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Relationship between Cell Size and Specific Thrombopoietin Productivity in Chinese Hamster Ovary Cells during Dihydrofolate Reductase-mediated Gene Amplification

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Abstract When parental Chinese hamster ovary (CHO) cell clones that are capable of producing thrombopoietin (TPO) were subjected to high methotrexate (MTX) concentrations, clonal variations in cell growth were apparent. In the clones that had no significant enhancement in specific TPO productivity (q_{Tpo}) when a higher level of MTX was administered, their growth was not depressed significantly nor their cell size changed significantly. On the other hand, those clones that showed a significant enhancement in q_{Tpo} at higher a MTX dosage, cell growth was depressed initially but recovered during successive sub-cultures. Furthermore, their cell size increased, which suggested that changes in cell size may be indicative of an enhanced q_{Tpo}. When the enhancement of the q_{Tpo} of 9 clones after a high MTX dosage was plotted against the extent of the increase of their size, there was a linear correlation (r^2=0.80, P<0.001, ANOVA), which suggested that an enhancement of q_{Tpo} after high MTX administration can be measured by the increase in their cell size. Taken together, our data demonstrate that the selection of amplified CHO cell clones with enhanced q_{Tpo} can be done based upon their increased size and growth pattern. This facilitates the development of highly productive recombinant CHO cell lines.

Keywords: cell size, CHO cells, gene amplification

INTRODUCTION

To obtain high-level expression of heterologous proteins, the most popular mammalian expression systems in the pharmaceutical industry use expression vectors that contain the dihydrofolate reductase (DHFR) gene as the selective gene and DHFR-deficient Chinese hamster ovary (CHO) cells as the host cell line [1,2]. This gene amplification procedure is based upon the DHFR gene coding for the DHFR enzyme. Methotrexate (MTX) binds to the DHFR enzyme, but DHFR-deficient CHO cells that have taken up an expression vector containing the DHFR gene can develop resistance to it by an amplification of the DHFR gene. Concomitantly, the specific gene of interest that is co-linked with the DHFR gene in the same expression vector or adjacently resides in the host chromosome is amplified [3,4].

When recombinant CHO (rCHO) cell clones have been subjected to successive rounds of selection in media that contain stepwise increments of MTX concentrations, it has been found that the specific productivity (q) does not always increase linearly by increasing the MTX dosage and the enhancement of q varies signi fycantly among the clones [5-7]. Thus, extensive efforts are required for screening amplified clones that have an enhanced q.

In our laboratory, we transfected the thrombopoietin (TPO) expression vector that contained the DHFR gene into the DHFR-deficient CHO cells in order to develop rCHO cells that produce TPO. When these parental clones were subjected to successive rounds of selection in media that contained stepwise increments of MTX concentrations, changes in their cell size became noticeable. Cells of these clones, particularly those that had an enhanced q_{Tpo}, tended to be bigger after higher MTX dosages. If the selection of amplified clones that had an enhanced q can be made based upon their size, automated cell sorting using a flow cytometry based on their cell size will facilitate the development of methods for creating highly productive rCHO cells. In the present study, the cell sizes of the parental clones that were producing TPO were monitored along with the increasing levels of MTX in an attempt to determine the relationship between cell size and q_{Tpo} during the course of DHFR-mediated gene amplification.

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MATERIALS AND METHODS

Cell Line Development and Cell Culture

Parental CHO cells that expressed a TPO were made

by transfecting 2 μg of a TPO expression plasmid into DHFR-deficient CHO cells (DUKX-B11, ATCC CRL-9096) using LipofectAMINE™ (20 μg, Gibco-BRL, Grand Island, NY, USA). Drug selection was carried out by seeding 10^3 cells/well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing IMDM (Gibco-BRL) supplemented with 10% d-FBS (Gibco-BRL) and 500 μg/mL of zeocin (Invitrogen, Carlsbad, CA). Zeocin was used only in the first selection. The culture supernatant of the zeocin-resistant clones that were isolated on 96-well tissue culture plates was tested for TPO production by an ELISA procedure.

Based on the TPO titers, 51 high producing parental clones were selected for gene amplification and were subjected to increasing levels of MTX (Sigma) administration (0.02 and 0.08 μM). The cells were sub-cultured after they had reached confluency by trypsinization.

To determine the changes in cell size, cell growth and TPO production during the adaptation to higher MTX levels, monolayer cultures of several clones were performed in 60 mm tissue culture dishes (Nunc) containing 5 mL of IMDM with 10% dFBS and the corresponding level of MTX in a humidified 5% CO_2 incubator at 37°C. The clones used for this experiment are shown in Fig. 1. Approximately 1.0×10^5 cells/mL in the exponential phase of growth were seeded into 60 mm tissue culture dishes. The cells were re-fed every 2-3 days with fresh medium. Culture dishes were harvested periodically to determine the cell density. The supernatants of the cultures were removed and kept frozen at –70°C for the TPO assay.

Analytical Methods

The concentrations of both the suspended cells and the adherent cells were estimated using a hemocytometer. Viable cells were distinguished from dead cells by the trypan blue dye exclusion method.

After trypsinization, the size distribution of cells was measured using CASY® (Schärfe System, GmbH) with a 150 μm capillary. This system is described in detail elsewhere [8]. For this analysis, 100 μL aliquots from the cell cultures were diluted 1:100 in a phosphate buffered saline (PBS) based electrolyte and gently mixed. By repeated measurements of each sample, approximately 5000 particles were analyzed to achieve a sufficient level of statistical reliability. Based on these results, representative size distribution curves were obtained. Bimodal distributions were obtained during culture period. The lower peak is likely resulted from cell debris and dead cells [8]. Therefore, we focused on the variations in the cell diameter of viable cells (>8 μm) and thereby estimated their mean cell diameter.

The secreted TPO was quantified using an ELISA method, according to the protocol provided by R&D Systems (Minneapolis, MN, USA).

Evaluation of Specific Growth and Production Rates

The specific growth rate (μ) was based on data collected during the exponential growth phase [9]. Specific TPO productivity (q_{Tpo}) was evaluated as described earlier [9]. When the plot of the TPO titer versus the time integral of viable cells is fitted to a straight line with a regression coefficient that is close to one, the slope represents the q_{Tpo}.

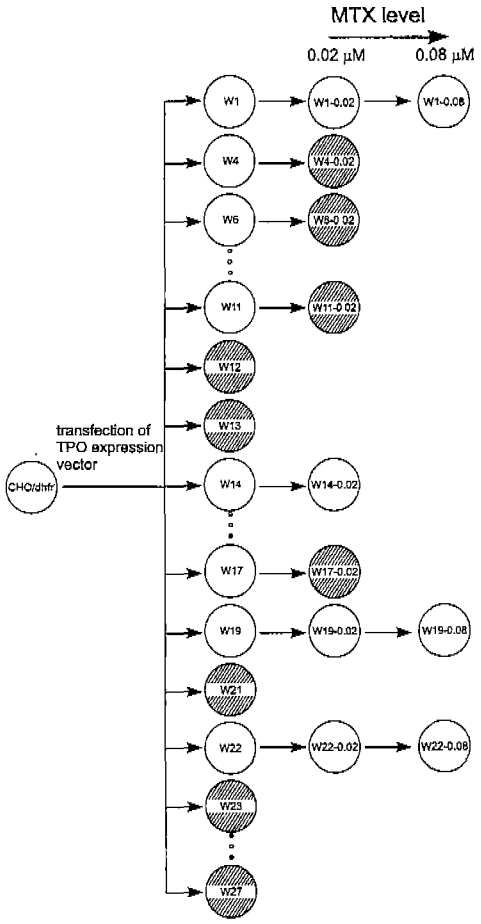

Fig. 1. Schematic representation of the procedure used for the establishment of TPO producing clones. The nine clones used in this experiment are shaded.

RESULTS AND DISCUSSION

Characteristics of Clones Exposed to a Higher Level of MTX

When highly productive parental clones that were selected by IMDM with 10% dFBS and 500 μg/mL zeocin were subjected to increasing levels of MTX concentrations, clonal variations in cell growth were apparent. Some clones grew without a significant depression. On the other hand, some clones displayed a significant growth depression initially but recovered cell growth capacity after successive sub-cultures. In addition, parental clones that displayed the suppressed growth rate

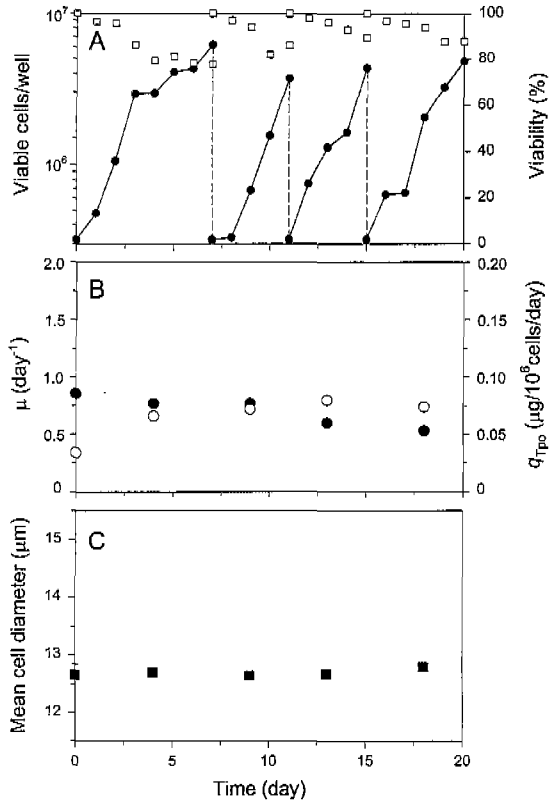

Fig. 2. Cell growth, μ, q_{Tpo}, and cell size profiles of a representative clone (W12) at 0.02 μM MTX level. *A*, Cell growth profiles; *B*, μ (closed circle) and q_{Tpo} (open circle); *C*, Mean cell diameter. In *A*, the closed circle and the open square represent the viable cell concentrations and the cell viabilities, respectively. In *A* and *B*, the values on day 0 are from the culture without MTX.

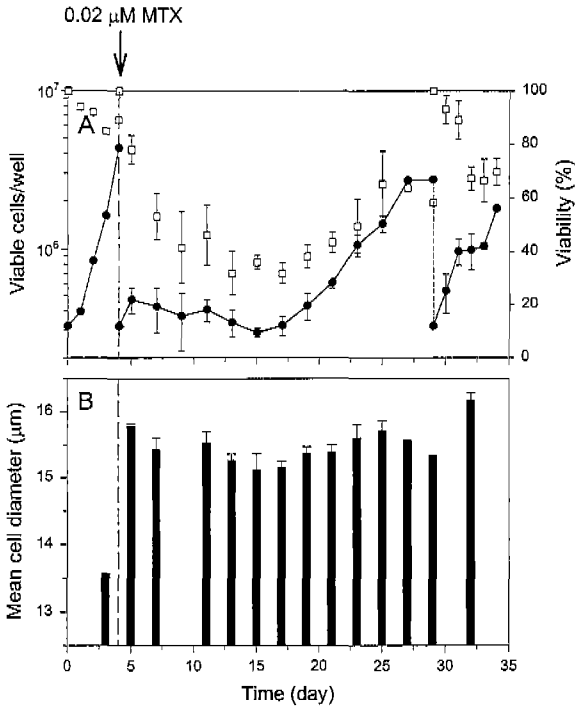

Fig. 3. Cell growth and cell size profiles of a representative clone (W21) that displayed severe growth depression at higher MTX concentrations. *A*, Cell growth profiles; *B*, Mean cell diameter. In *A*, the closed circle and the open square represent the viable cell concentrations and the cell viabilities, respectively. On day 4, the cells were sub-cultured into the medium containing 0.02 μM MTX, as indicated by the arrow. Error bars represent the standard deviation as determined from triplicate dishes.

looked larger in microscopic observations. If changes in cell size are indicative of an enhanced q_{Tpo} at a higher MTX level, this can be used to facilitate the screening for highly productive rCHO cells. Therefore, we closely monitored changes in the cell growth and size of some representative clones as shown in Fig. 1.

Fig. 2 shows an example of typical cell growth in clones that did not show a suppressed cell growth after a higher MTX administration level. When the parental clone W12 was subjected to 0.02 μM MTX, the cell growth was not depressed and cell viability was maintained over the 78% level (Fig. 2A). Compared with μ (=0.86 day^{-1}) and q_{Tpo} (=0.03 $\mu g/10^6$cells/day) in medium that did not have MTX, μ was decreased slightly and q_{Tpo} was increased by less than 2.4-fold at a 0.02 μM MTX concentration (Fig. 2B). MTX-resistances that appeared not to be caused by gene amplification events may be attributed in part either to a mutation in the MTX transport system in cell membrane or a DHFR that had an altered affinity for MTX [10,11]. Cell sizes that were measured during the exponential phase of growth at each sub-culture did not change significantly (Fig. 2C). The mean cell diameter in instances of IMDM that did not have MTX was 12.66 μm while that at 0.02 μM MTX during successive sub-cultures was in the range of 12.64-12.70 μm.

Fig. 3 shows the typical cell growth of clones that experienced a severe growth depression and a colony formation which was followed by growth recovery after administration of a high MTX dosage. When the parental clone W21 was subjected to a 0.02 μM MTX concentration, cell growth was arrested immediately and the cell viability decreased to 32% until colonies appeared. After that, cells started to grow and reached confluency (Fig. 3A). Depending on the parental clones, it took 2-3 weeks to reach confluency after 0.02 μM MTX dosage. In succeeding sub-cultures, cells grew without showing a significant lag phase. Compared with μ (=0.62 day^{-1}) and q_{Tpo} (=0.08 $\mu g/10^6$ cells/day) in medium that did not receive MTX, the μ was decreased to 0.29 day^{-1} and q_{Tpo} was increased to 0.82 $\mu g/10^6$ cells/day at a 0.02 μM MTX concentration. More than a 10 fold-increase in q_{Tpo} was obtained.

Fig. 4 shows that the morphological changes in the cells are distinct. Cells became longer and larger after a

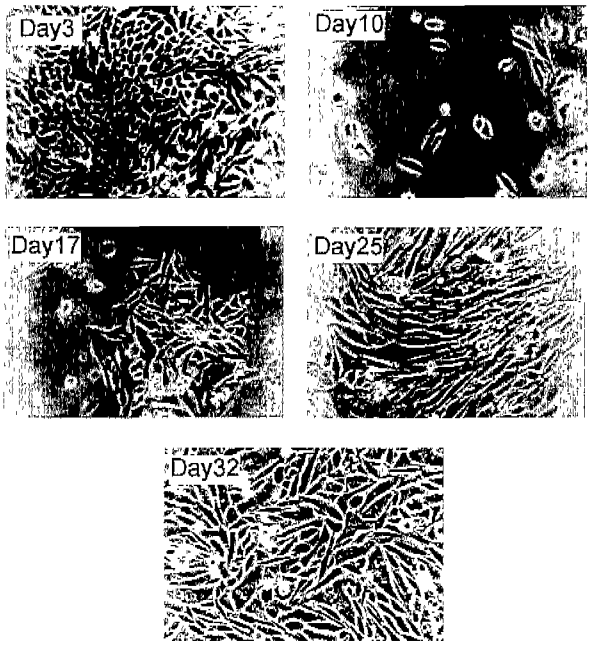

Fig. 4. Morphological changes in a representative clone (W21) that was subjected to a 0.02 μM MTX concentration. Days in photomicrographs correspond to the culture day in Fig. 3.

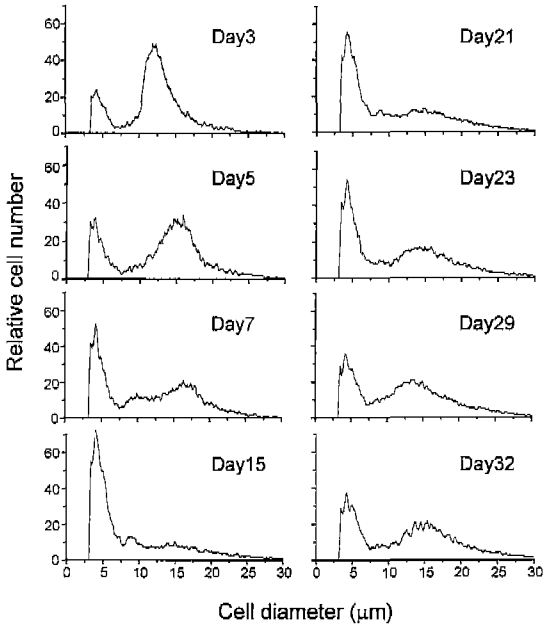

Fig. 5. Size distributions of a representative clone (W21) that was subjected to a 0.02 μM MTX concentration. The days in this figure correspond to the culture day in Fig. 3.

0.02 μM MTX administration. In order to quantitate changes in cell size during culture, the cell size distribution was measured. Fig. 5 shows a bimodal distribution of cell size. The peak with a smaller cell diameter represents dead cells and cell debris that had a diameter less than 8 μm. The peak with a larger cell diameter represents viable cells that had a diameter greater than 8 μm. The mean cell diameter of those greater than 8 μm was calculated and plotted in Fig. 3B. When subjected to a 0.02 μM MTX concentration, the cell diameter increased from 13.58 μm to 16.17 μm. This result suggests that an increase in cell size may be indicative of an enhanced q_{Tpo} that has resulted from gene amplification.

When cells after an administration of a 0.02 μM MTX dosage were subjected to a 0.08 μM MTX concentration, cells that displayed an enhanced q_{Tpo} also experienced severe growth arrest and colony formation which was followed by growth recovery and an increase in their size (data not shown).

Relationship between Cell Size and q_{Tpo} in CHO Cells during DHFR-mediated Gene Amplification

To determine whether changes in cell size are indicative of an enhanced q_{Tpo} resulting from gene amplification, changes in the mean cell diameter of 9 clones that subjected to a higher MTX level were measured. The growth pattern of the W11-0.02 clone at higher MTX level was similar that of the W12 clone while the growth pattern of 6 clones was similar to that of the W21 clone. To determine the correlation of q_{Tpo} with the mean cell diameter during the exponential phase of growth of various clones, the following two plots were made. Since cell size depends on the growth phase [8, 12, 13], we used the cell size measured in the exponential phase of growth for these plots.

Fig. 6A shows the plot of q_{Tpo} versus the mean cell diameter of various clones after administration of various MTX concentrations. Cells with a diameter in the range of 12-14 μm had a lower q_{Tpo}, compared with cells with a diameter greater than 14 μm. However, cells with a diameter greater than 15 μm had a broad range in their q_{Tpo}. Consequently, it is difficult to select highly productive clones between the various clones based their absolute cell size. However, enhancement of the q_{Tpo} of each clone was related to the amount of increase in cell sizes. This relationship is shown in Fig. 6B. For each clone, the values of q_{Tpo} and the mean cell diameter at higher MTX concentrations were adjusted to those that were treated with a lower MTX concentration. The enhancement of the q_{Tpo} of higher MTX concentrations was proportional to the amount of increase in their cell size, as shown by analysis of variance (r^2=0.80, P<0.001, ANOVA). This result suggests that enhancement of the q_{Tpo} after administration of higher MTX concentrations can be judged by the increase in their cell size. Furthermore, because the amount of increase in cell size is large enough to be noticeable by microscopic observation, efforts to screen clones that had an increased q_{Tpo} can be eased significantly.

In conclusion, CHO clones that had an increased q_{Tpo} displayed increased cell sizes when subjected to higher MTX concentrations. Thus, the selection of amplified CHO clones with enhanced q_{Tpo} can be made based on

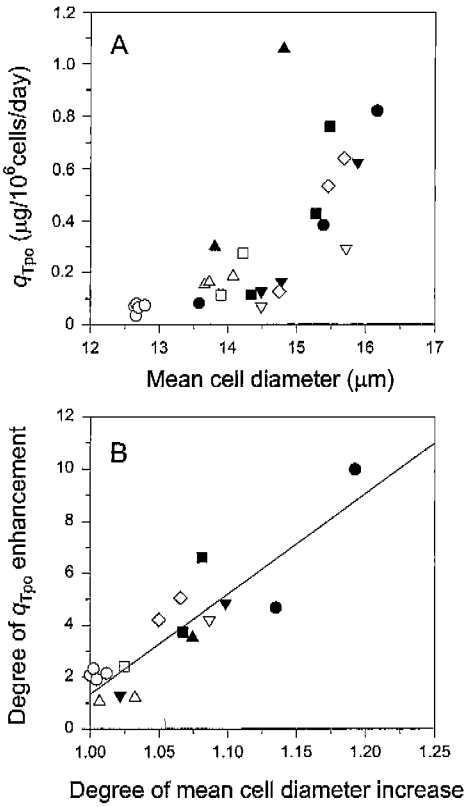

Fig. 6. Relationship between cell size and the q_{Tpo}. *A*, Plot of the q_{Tpo} versus mean cell diameter of various clones at various MTX concentrations; *B*, Linear regression plot of the enhancement of q_{Tpo} of each clone that was subjected to higher MTX concentration versus amount of increase in its mean cell diameter. Each symbol represents a specific clone that is shown in Fig. 1: W12 (○), W13 (▼), W21(●), W23 (▲), W27 (□), W4-0.02 (◇), W6-0.02 (▽), W11-0.02 (△), W17-0.02 (■). The same symbol was used for representing the specific clone that was subjected to a higher MTX concentration in Fig. 6A. The degree of the q_{Tpo} enhancement and the mean cell diameter increase was determined by dividing the values of q_{Tpo} and the mean cell diameter at higher MTX concentrations by those at lower MTX concentrations.

their increased size and growth pattern. This facilitates the development of highly productive rCHO cells.

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