (Tokashiki *et al.*, 1990), flat membranes (Banik and Heath, 1995), porous matrix and encapsulation. Among these devices, spin filter stands out as the most common and simplistic implementation.

A large number of cells have been successfully grown on microcarriers (Nilsson, 1989). Because of the size difference between cell attached microcarriers and floating cells, microcarrier culture greatly eases viable cell retention and selective removal of detached non-viable cells in a perfusion culture, such as a spin filter perfusion bioreactor. Several therapeutic proteins, such as tissue plasminogen activator, human β interferon and Erythropoeitin, have been produced at around 20 L scale from microcarrier processes, and Aujeszky vaccine, foot-and-mouth vaccine, and Polyomyelitis vaccine have been made in 150–1000 L microcarrier cultures (Miller, 1989). However, few of the processes have yet been transposed to commercial production due to the difficulties encountered in scale-up and the implementation of process control.

We demonstrate microcarrier perfusion cultures of an anchorage-dependent CHO cell have been successfully carried out in 3L and 15L scale to produce therapeutic proteins in quantity. By proper medium formulation, autonomous microcarrier-to-microcarrier cell transfer facilitated microcarrier culture scale-up, and made microcarrier addition and replenishment possible. Improvements to current process control have been revealed. The microcarrier CHO cell perfusion process was shown feasible to produce therapeutic protein in

large scale. Specific productivity was stable during a total of more than two month operation to meet regulatory requirements. Guidelines for future process scale-up and optimization were established.

Materials and Methods

M-CIF, cell line, media and cell cultures

Monocyte-colony inhibition factor (M-CIF) has been identified and characterized at Human Genome Sciences, Inc. (Kreider *et al.*, 1996), which is identical in sequence to recently published HCC-1. M-CIF consists of 74 amino acids and has a molecular weight of 8.673 kD. M-CIF is a new member of the β-chemokine family with no chemotactic activity to monocytes/macrophages and some degree of chemotactic activity to T lymphocytes. It is inactive on most leukocytes except that it induces monocytes/macrophages for intracellular Ca^{++} change via receptors shared with MIP-1α and RANTES. The *in vivo* results show that M-CIF has a significant protective effect on LPS-induced macrophage-mediated lethal sepsis (Zhang *et al.*, 1996), which is at least in part due to its down-regulation with TNF-α and up-regulation with IL-10 in vivo. In addition, the protein may have potential medical application in areas such as arthritis.

A CHO clone, CHO.M-CIF.46, was developed at Human Genome Sciences, Inc. (HGS) by transfecting CHO-DG44 host (Urlaub *et al.*, 1983; Urlaub *et al.*, 1986) with our expression vector containing the gene of M-CIF. The CHO.M-CIF.46 clone was partially amplified to 20 μM methotrexate (MTX).

Routine T-flask cell passage was carried out upon 80–90% confluence every 3–5 days at 1:3–1:4 split ratio in MEM alpha$^-$ medium supplemented with 5% dialyzed fetal bovine serum (DiFBS). Seed microcarrier cultures were prepared and maintained under MTX selection in an in-house made completely modified MEM (CM-MEM) medium containing 5% DiFBS. Expansion of seed microcarrier culture and bioreactor culture were made in another in-house made HGS-CHO-3 medium, containing 1% regular fetal bovine serum (FBS) and boosted with glucose and glutamine. No methotrexate was present in the HGS-CHO-3 medium.

Cytodex I microcarriers (Pharmacia, Uppsala, Sweden) were hydrated in calcium and magnesium-free phosphate-buffered saline (PBS) overnight and autoclaved (121 °C, 60 min). Before use, PBS was decanted and the microcarriers were washed and conditioned in a fresh serum-containing medium. 2.5 g/L microcarrier load was used for all spinner cultures. Inoculation density was maintained at 1–2 $\times$ 10^5 cells/ml.

Perfusion bioreactors

One 3L Applikon bioreactor (Applikon Inc., Foster City, CA) and one 15L BIOSTAT® MD10 bioreactor (B. Braun Biotech Inc., Allentown, PA) equipped with a conical bottom stainless steel spin filter mounted on their agitation shafts were used to set up two microcarrier perfusion systems. Fresh medium was dispensed into extra-spin filter space and microcarrier-free spent medium was withdrawn from within the spin filter. After comparison of different pore sizes, spin filters with 75 μm pore size were chosen for the microcarrier perfusion cultures.

The 3L perfusion bioreactor was operated at a working volume of 2 L. A 500 ml spinner microcarrier culture was used to inoculate the bioreactor. The microcarrier perfusion culture was controlled at pH between 7.0–7.2, dissolved oxygen (DO) of 30% air saturation and temperature of 37 °C by Applikon ADI 1030 BioController, and agitation of 50 rpm by Applikon ADI 1012 motor controller. Process data were logged to a computer every 10 min. Glucose and lactate concentrations were monitored on-line hourly by YSI 2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, Ohio). Cell density and ammonia concentration were analyzed daily. Samples were centrifuged and stored in a –20 °C freezer for product concentration analysis.

The 15L perfusion bioreactor was operated at a working volume of 8 L. A digital control unit (DCU) was used to control pH, agitation, DO and temperature at 7.0–7.2, 45 rpm, 30% air saturation and 37 °C, respectively. Samples were taken daily for the measurement of glucose, lactate, ammonia and cell density.

Assays

Cell density was determined by spinning down cells and microcarriers followed by crystal violet stain and haemocytometer count for total cell density in numbers of cell per ml. Previous results and occasional viability measurements showed culture viability of over 95% at all time. Glucose and lactate were analyzed by YSI

2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, OH). Ammonia was measured by Kodak IBI Biolyzer (New Haven, CT). M-CIF concentration was analyzed by reverse phase HPLC (Waters, Milford, MA).

Productivity calculation and statistical analysis

Assuming a product is stable, a material balance on the product in a perfusion bioreactor can be described as

$$[P(t_{i+1}) - P(t_i)] + [P_H(t_{i+1}) - P_H(t_i)] = \int_{t_i}^{t_{i+1}} C(t) \cdot q(t) \cdot dt \quad (1)$$

where

$P(t_i)$ = the total amount of the product in the bioreactor at time t_i, g or mg;

$P_H(t_i)$ = the total amount of the product in the harvest at time t_i, g or mg;

$C(t)$ = the total number of cells in the bioreactor at time t, billion cells;

$q(t)$ = the specific productivity of cells at time t, g/billion cells/day or mg/billion cells/day;

t = time, day.

Further assuming the specific productivity changes little within a small time interval and can be represented by an average specific productivity ($\overline{q}(t_i, t_{i+1})$), and assuming the total number of cells is close to a linear profile within the time interval, the following equation can then be derived from the Equation (1) to estimate average specific productivity

$$\overline{q}(t_i, t_{i+1}) = \frac{[P(t_{i+1}) - P(t_i)] + [P_H(t_{i+1}) - P_H(t_i)]}{\frac{C(t_{i+1}) + C(t_i)}{2} \cdot (t_{i+1} - t_i)} \quad (2)$$

In a batch culture, the product in the harvest (i.e. $P_H(t)$) is zero.

Glucose uptake and lactate generation data were analyzed for statistical significance. P value answers whether null hypotheses are true and what is the probability that two random samples would have means far apart. A P value less than 0.001 was rendered 'extremely significant', indicating either a difference exists in the overall population, or a very rare chance event or a coincidence has occurred (Ross, 1987).

Results and Discussion

Cell growth, metabolism and protein expression in 3L microcarrier perfusion culture

The CHO host (DG44) has been used for the development of many recombinant CHO lines at HGS. Unlike other novel proteins, M-CIF gene was difficult to amplify in CHO cells under methotrexate selection. A partially amplified clone was used in this study. In a typical batch culture, cell density reached 6×10^5 cells/ml in 4 days from initial 2×10^5 cells/ml with a final M-CIF titer of 5 mg/L. Biological activity assay of M-CIF purified from small batches of microcarrier cultures was positive (data not shown). Microcarrier cultures were suitable to produce M-CIF. The ability of the CHO cells to transfer directly from microcarriers to microcarriers in the formulated CM-MEM and HGS-CHO-3 media eliminated the step to remove cells from microcarriers and facilitated seed culture preparation, maintenance and scale-up.

To produce M-CIF in quantity, a large volume of microcarrier batch culture would be required. An alternative was to use a cell retention device to achieve a high cell density and long-term on-going production of M-CIF. Spin filters, commonly made of stainless steel mesh, have a solid track of record of good biocompatibility, ease of sterilization, reusability, easy cleaning, and long process duration (Avgerinos *et al.*, 1990; Fenge *et al.*, 1993; Emery *et al.*, 1995). Also the implementation of a spin filter requires a minimal modification to an existing stirred tank bioreactor.

After the inoculation with a 500 ml spinner, the 3L bioreactor was operated for 4 days in a batch mode. Perfusion started on the fourth day of the batch culture. Fig. 1 shows the results from the 3L microcarrier perfusion run. Cell density kept increasing as perfusion brought in fresh medium and carried away spent medium. The CHO.M-CIF.46 cells were able to reach a final cell density of more than 6×10^6 cells/ml, a 10-fold increase compared to batch cultures.

Two subsequent microcarrier additions were made from initial 2.5 g/L to 5 g/L at 235 h and from 5 g/L to 10 g/L at 282 h in order to provide surface for cells to grow. As cell density kept increasing, the surface area at 10 g/L microcarrier load was still limiting. Some floating cells formed cell aggregates. The resulting high cell density increased oxygen demand. Frequent direct sparging with pure oxygen induced the formation of microcarrier clumps. A further increase in microcarrier load beyond 10 g/L is not recommend-

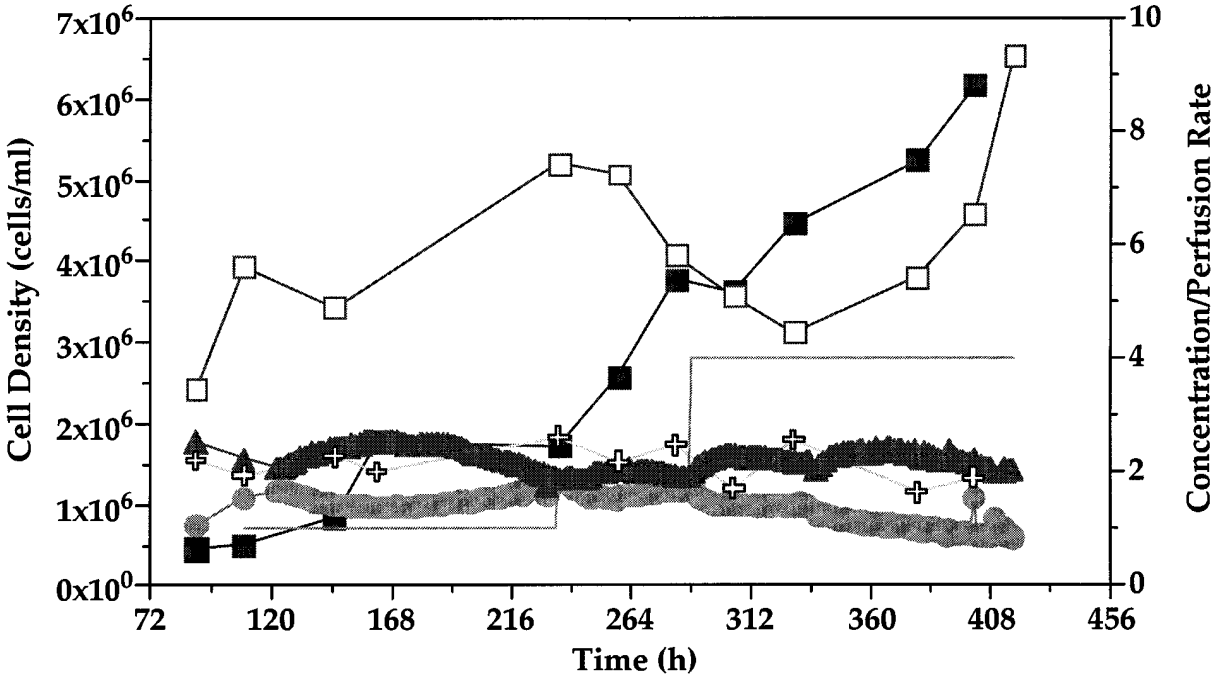

Figure 1. Cell growth, metabolism and protein expression in a 3L microcarrier perfusion bioreactor, (■) cell density (cells/ml), (▲) glucose concentration (g/L), (●) lactate concentration (g/L), (—) perfusion rate (L/day), (+) ammonia concentration (mM), and (□) M-CIF concentration (mg/L). Microcarrier load was changed from 2.5 g/L to 5 g/L at 235 h and to 10 g/L at 282 h. Perfusion started 1 L/day at 109 h and increased to 2 L/day at 235 h and 4 L/day at 288 h.

ed. Two possible solutions are the use of macroporous microcarriers for more surface area and bubble-free aeration (Fenge *et al.*, 1993). The replenishment with fresh microcarriers could be another alternative.

Glucose, lactate and ammonia were monitored throughout the run as indicators for perfusion rate adjustment. As shown in Fig. 1, glucose concentration increased and lactate decreased with every perfusion rate increase at 109 h, 228 h, 235 h and 288 h. Soon after each perfusion rate increase, the glucose dropped and the lactate rose in response to increasing glucose consumption and lactate generation. Ammonia maintained around 2 mM throughout the perfusion culture.

Changes in cell physiology may be reflected in specific nutrient uptake and/or by-product generation rates. Specific glucose uptake and lactate generation rates were calculated as described in Materials and Methods section and are shown in Fig. 2. Within the first two days of the perfusion culture, specific glucose uptake rate was high (2.137±0.889 g/billion cells/day) (mean ± standard deviation). Then, glucose uptake rate kept relatively constant at 0.918±0.383 g/billion cells/day. At the end of the perfusion run, glucose uptake rate slightly increased to 1.299±0.728 g/billion cells/day, corresponding to the formation of microcarrier clumps and cell aggregates. With these mean values, standard deviations and sample sizes, both standard T test and Welch T test rendered extremely significant differences among the means of any two groups of the three glucose uptake stages, using unpaired and two-tailed P models. These results suggest that when a stable perfusion environment was established and maintained, glucose was utilized more efficiently. The specific lactate production rate remained relatively constant at 0.645±0.339 g/billion cells/day, despite of varying cell density, perfusion rate and microcarrier load. High initial glucose consumption was also documented in the cultures of CHO-K1 (Hayter *et al.*, 1991; Ogata *et al.*, 1993). Further, high product formation rates occurred at a small ratio of lactate production rate to glucose consumption rate (Ogata *et al.*, 1993). Specific glucose uptake and lactate generation rates could be used as a monitoring system during long-term perfusion operation.

During the perfusion operation, M-CIF concentration was in a range from 4 to 10 mg/L as shown in Fig. 1. The expression level of M-CIF was low due to the fact that the CHO.M-CIF.46 clone had not been fully ampli-

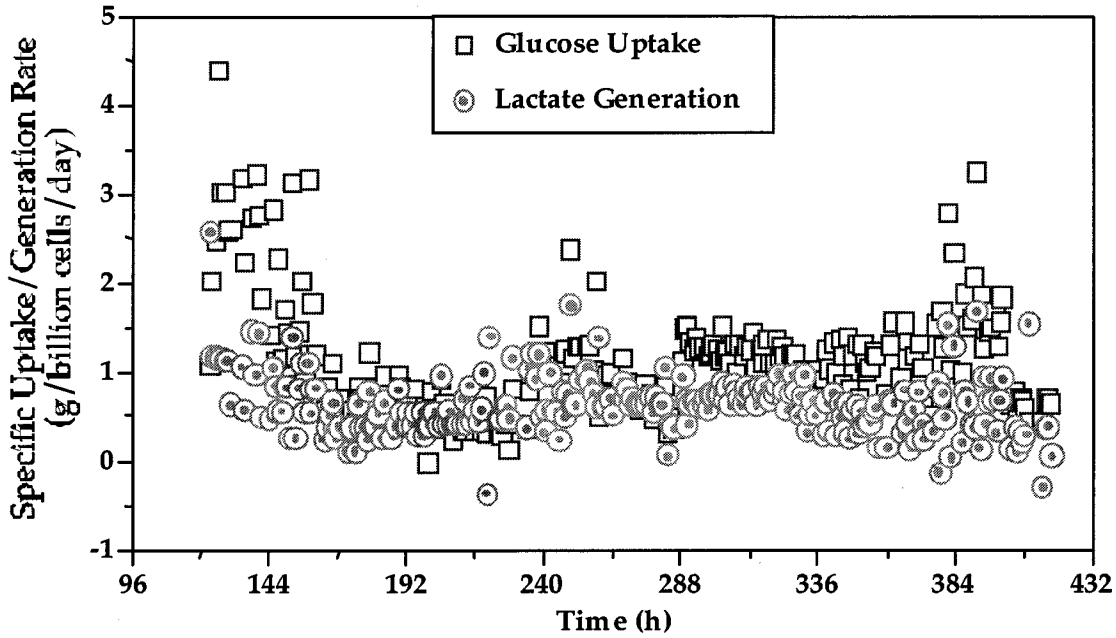

Figure 2. Specific glucose uptake and lactate generation rates in the 3L microcarrier perfusion culture.

fied. Other fully amplified recombinant CHO clones such as the one producing stanniocalcin had an expression level up to 300 mg/L (Zhang, unpubl.). However, the specific M-CIF productivity was maintained at a relatively constant level around 2.69±0.53 mg/billion cells/day (see data in Fig. 4B). A total volume of 60 liter was harvested and 259 mg of M-CIF was purified.

Scale-up of microcarrier perfusion culture

The whole 2 liter microcarrier culture was subsequently used to seed a 15L perfusion bioreactor with a working volume of 8 L. The spin filter in the 3L bioreactor was partially clogged at around 422 h of the run. The 3L bioreactor was then run in a repeated batch mode for 10 days with three quarters of medium replacement daily. The 15L perfusion bioreactor was initiated in a batch mode at a microcarrier load of 5 g/L. Perfusion started on the fifth day at 4 L/day.

Several efforts were made during the microcarrier perfusion operation based on the results of the 3L run. It was speculated that spin filter clogging was attributed to the floating cells more close in size to spin filter openings. Approaches, like providing enough microcarriers, removing floating cells and microcarrier replenishment, would then extend the duration of microcarrier perfusion culture.

To provide more microcarrier surface area, the microcarrier load was increased from 5 to 10 g/L on day 8, when considerable amount of floating cells appeared. At the 10 g/L microcarrier load, formation of microcarrier clumps posed a problem, similar to the 3L perfusion run. In addition, the impeller used in the 15L bioreactor was not optimized for a microcarrier culture at high microcarrier load. Normally, two large pitch-blade impellers were installed in MD10 bioreactors for mammalian suspension cultures. The top impeller was removed to accommodate the spin filter and only the bottom impeller was left for agitation. A 3-blade segment impeller should be tested and might be suitable for a microcarrier culture (Fenge *et al.*, 1993).

Another effort was made to extend perfusion operation by replenishing microcarriers. On day 20, the microcarrier load was reduced to 5 g/L with 50% microcarrier replenishment. Additional 50% microcarrier replenishment was made on day 27. The 15L bioreactor was operated for 35 days without further microcarrier replenishment. Microcarrier replenishment can be an effective way to extend spin filter perfusion culture.

Fig. 3 shows the results of cell growth, metabolism and M-CIF expression from the 15L microcarrier perfusion run. Perfusion started at 4 L/day on day 5 and its rate was changed to 8 L/day on day 10, 12 L/day

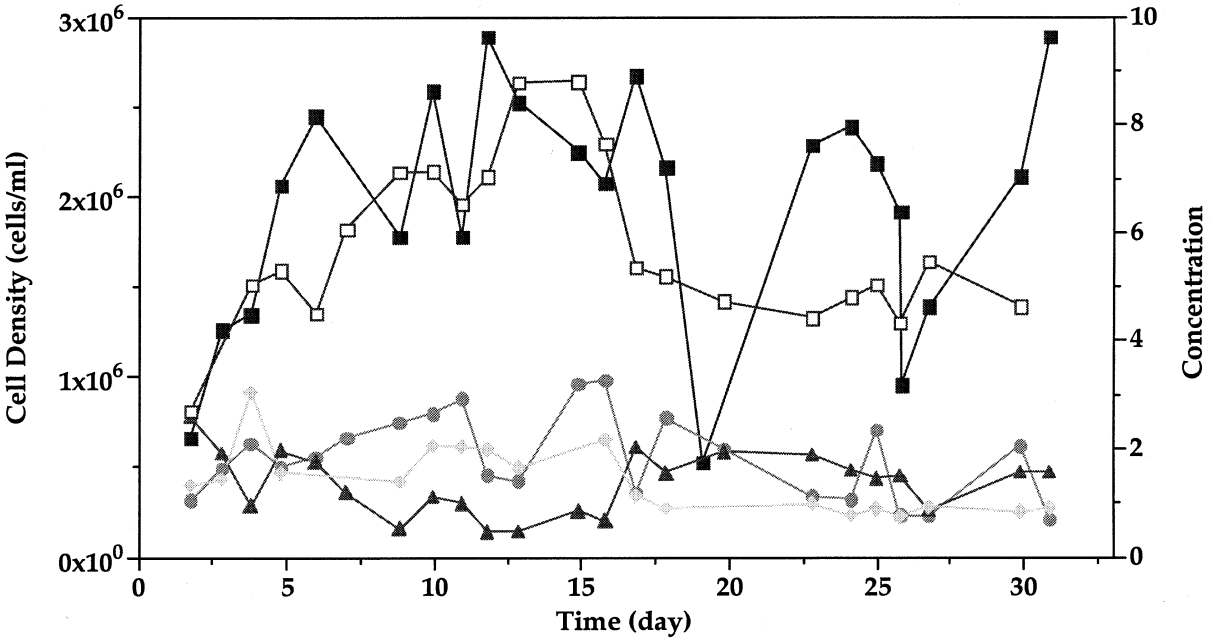

Figure 3. Cell growth, metabolism and protein expression in a 15L microcarrier perfusion bioreactor. Symbols: (■) cell density (cells/ml), (▲) glucose concentration (g/L), (●) lactate concentration (g/L), (♦) ammonia concentration (mM), and (□) M-CIF concentration (mg/L). Microcarrier replenishment was made on day 20 and 27 to extend the perfusion operation.

Table 1. Scalability of microcarrier perfusion bioreactors

Perfusion bioreactor	Working volume (V_W) (L)	Harvest volume (V_H) (L)	Ratio V_H/V_W	q_{M-CIF} (mg/10^9 cells/day)
3L	2	60	30	2.69±0.53
15L	8	300	37.5	2.61±0.61
60L	50	1500*	30*	2.65±0.60*

* Predicted values.

on day 12, 16 L/day on day 17, 12 L/day on day 18, and 8 L/day on day 22. Cell density was maintained between 1.5×10^6 and 3.0×10^6 cells/ml except on day 20 and 27 when microcarrier replenishment was made. By controlling a glucose level between 0.5 and 2.0 g/L, lactate and ammonia levels were maintained within the desired ranges during the run. M-CIF was produced at a level between 4 and 9 mg/L, similar to the 3L perfusion run. A total volume of 300 L was harvested from the 15L microcarrier perfusion system and 1.57 g of M-CIF was purified for animal studies.

The scalability of a microcarrier perfusion system could be characterized by the ratio of total volume harvested from the system (V_H) to the working volume of the system (V_W) as well as the specific protein productivity (q). Table 1 summarizes the scalability of the microcarrier perfusion system developed at 3L and 15L scales. Based on a V_H/V_W ratio of 30 and a (q) of 2.65±0.60, a 60L perfusion bioreactor would yield a total volume of 1500 liter. Compared to a batch culture process, a perfusion system represents a significant reduction in the capital investment on bioreactors and in the building space. A 1:4 cell split ratio of from 500 ml to 2 L working volume, and from 2 L to 8 L was shown working in the successive scale-up. The split ratio will be tested in the scale-up to the 60L perfusion culture.

Long-term stable production of M-CIF

Fig. 4A shows the cumulative production of M-CIF. The calculation of cumulative production was based

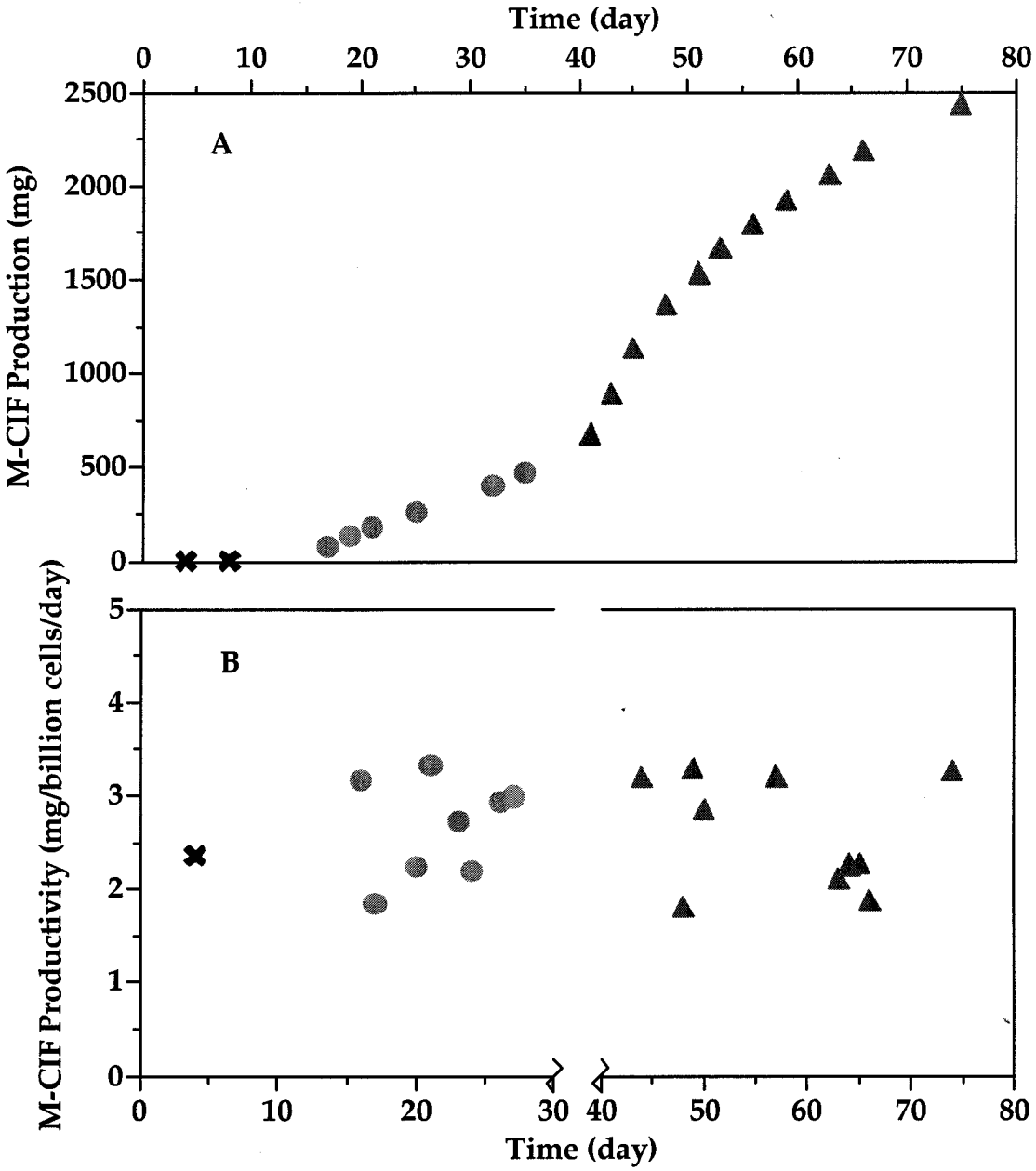

Figure 4. (A) Cumulative production of M-CIF in a spinner culture, 3L and 15L cultures. (B) Stable long-term production of M-CIF from microcarrier CHO cultures. Specific M-CIF production rate in (×) the spinner culture (mg/billion cells/day) (●) 3L perfusion culture (mg/billion cells/day) and (▲) 15L perfusion culture (mg/billion cells/day).

on the harvest pool volume and product concentrations. Over a period of more than two months, a total of 2.4 g of M-CIF was produced, among which 259 mg and 1.57 g were purified from the 3L and 15L harvest, respectively.

Average specific productivity was calculated by Equation (2) and is shown in Fig. 4B. Given the assumptions made in average specific productivity estimation, perfusion data available on any two consecutive days without large variations in process parameters, such as the changes in microcarrier load and

cell density, were chosen for the calculation. All data scattered between 1.8 and 3.4 mg/billion cells/day. Over the two month period where average specific productivity data were available, no significant productivity loss was noticed and M-CIF production was stable. The specific productivity from the 3L and 15L runs was averaged to be 2.69 ± 0.53 and 2.61 ± 0.61 mg/billion cells/day respectively. If cell density was taken into account, volumetric productivity would be much higher from a perfusion culture than from a batch culture. The specific productivity was similar to that from other CHO cell lines and products (Avgerinos *et al.*, 1990; Ogata *et al.*, 1993). Recombinant human soluble thrombomodulin (rsTM) was produced at 2–4 mg/billion cells/day by a repeated batch culture of engineered CHO-K1 cells on macroporous beads (Ogata *et al.*, 1993). Recombinant single chain urokinase-type plasminogen activator (rscuPA) was produced at 3.5–7.0 mg/billion cells/day from an aggregated perfusion culture of CHO CGM-113 cells (Avgerinos *et al.*, 1990). More importantly, all three batches of M-CIF purified separately from the microcarrier spinner batch culture, 3L and 15L microcarrier perfusion runs showed the same biological activities in animal studies (data not shown). The microcarrier perfusion culture was feasible to produce M-CIF from the engineered CHO cells.

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References

Avgerinos GC, Drapeau D, Socolow JS, Mao JI, Hsiao K and Broeze RJ (1990) Spin filter perfusion system for high density cell culture: Production of recombinant urinary type plasminogen activator in CHO cells. Bio/Technology 8(1): 54–58.

Banik GG and Heath CA (1995) Partial and total cell retention in a filtration-based homogeneous perfusion reactor. Biotechnol Prog 11(5): 584–588.

Cartwright T (1994) Introducing the animal cell as a bioreactor. In: Cartwright T (ed.) Animal Cells as Bioreactors. (pp. 1–10) Cambridge University Press, Cambridge, UK.

Emery AN, Jan DCH and al-Rubeai M (1995) Oxygenation of intensive cell-culture system. Appl Microbiol Biotechnol 43: 1028–33.

Fenge C, Klein C, Heuer C, Siegel U and Fraune E (1993) Agitation, aeration and perfusion modules for cell culture bioreactors. Cytotechnology 11(3): 233–244.

Griffiths JB (1988) Overview of cell culture systems and their scale-up. In: Spier RE and Griffiths JB (eds.) Animal Cell Biotechnology, Vol. 3 (pp. 179–220) Academic Press, London.

Hansen HA, Damgaard B and Emborg C (1993) Enhanced antibody production associated with altered amino acid metabolism in a hybridoma high-density perfusion culture established by gravity separation. Cytotechnology 11(2): 155–166.

Hayter PM, Curling EM, Baines AJ, Jenkins N, Salmon I, Strange PG and Bull AT (1991) Chinese hamster ovary cell growth and interferon production kinetics in stirred batch culture. Appl Microbiol Biotechnol 34: 559–564.

Himmelfarb P, Thayer PS and Martin HE (1969) Spin filter culture: The propagation of mammalian cells in suspension. Science 164: 555–557.

Kreider BL, Zhang J, Li H, Su J, Oelkuct M, Gentz R, Garotta G and Patel VP (1996) A beta-family chemokine which specifically inhibits M-CSF mediated colony formation. Eur Cytokine Netw 7(3): 493.

Miller AO, Menozzi FD and Dubois D (1989) Microbeads and anchorage-dependent eukaryotic cells: the beginning of a new era in biotechnology. Adv Biochem Eng Biotechnol 39: 73–95.

Nilsson K (1989) Microcarrier cell culture. Biotechnol. Genetic Eng. Rev. 6 (September): 403–439.

Ogata M, Wakita K, Kimura K, Marumoto Y, Oh-i K and Shimizu S (1993) High-level expression of recombinant human soluble thrombomodulin in serum-free medium by CHO-K1 cells. Appl Microbiol Biotechnol 38(4): 520–5.

Omasa T, Kobayashi M, Nishikawa T, Shioya S, Suga KI, Uemura SI, Kitani Y and Imamura Y (1995) Enhancement of antibody production by growth factor addition in perfusion and hollow-fiber culture systems. Biotechnol Bioeng 48: 673–680.

Ross, SM (1987) Introduction to Probability and Statistics for Engineers and Scientists. John Wiley & Sons, New York.

Searles JA, Todd P and Kompala DS (1994) Viable cell recycle with an inclined settler in the perfusion culture of suspended recombinant Chinese hamster ovary cells. Biotechnol Prog 10(2): 198–206.

Tokashiki M, Arai T, Hamamoto K and Ishimaru K (1990) High density culture of hybridoma cells using a perfusion culture vessel with an external centrifuge. Cytotechnology 3: 239–244.

Urlaub G, Kas E, Carothers AM and Chasin LA (1983) Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. Cell 33(2): 405–412.

Urlaub G, Mitchell PJ, Kas E, Chasin LA, Funanage VL, Myoda TT and Hamlin J (1986) Effect of gamma rays at the dihydrofolate reductase locus: Deletions and inversions. Somat Cell Mol Genet 12(6): 555–566.

Zhang J, Kreider BL, Li H, Su J, Zhang JL, Gentz R, Garotta G and Antonaceio M (1996) Preventative effect of a β-chemokine on LPS-induced septic shock. Eur Cytokine Netw 7(3): 207.

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