Application of a reversible immortalization system for the generation of proliferation-controlled cell lines

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Abstract

To employ physiological mechanisms to control cell growth primary cells were reversibly immortalized using the SV40 TAg. The cells showed a fibroblast-like morphology. When the expression of the TAg was turned off, the cells arrested in the G0/G1 cell cycle phase. The cell culture could be kept for over 1 week in the proliferation-controlled state while the growth arrest remained fully reversible. The regulation was highly efficacious in that the arrested cell population did not spontaneously resume growth, suggesting that in the absence of the immortalizing gene expression endogenous growth-control mechanisms can keep these cells in a viable state for a prolonged time. Recombinant protein expression increased in growth-controlled cells when compared to conventionally cultured cells. Analysis of a secreted pharmaceutical protein revealed high product integrity without any signs of degradation. Therefore, it is feasible to apply genetic regulation of cell immortalization to obtain proliferation-controlled cell lines and this technique may be of interest to generate novel biotechnological producer cells.

Abbreviations: DMEM – Dulbecco's modified Eagle's medium; Dox – doxycycline; eGFP – enhanced green fluorescent protein; EPO – recombinant human erythropoietin; FCS – fetal calf serum; IRES – internal ribosomal binding site; LTR – long terminal repeat; MEF – murine embryonic fibroblast cell; neo – Neomycin phosphotransferase; TAg – SV40 virus large T-antigen; w.t. – wild type

Introduction

Pharmaceutical proteins are conventionally produced by cultured mammalian cells due to superior *in vivo* product activity, stability and human compatible post-translational modifications. Multiple requirements have led to the preferential use of a small number of producer cells that are routinely employed as pharmaceutical protein producer cells (Andersen and Krummen 2002). In

addition to efficient protein production, biotechnological cell lines should be free of pathogens and must not produce any potentially harmful or infective virus. Moreover, the cells must not only survive but also proliferate and efficiently produce under the influence of multiple stress factors in the fermenter, such as low or very high cell densities, cultivation in suspension culture and in serum-free or preferably even protein-free medium. Towards the end of conventional batch or fed batch

production processes, waste products such as ammonia and lactate accumulate, the medium gets depleted of nutrients, which has been shown to affect protein quality (Gawlitze et al. 1995, 1998). The contents of the dead and lysed cells are released to the cell culture supernatant, leading to contamination of the product. Most of the presently popular mammalian producer cell lines have been optimized with respect to technological requirements by adaptation and maintenance for extended periods of time under the appropriate culture conditions, while adaptation of the cellular physiology by genetic engineering is still in its infancy (Grabenhorst et al. 1995; Monaco et al. 1996; Irani et al. 1999, 2002; Umaña et al. 1999; Davies et al. 2001; Fogolin et al. 2004; for a review see Grabenhorst et al. 1999). However, due to comparatively high costs and low productivities of cultured mammalian cells, there is a constant pressure to further improve the producer cell lines as well as the protein production processes.

It has been proposed that by controlling cell growth during the production process, a more physiological situation could be mimicked in a technical environment, which is expected to increase the cells performance (Fussenegger et al. 1999). According to this proposal, cells are first grown until an optimal cell density is reached, then further cell growth is restricted and the cells can devote their resources to produce higher levels of recombinant protein. This would then stabilize the culture and prolong the productive time period. Since even in the most efficient producer cell clones the recombinant protein amounts to only a small fraction to the total protein synthesized, these have nevertheless in many cases a reduced growth rate in comparison to the parental low or non-producer cells. This phenomenon has been described as metabolic load and is likely to be at least partially due to stress responses (Rutkowski and Kaufman 2004). Since uncontrolled growth favors the accumulation of the most rapidly growing cells, this results in a selection bias for cells to reduce the metabolic load, leading to an significant decrease in the productivity with increasing cultivation time. This effect occurs in many production processes and is in part exacerbated by the requirement that the final fermentation steps are done in the absence of toxic selection drugs. A proliferation-controlled process would reduce such a drift and may therefore improve the productivity. In addition, the reduced nutrient

consumption and reduced waste product accumulation due to the growth arrest are expected to result in more homogenous fermentation conditions and therefore to increase product homogeneity and product quality.

Various attempts have been made to establish such a proliferation-controlled producer cell line, however, despite much progress to meet all of the multiple and complex requirements of an industrial production process, no such system has yet been developed to the stage of a commercial application (Geserick et al. 2000; Meents et al. 2002a, b; Watanabe et al. 2002; Chilov et al. 2003; Ibarra et al. 2003; Bi et al. 2004; for a review see Fussenegger et al. 1999). In these approaches, growth-control systems were superimposed on established cell lines that have previously been selected extensively to achieve maximal growth rates and that have lost the endogenous cell growth-control mechanisms of primary cells. However, the generation of new regulated cell lines from primary cells may allow the selection of a cell line that shows optimal properties specifically under proliferation-controlled conditions. As a first step to this aim we investigated the feasibility of this approach. Primary cells were reversibly immortalized by a sophisticated autoregulated multi-gene expression system. To study the influence of growth regulation on recombinant gene expression, a cell line was employed whose proliferation was strictly dependent on the addition of the non-toxic inducer doxycycline to the medium. The cells increased the productivity under proliferation-controlled conditions when secreted alkaline phosphatase (SEAP) was expressed as a model protein from a constitutive promoter. As a relevant pharmaceutical product, recombinant erythropoietin (EPO) could be expressed under proliferation-controlled conditions. The reversible immortalization strategy could therefore be applied to generate novel producer cell lines that allow to adjust the cell growth rate to optimize the production process.

Materials and methods

Immortalization procedure

Primary cells were isolated from 13.5-day-old Balb/c embryos and immortalized as described

(May et al. 2004). Forty eight hours after transfection with the immortalization construct (Figure 1a), the cells were incubated in the presence of 0.4 mg/ml G418 (Gibco BRL Live Technologies, Karlsruhe, Germany) to select stably transfected cell clones. For selection of pM5SEAP transfected LT1 cells puromycin dihydrochloride (Sigma Aldrich Chemie GmbH, Deisenhofen, Germany) was used at a concentration of 1 $\mu\text{g}/\text{ml}$.

Cell culture conditions

LT1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) with 10% fetal calf serum (FCS) (Cytogen, Berlin, Germany), 2 mM L-glutamine, penicillin (10 U/ml), streptomycin sulfate (100 $\mu\text{g}/\text{ml}$), 1 mM non essential amino acids and 0.1 mM β -mercaptoethanol. Doxycycline was added to a concentration of 2 $\mu\text{g}/\text{ml}$ as indicated. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Cell proliferation

For determination of the cell proliferation rate 1.5×10^4 cells were plated per 3.5 cm dish and grown for 4 days. The cell number was determined with the CASY1 DT cell counter (Schaefer Systems, Reutlingen, Germany). Growth kinetics were determined for three independent cultures of each cell line and cells were counted twice in each sample. For cell cycle analysis cells were detached with trypsin at day 3 after transfer to the respective culture conditions. Then the cells were washed with ice-cold PBS and fixed with ice-cold methanol:PBS (4:1 volumes, respectively). The cells were incubated in 0.1% saponin dissolved in PBS. DNA was stained with 0.02 mg/ml propidium iodide, 1 mg/ml RNase S (Sigma) in 0.1% saponin dissolved in PBS for 30 min. Flow cytometric analysis was done with a FACSCalibur (Becton Dickinson, Heidelberg, Germany) using the program ModFit.

Determination of SEAP activity

SEAP activity was measured using the chemiluminescent SEAP Reporter Gene Assay (Roche

Diagnostics, Mannheim, Germany). Briefly culture medium of the LT1SEAP cells was heated for 30 min at 65 °C. After 1 min of centrifugation at 14,000 $\times$ g, the supernatant was transferred to a fresh tube and diluted with the dilution buffer. This was mixed with an equal volume of inactivation buffer. After 5 min the substrate was added and incubated for additional 10 min. Luminescence was measured using a Berthold Lumat LB9501 (Berthold, Wildbad, Germany). The light signal was integrated for 5 s. To calculate the productivity the medium was completely replaced with fresh medium and after 24 h incubation time the supernatant was removed, treated as described above. The cells were detached and the cell number was determined. The relative light units were then divided by the cell number of the respective culture.

Recombinant adenovirus

Recombinant adenovirus vectors are derived from E1/E3 deleted adenovirus type 5 cosmid vectors by cloning of an expression cassette, consisting of CMVie-promoter, coding region, and SV40 poly-A site, into an adeno-cosmid vector. The expression cassette for enhanced green fluorescent protein (eGFP) was amplified by PCR from pEGFP-C1 (Clontech Laboratories, Palo Alto, CA) using primers with added SmaI and Psp1406I restriction enzyme recognition sites as well as ClaI and XbaI sites to the 5'- and 3'-ends, respectively. The resulting PCR DNA fragment was cloned into pGEM-T vector (Invitrogen, Carlsbad, CA) to yield the plasmid pGEMeGFP. Excision of the expression cassette with Psp1640I/XbaI and insertion into ClaI/XbaI-digested pAdcos45 vector yielded pAdcos45eGC1. For the construction of the erythropoietin encoding expression cassette the erythropoietin coding region of EPO was inserted in front of the IRES element of the plasmid pGEM IRESeGFP. The PCR DNA fragment carrying the dicistronic expression cassette was cleaved with Psp1640I/XbaI and inserted into pAdcos45 to yield pAdcos45epoieG. Recombinant adenovirus was produced after transfection of cosmid DNA into 293LP cells (Microbix Biosystems, Ontario, Canada) as described (Wiethe et al. 2003). Recombinant adenoviruses were propagated and purified according to Graham and Prevec (1991). Horse serum was replaced by FCS. Virus particles

in infected cells were harvested from freeze-thaw lysates that were incubated with Benzonase (Merck, West Point, PA) according to manufacturer's recommendation to reduce sample viscosity. Purified virus was isolated by two rounds of CsCl density gradient centrifugation, and stored at 4 °C. The virus titer was determined by plaque assay on 293LP cell cultures (Mittereder et al. 1996). For gene transfer the cell culture medium was removed and replaced by recombinant adenovirus diluted in PBS, 2% FCS. Cells were overlaid with 100 μ l virus suspension per 1 cm² of surface area containing the number of plaque forming units indicated. Adsorption of adenovirus was done at room temperature with gentle rocking on a mechanical rocker platform for 60 min. Then cell culture medium was added and incubation continued in a humidified 5% CO₂ atmosphere at 37 °C. Infection was monitored by fluorescence microscopy of eGFP expressing cells. Mock-infections were done by performing the same procedure but the solution added did not contain any virus particles.

EPO analysis

Serum-free MEM cell culture supernatant of EPO producing cells was collected from tissue culture plates (Costar 3524; Corning Inc., Corning, NY) and analyzed by polyacrylamide gel electrophoresis and Western blotting as described in (Schlenke et al. 1999). SDS-PAGE and Western blotting SDS-PAGE was carried out on 15% polyacrylamide gels with 3% stacking gels (Laemmli 1970). Proteins were visualized by Coomassie brilliant blue staining. For Western blot analysis, the proteins were transferred to nitrocellulose (Millipore, Eschborn, Germany) in a semi-dry instrument (Bio-Rad, Munich, Germany). The membrane was blocked with Tris-buffered saline containing 10% horse serum and 3% BSA for 1 h and incubated overnight with a rabbit anti-EPO anti-serum (Fibi et al. 1995) in blocking buffer at 1:1000 dilution. The second antibody, anti-rabbit immunoglobulin coupled to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands), was used at a 1:1000 dilution. The blots were developed with Tris-buffered saline containing 0.5 mg/ml of 4-chloro-1-naphthol solubilized in methanol and 0.2% perhydrol.

Results

Establishment of a proliferation-controlled cell line

To explore the feasibility to establish a proliferation-controlled cell line an autoregulated construct (Figure 1) was used to immortalize primary embryonic cells (May et al. 2004). A cell clone LT1 was isolated whose proliferation was strictly dependent on the presence of the rtTA inducer doxycycline. In the presence of Dox the cells continued to grow, while in the absence of Dox, the cell density remained almost constant (Figure 2). No signs of apoptosis were apparent by light microscopic analysis and by propidium iodide staining. The cell morphology changed to a spread out shape and a granular appearance. To determine the cell cycle distribution of the proliferation-controlled cells, we analyzed the DNA content of the cells by flow cytometry. In the absence of Dox, the cells accumulated specifically in the G0 or G1 phase of the cell cycle as indicated by the DNA profile (Figure 3). Flow cytometric analysis also showed that the forward light scattering intensity that is dependent on the cell volume showed a minor increase upon growth arrest (Figure 4). However, the sideward scatter increased. This is consistent with the granularity in the growth arrested state that was observed by light microscopic examination. A similar granularity was observed in primary cells at high passage numbers. However, the granularity was not associated with a decrease in cell viability since the reversibly immortalized cells could resume growth without delay when Dox was added to the cell culture medium (May et al. 2004).

Effects of cell growth-control on recombinant gene expression

To quantify the influence of arresting proliferation on recombinant gene expression from a constitutive promoter, the cells were stably transfected with a SEAP construct that also conferred resistance to the antibiotic puromycin (Figure 1b). Resistant cells were selected and grown as a pool of transfectants. SEAP activity in the cell culture supernatant was determined in the presence or absence of doxycycline. Interestingly, the comparison of the expression levels

Figure 1. Schematic representation of the vectors used. (a) The plasmid vector pLTRGFPN contains a bi-directional tetracycline dependent promoter PbitTA that drives the expression of two divergent mRNAs encoding the TAg and the reverse transactivator rtTA2M2, respectively (Urlinger et al. 2000), and a fusion protein of eGFP and neo (Colbère-Garapin et al. 1981). The individual components of pLTRGFPN are derived from the following constructs: the bi-directional doxycycline regulated promoter PbitTA stems from pBI-I (Baron et al. 1995), the SV40 TAg is followed by SV40 p(A) polyadenylation signal sequences; the rtTA2M2 sequences are derived from pUHrt62-1 (Urlinger et al. 2000) an encephalomyocarditis virus internal ribosomal entry site is derived from pEMCVhyg (Verhoeven et al. 2001); the reading frame encoding an enhanced green fluorescent protein was fused at the C-terminus without the stop codon in frame to the N-terminus of the selection marker gene neo followed by a SV40 polyadenylation signal. (b) Plasmid vector pM5SEAP. The constitutive 5' LTR promoter drives the expression of the SEAP reporter gene as well as the puromycin resistance gene, the 3' LTR functions as poly (A) addition site. (c) Expression unit of the adenoviral vectors. EPO and eGFP are expressed as a dicistronic mRNA from the constitutive CMV promoter (PCMV). (d) CMV promoter-eGFP expression cassette of the adenoviral vector construct.

showed a twofold higher productivity of the proliferation-controlled cells (Figure 5). This is consistent with previous results in unrelated cell growth-control systems and supports the notion that proliferation-control leads to productivity increases (Fussenegger et al. 1998; Kaufmann et al. 2001; Schlatter et al. 2001; Meents et al. 2002b; Bi et al. 2004).

EPO production in proliferation-controlled cells

EPO is a well-characterized pharmaceutical protein product of cultured mammalian cells

Figure 2. Efficient doxycycline-dependent proliferation-control of LT1SEAP cells. For the determination of the cell density 2×10^4 LT1SEAP cells were plated and incubated without (black squares) or with doxycycline (circles) in the cell culture medium. Samples for determination of cell density were taken at the time points indicated. Cell numbers were determined by counting two times each sample from three parallel cultures for each condition. The standard deviation was below 15% for all values shown.

Figure 3. LT1 arrest growth specifically at the G1/G0 cell cycle phase. Cells were grown for three days in the absence (–) or in the presence of Dox in the cell culture medium (+ Dox). For flow cytometric analysis of the cellular DNA content the cells were incubated with trypsin, washed, fixed, permeabilized and stained with propidium iodide. The intensity of the fluorescence per cell is given on the x-axis (FL2A); the fraction of cells with a given fluorescence intensity is indicated on the y-axis.

(Takeuchi et al. 1989; Yamaguchi et al. 1991; Nimtz et al. 1993; Schlenke et al. 1999). To examine if the proliferation-controlled cells could be used for recombinant gene expression, LT1 cells were genetically modified using an adenoviral vector carrying a recombinant EPO expression cassette. The EPO vector and a control construct carry the eGFP gene as an easily detectable marker protein (Figure 1c, d). As expected for adenoviral vectors, recombinant gene expression was very efficient in both primary cells and in

Figure 4. Cell morphology of proliferation-controlled cells. LT1SEAP cells were cultivated for 3 days in the presence (black curve) or absence of Dox in the cell culture medium (gray curve). The cells were enzymatically detached with trypsin. Propidium iodine staining was used to exclude dead cells from the analysis. The forward (FSC-H) and sideward light scattering profiles (SSC-H) were determined (a and b, respectively). The two-dimensional representation is shown below: LT1SEAP in the presence (c) or absence of Dox (d).

Figure 5. Enhanced productivity in proliferation-controlled cell cultures. SEAP activity was determined per day from the supernatants of growth arrested (–Dox) and proliferating cells (+Dox). The productivity of growth arrested versus proliferating cells is shown. The RLU ratio was calculated as the ratio (–Dox/+Dox) of the average relative light units in the cell culture supernatant per cell per day at the time points indicated.

proliferation-controlled cells. A high proportion of cells infected with either construct exhibited easily detectable green fluorescence, whereas

mock-infected cells did not (Figure 6). This is important since a low transfection efficiency could introduce a bias due to the selective expression in a special cell sub-population. For reasons that were not further investigated, cells infected with the adenoviral vectors appear rounded when compared to mock-infected cells without the virus (Figure 6). Cells were maintained in serum-free medium in the presence and subsequently in the absence of doxycycline. Proteins that were secreted by the cells were precipitated from the supernatant and visualized by gel electrophoresis and Western blot analysis. A band corresponding to the molecular weight expected for glycosylated EPO (approximate apparent molecular weight of 36 kDa in SDS-PAGE analysis) reacted with EPO-specific antibodies (Figure 7). EPO produced from growth-controlled cells or from the uncontrolled proliferating culture could not be distinguished, demonstrating that full length EPO protein could be expressed during

Figure 6. Recombinant adenovirus-mediated gene expression in growth-regulated cells. LT1 cells were infected with Adcos45eGFPC (A1, B1), Adcos45epoieG (A2, B2) or mock-infected (A6, B6). LT1 cells were cultivated for 24 h in the presence (A) or absence (B) of doxycycline, respectively. The cells were photographed with visible light (A and B1, 2, and 6); green fluorescence was examined using UV light excitation (A and B1G, 2G and 6G).

Figure 7. EPO production in proliferation-controlled cells. Proteins from Adcos45epoieG infected LT1 cell culture supernatants were separated by SDS-PAGE, blotted to nitrocellulose membranes and hybridized to anti-EPO antibodies. Cells were cultivated for 2 days in the presence of Dox (lane 1); subsequently, the medium was replaced by fresh medium without Dox and the cells incubated for additional 3 days (lane 2); cell culture supernatant from a mock-infected LT1 cell culture (lane 3); 10, 25 and 50 ng of purified EPO (lanes 4, 5 and 6, respectively).

proliferation-controlled conditions with no apparent changes in the apparent molecular weight.

Discussion

Due to the multiple requirements only a few biotechnological cell lines are widely used. However, recently a novel producer cell line has been introduced successfully that was established by pursuing a rational immortalization strategy (Fallaux et al. 1998), showing that the generation of new commercial producer cell lines is feasible. Numerous attempts have been made to control the cell growth during the production process. Therefore, a novel producer cell line whose growth rate can be adjusted according to the needs would be of

considerable interest. As a first step and as a proof of principle, we investigated the feasibility to generate new cell lines that make use of the cells own control mechanisms to regulate proliferation. By employing a regulated immortalizing gene expression system we show that cell lines can be derived from primary cells that show a very tight growth regulation. Primary cells can survive in culture for long periods of time without proliferation, a property that is used in applications such as the co-cultivation of growth arrested primary fibroblast feeder cells with stem cells. This has motivated us to investigate endogenous growth-control mechanism rather than using sub-optimal cell culture conditions or overexpression of inhibitory molecules that may impair cell viability (Sekiguchi and Hunter 1998; Schroeder et al. 2002). It has been demonstrated that the doxycycline induction system is non-toxic and is compatible with self-regulatory growth-control during fermentation (Mazur et al. 1999). The immortalization vector used here is based on an autoregulated expression system with a bi-directional promoter (May et al. 2004). The cell lines derived by such vectors allow adjusting cell proliferation as an additional parameter in the production process to optimize product yield and product quality. The SV40 large T-antigen was chosen for this study, since it is a well-characterized immortalizing gene that has been shown to be compatible with the reversion of the immortalized phenotype upon reduction of the TAg-activity (Jat and Sharp 1989; Westerman and Leboulch 1996; Ali and DeCaprio 2001; Obinata 2001; O'Hare et al. 2001; Lidington et al. 2002). Alternatively, the autoregulated, multivalent expression vector allows to investigate the suitability of other single or

combinations of multiple immortalization genes in place of the TAg, such as the adenoviral E1 sequences that have been used to establish biotechnological producer cells (Fallaux et al. 1998).

Our results demonstrate that strictly growth regulated cell lines can be obtained by this approach. The phenotype of the arrested cells resembles that of primary cells that have reached the proliferation limit. However, the fact that the cells can resume proliferation even after 1 week of growth arrest argues against a senescent or apoptotic state. In growth-controlled cells, recombinant gene expression was enhanced. In fact, many of the previous investigations of unrelated proliferation-control systems in a biotechnological context have found an increased productivity (per cell) during growth arrest (Fussenegger et al. 1999; Ibarra et al. 2003; Bi et al. 2004). This is in accordance with the notion that growth-controlled producer cells devote more resources to recombinant protein production. As an alternative explanation, since there is also a increase in the cell size, the gains in productivity might well be a consequence of an increased average cell mass (Lloyd et al. 2000).

Growth arrest is thought to prolong the productive period in genetically unstable systems, and it could therefore extend the time of recombinant gene expression from non-replicating episomal expression vectors. However, the experimental conditions used here do not allow to draw any conclusions in this respect. Concerning protein quality, in addition to the medium composition, the cell physiology can affect the post-translational protein modifications. Both the reduced medium consumption and a uniform physiological state such as the cell-cycle-specific growth arrest of the LT1 cells are expected positively affect product homogeneity.

In conclusion, this study shows that a reversible immortalization-based proliferation-control system is compatible with recombinant pharmaceutical product expression. Future development of this technique will focus on biotechnological applications and aim at the generation of regulated cell lines with a higher production capacity and with human compatible protein glycosylation.

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