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Development and improvement of a serum-free suspension process for the production of recombinant adenoviral vectors using HEK293 cells

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Abstract

Human Embryonic Kidney 293 (HEK293) cells were adapted into a serum-free suspension medium through steps of gradual serum weaning for the production of adenoviral (AdV) gene therapy vectors. The presence of sodium heparin in the medium formulation reduced cell clumping dramatically in suspension culture. The adapted cells were ready to grow either in serum-containing medium as an attached culture or in serum-free medium in suspension culture. A scalable production process was developed in shake flasks and was then evaluated in stirred tank bioreactors. This process includes a growth phase in batch-mode followed by a production phase involving medium perfusion and supplementation. Fortification with calcium chloride post viral inoculation resulted in an increase in virus production by at least one fold. Addition of stimulating agents such as sodium butyrate, N-acetyl-L-cysteine (NAC), dimethyl sulfoxide (DMSO), or ethyl alcohol post infection was shown to further improve virus production in a dose-dependent manner. The serum-free suspension process described here should be suitable for the manufacturing of other E1-deleted AdV vectors and could potentially be used for the production of recombinant proteins by HEK293 cells.

Introduction

Replication-defective adenoviral (AdV) vectors have been widely used as an efficient delivery vehicle of gene targeting in a number of research and clinical applications (Benihoud et al. 1999; Katayose and Seth 1999; Graham 2000; Russell 2000). The use of recombinant adenovirus as a gene therapy vector has several advantages. It allows potentially larger DNA inserts, ease in producing high titer of the vector, efficient gene transfer to non-dividing cells in a broad range of cell types and avoidance of the concern of mutagenesis caused by chromosomal integration (Anderson 1998; Amalfitano 1999; Benihoud et al. 1999). Group C adenovirus has been the most common AdV vectors both *in vitro* and *in vivo* (Shenk 1995; Russell 2000). The coding sequences such as the E1 region, which is essential for virus replication, has been deleted from these vectors so that they are replication-defective in normal human cells. *In vitro* amplification of these vectors, therefore, requires packaging cell lines that provide the missing components *in trans*. The Human Embryonic Kidney 293 (HEK293) cell line or its derivatives have been historically the common choice (Graham et al. 1977; Shenk 1995; Robbins and Ghivizzani 1998) for the production of such vectors.

Manufacturing of a large quantity of recombinant product can be achieved in a stirred tank bioreactor, a stationary attached culture or a hollow-fiber system (reviewed by Hu and Aunins (1997)). The serum-free suspension process is often the preferred mode of production due to its potential ease in scale-up operation, simplicity in downstream processing and re-

duced risk of virus contamination. The HEK293 cell and its derivatives, however, have a tendency to form large cell aggregates in suspension culture (Peshwa et al. 1993; Garnier et al. 1994; Nadeau et al. 1996; Cote et al. 1998; Schoofs et al. 1998; Iyer et al. 1999; Tsao et al. 1983). Severe and uncontrolled cell clumping may lead to difficulties in long-term cell propagation and altered cell metabolism (Spier 1997; Schoofs et al. 1998; Kallos et al. 1999). Furthermore, cells inside the clumps may not be efficiently infected at initial viral inoculation for viral vector production. Significant progress has been made in recent years to address the problem of HEK293 cell aggregation in suspension culture. General approaches include media development, cell line adaptation through gradual weaning steps, clonal selection and controlled mechanical agitation (summarized in Cote et al. (1998)).

HEK293, originally derived from attached culture, tend to produce a lower level of adenoviral particles once grown in serum-free suspension culture (Iyer et al. 1999). Since the volumetric titer is the product of the virus particles produced per cell and viable cell density achieved in the production vessel, optimization of specific virus productivity in serum free suspension culture becomes very important. The manufacturing process for an AdV vector can be divided into two stages, namely cell expansion and post infection virus production. Commercial medium and published formulation seem to be able to support HEK293 growth with satisfactory results (Berg et al. 1993; Nadeau et al. 1996; Cote et al. 1998; Schoofs et al. 1998; Iyer et al. 1999). However, changes in the metabolic profile and nutrient requirement of the host cells after virus infection have not been extensively studied. Such changes could be further complicated by the type of vector used and by the effect of the transgene it carries.

Here we report the development of a serum-free suspension process with HEK293 cells for the production of a recombinant adenovirus vector expressing the wild type human p53 gene. HEK293 cells were adapted into serum-free suspension culture by gradual serum weaning steps. A commercial medium was modified in order to achieve better control of cell clumping and optimized growth. Medium exchanges were performed at and post infection. Several medium supplements added post infection were found to improve virus production significantly. The baseline process developed in shake flasks was confirmed in 2-l stirred tank bioreactors. The process described here should be suitable for the production of other E1-deleted AdV gene therapy vectors and potentially for the expression of recombinant proteins by infection of HEK293 cells with AdV vectors.

Materials and methods

Cell culture medium

Serum-free growth medium (denoted as 293-4-08) was prepared by supplementing IS293-V (Irvine Scientific, 99278) with 0.1% pluronic F-68, 10 mM Tris.HCl (pH 7.4, Biowittaker, 16-014V), 1x Trace Elements A, B and C (Mediatech, MT 99-182, MT 99-175, and MT 99-176), 13.4 mg l^{-1} ferrous gluconate (Fluka, 44948) and 100 μg ml^{-1} sodium heparin (Sigma, H1027). Sodium heparin was not included in the media used for perfusion either at or post infection. This heparin-free medium was denoted as 293-4-06. Medium supplements added post infection to improve virus production were obtained from: sodium butyrate (Sigma, B5887); N-acetyl-L-cysteine (NAC) (Sigma, A8199); dimethyl sulfoxide (DMSO) (Sigma, D8779); ethyl alcohol (Pharmco Products, Inc., 111USP200CSPT); calcium chloride (Sigma, C7902).

Cell line and suspension adaptation

HEK293 cells were originally obtained from Magenta Corporation (Rockville, MD) and were maintained in tissue culture flasks with vent cap (Corning, 430641) in a modified DMEM medium (HyClone, SH3A435) supplemented with 10% Bovine Calf Serum (HyClone, SH30072) and 110 mg l^{-1} sodium pyruvate (GIBCO BRL, 11360-070). The medium was denoted as 293-1-RO3. The attached cell culture was adapted into serum-free suspension medium by cultivating the cells in a sequential 1:2 dilution of the serum-containing 293-1-RO3 medium with the serum-free 293-4-08 medium. The resulting medium mixture contained 5, 2.5 and 1.25% bovine calf serum, respectively. Cells were allowed to adapt at each serum level for 2 to 3 passages. The attached culture was transferred into Erlenmeyer flasks with vent cap (Corning, 431144) when the serum concentration reached 1.25%. Serum weaning was then resumed in shake flask until the culture growth rate was maintained at ~ 0.022–0.025 h^{-1}. in serum-free 293-4-08. The shake flasks were agitated at 110 rpm on an orbital shaker (Forma, Model 4530). All cell cultures were main-

tained in a 37 °C tissue culture incubator supplied with 5% CO_2. Cell banks were prepared and stored in liquid nitrogen with freezing media that contains 80% IS293-V, 10% DMSO (Sigma, D2650) and 10% Bovine Calf Serum.

Cell counting and culture monitoring

Cell counting and viability assessment were obtained by a combination of standard hemacytometer count with Trypan Blue dye exclusion and a Nicomp AccuSizer 780 Optical Particle Sizer (Particle Sizing Systems, Langhorne PA) that measures cumulative volume of the cell aggregates for an estimation of total cell counts (Tsao et al. 2000). Culture pH was measured offline by an ABL5 Blood-Gas Analyzer (Radiometer Medical A/S, Copenhagen Denmark). Offline measurement of culture glucose, lactate, glutamine was obtained by a YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH).

Adenoviral vector production in shake flasks

HEK293 cells were typically allowed to grow to approximately 2.2–2.5 $\times$ 10^6 cell ml^{-1} prior to infection in 293-4-08 in batch mode. Before virus inoculation, a medium exchange of approximately 90% of the original culture volume was performed with 293-4-06 by centrifugation at 850 rpm for 10 min (Beckman, Model Allegra 6R). Virus was inoculated at a final concentration of 1 $\times$ 10^8 virus particles (vp) ml^{-1}. That is equivalent to approximately a 40 to 1 ratio of virus particles to cells. Immediately after infection, the CO_2 level of the incubator was reduced to 0% to maintain a neutral pH post infection. In addition, the agitation rate of the shaker was lowered to 105 rpm from 110 rpm. Approximately 20 h post infection, another 90% medium change with 293-4-06 was performed by centrifugation. Three ml of culture samples was collected at 24, 48 and 72 h post infection for the quantification of virus produced.

Adenoviral vector production in bioreactors

The scale-up development of virus production was performed in Braun Biostat 2-litre Bioreactors, connected to a DCU3 control module and a gas mixer (1057-C) (B. Braun Biotech, Allentown PA). The bioreactors were fitted with an internal spin filter with a mesh size of 20 microns and equipped with a 3″ pitch blade impeller (B. Braun Biotech, Allentown PA). The culture temperature was maintained at 37 °C with a heating blanket (#880773/7) (B. Braun Biotech, Allentown PA). Dissolved oxygen was maintained at 40% of air saturation by a single loop controller. The flow rate of air in the headspace was maintained at 0.1 l min^{-1}. The bioreactor tanks were inoculated with cells from shake flasks with targeted initial seeding density of 0.6 $\times$ 10^6 cell ml^{-1} in 293-4-08 medium. The total working volume was 2000 ml. The agitation rate was maintained at 150 rpm during the subsequent growth phase. When cell density reached approximately 2.5 $\times$ 10^6 cell ml^{-1}, a perfusion with 3.8 l of 293-4-06 medium was performed at a rate of 2.5 l h^{-1}. Virus was inoculated at a final concentration of 1 $\times$ 10^8 vp ml^{-1} immediately after the perfusion. The agitation rate was reduced to 120 rpm after virus inoculation. Approximately 20 h post virus inoculation, another perfusion with 2 l of 293-4-06 medium was conducted at a rate of 0.7 l h^{-1}. The pH was kept above 6.8 during the entire culture period with 1 M Na_2CO_3 solution (Sigma, S9140).

Sample preparation and analysis of viral production

Collected culture samples were treated by freeze/thaw in a Dry Ice – Ethanol bath and a 37 °C water bath for three times, followed by centrifugation at 3000 rpm for 20 min in a bench-top centrifuge (Beckman GS-6R, Palo Alto, CA). The supernatant was then treated with 50 units ml^{-1} Benzonase (EM Industries, 1016979M) at room temperature for 1 h. The virus particles were measured by an analytical anion-exchange HPLC method with a 1-ml Resource Q column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) (Shabram et al. 1997).

Results and discussion

Effect of sodium heparin on growth behavior of HEK293 in serum-free suspension culture

One of the major obstacles in growing HEK293 cells in suspension is their tendency to form large cell aggregates. In some severe cases, these clumps can be as large as millimeters in diameter (Cote et al. (1998); our observation). Control of HEK293 cell clumping can be achieved via several approaches (Cote et al.

1998). Reduction of divalent ions such as calcium or magnesium alone has only limited effect on minimizing cell clumps in our experience. Mechanical shear by itself is insufficient to break up cell clumps without causing cell damage or slower cell growth. Alternatively, certain sulfated polyanions have been shown to be able to induce single-cell suspension of various cell types (Rochon et al. 1994; Dee et al. 1997a, 1997b). Among our earlier attempts to reduce HEK293 aggregation in serum-free suspension culture, dextran sulfate (0.1 g l^{-1}) was shown to significantly suppress cell clumping. However, this was accomplished at the expense of lower culture viability. More importantly, the specific productivity of recombinant adenoviral vectors was decreased as much as four-fold as compared with a microcarrier process with a serum-containing medium (Data not shown).

Heparin and other sulfated glycosaminoglycans have been shown to suppress the aggregation of certain cell types (Rochon et al. 1994; Dee et al. 1997a; Irvine Scientific, Inc. 1997) but induce cell clumping among others (Eldor and Weksler 1979; Reches et al. 1979; Bazzoni et al. 1993; Kameneva et al. 1994; Stanley et al. 1995). Specifically, heparin is believed to block L-selectin-dependent neutrophil aggregation and is recommended by the medium manufacturer to reduce HEK293 clumping in serum-free suspension culture (Rochon et al. 1994; Irvine Scientific, Inc. 1997). We, therefore, tested the effectiveness of sodium heparin for its anti-clumping effect on HEK293 cells as well as its potential influence on cell growth. In this experiment, HEK293 cells were first adapted into serum-free suspension culture by stepwise gradual serum weaning. The suspension-adapted HEK293 cells were then passaged by subculturing in serum-free medium with and without sodium heparin (Figure 1). The starting cell density after each culture split was kept at 0.5×10^6 cell ml^{-1}. The cultures were maintained in batch mode with 5% CO_2 at 37 °C. Cell densities and aggregation profiles were monitored by particle size analysis. In this measurement, culture samples with cell clumps were passed through a particle size analyzer that detects and measures the size of individual particles (Tsao et al. 2000). First, the cumulative particle volume derived from this measurement was converted to cell density based on the average single cell volume (Figure 1A). Second, the median cell aggregation (cells/aggregate) was calculated from the size distribution of the particle population (Figure 1B). This value indicates that 50% of the entire cell population is growing in clumps equal to or smaller than this number and was then used to describe the aggregation profile of the sample. Our data showed that even though the presence of sodium heparin slightly decreased the cell growth rate and lowered peak cell density, the aggregation profiles of both cultures displayed consistent and dramatic differences (Figure 1). The median cell aggregate size was reduced by more than 50% by the addition of sodium heparin (Figure 1B). Additionally, the cell viability of both cultures was greater than 95% during the entire course of the experiment. Finally, there were no significant changes in the metabolic profiles (such as culture pH, glucose consumption and lactate accumulation) when sodium heparin was present in the culture medium (Data not shown).

Comparison of growth between suspension adapted vs unadapted HEK293 cells in serum-free suspension culture

We noticed that HEK293 cells respond to the suspension adaptation differently from experiment to experiment during serum weaning. To better understand the changes associated with suspension adaptation, the growth kinetics of HEK293 cells under different adaptation schemes was compared. First, the original HEK293 cells grown in serum-containing 293-1-RO3 was adapted into the serum-free suspension medium 293-4-08 (containing 100 μg ml^{-1} sodium heparin) by stepwise serum weaning to generate a culture, named 293S. Second, these fully adapted suspension HEK293 cells (293S) were re-adapted to attachment culture in the serum-containing medium (293-1-RO3). After 7 passages, these cells were transferred into suspension culture directly, bypassing the gradual serum weaning. The resulting culture was named as 293S-AS. Third, the unadapted original HEK293 cells grown in 293-1-RO3 were switched abruptly into the serum-free medium (293-4-08), in parallel to 293S-AS cells, to generate a new suspension culture denoted as 293A-S. The subsequent experiment was conducted by subculturing these HEK293 cells in 293-4-08 in batch mode.

The 293A-S displayed a slower growth rate than both of the suspension adapted cultures, 293S and 293S-AS, for at least 5 passages (Figure 2; Figure 2B, ratio of 293S-AS/293A-S). On the other hand, serum-free adaptation of the HEK293 cells seems to result in

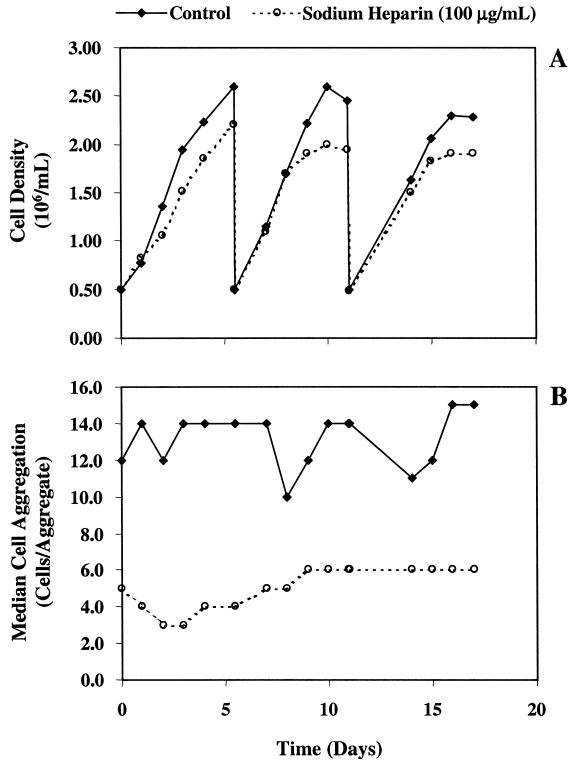

Figure 1. Effect of sodium heparin on HEK293 cells in serum-free suspension culture. HEK293 cells adapted in serum-free suspension culture were subcultured in batch mode in shake flasks. The control culture was maintained in 293-4-06 medium (with no sodium heparin). The test culture was passaged in 293-4-08 medium (100 μg ml^{-1} sodium heparin). The starting cell densities were kept at 0.5×10^6 cell ml^{-1} at each passage. Growth kinetics (A) and aggregation profile (B) of the cultures were monitored by particle size analysis. The median cell aggregate size indicates 50% of the entire cell population is growing in the clumps equal to or smaller than this number (presented as cells/aggregate).

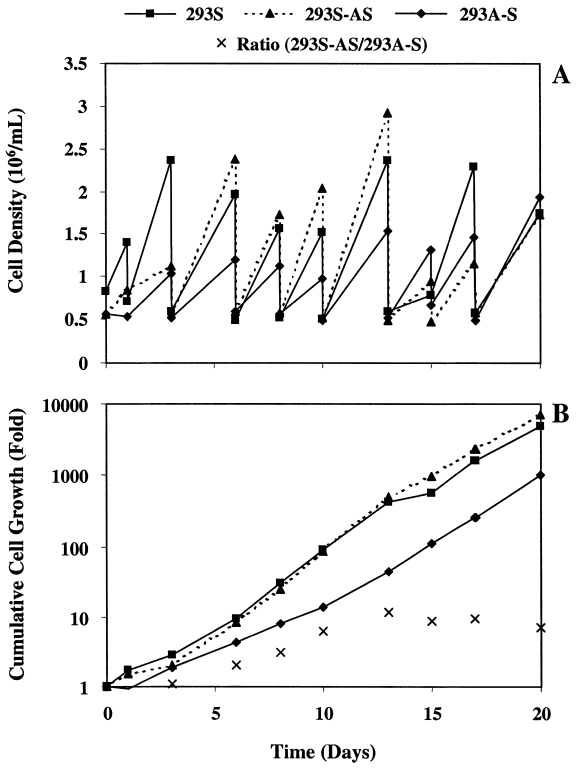

Figure 2. Comparison of growth kinetics of HEK293 cells under different adaptation schemes in serum-free suspension culture. 293S: suspension adapted HEK293 cells; 293A-S: the original HEK293 cells grown in serum containing medium as an attached culture were switched abruptly to serum-free suspension culture via a single step; 293S-AS: suspension-adapted HEK293 cells (293S) were cultured in attached mode in a serum containing medium for 7 passages before being transferred into serum-free suspension culture directly as with the 293A-S. The growth rates of these three cell types were compared by passaging the cultures in serum-free suspension medium 293-4-08. The ratio between 293S-AS/293A-S was calculated according to the cumulative cell growth of these two culture types.

a selected population of cells, represented by 293S-AS, that are ready to grow in either attached or suspension cultures (comparing 293S and 293S-AS in Figure 2) (Mather et al. 1997). This is because, similar to 293S, there is no apparent lag phase in growth rate of 293S-AS immediately after it was transferred into serum-free suspension. The selection of such a sub-population would facilitate genetic engineering of HEK293 for recombinant protein production or further engineering of the packaging cell line. The pre-adapted HEK293 should allow easy re-adaptation of selected clones back into suspension mode after manipulation in attached culture that is often maintained in serum-containing media.

Production of an adenoviral vector in serum-free suspension culture in shake flasks with various amounts of virus inoculum

Production of a recombinant E1-deleted AdV vector expressing a wild type p53 gene from the suspension adapted HEK293 cells were evaluated in shake flasks. Prior to viral infection, a medium exchange (90% of the culture volume) was performed with 293-4-06 (i.e. 293-4-08 without sodium heparin) through centrifugation. The cell density at infection is 2.0×10^6cell ml^{-1}. The cultures were inoculated with virus

stock immediately after the medium exchange at the concentrations indicated (Figure 3). Approximately 20 h post infection (p.i.), another medium exchange was performed in the same manner as the one prior to infection. Virus samples were collected at 48 h p.i.. Data in Figure 3 showed a dose-dependent virus production with the maximum reached at approximately 1×10^8 vp ml^{-1}. All of the subsequent infection experiments in shake flasks were conducted with 1×10^8 vp ml^{-1} virus inoculation at infection. The virus titer at 24 h p.i. was typically undetectable. Because of the difficulties in pH control in shake flask, which often dropped as low as pH 6.0, the virus titer at 72 h p.i. was generally lower than that at 48 h p.i..

Effect of calcium fortification post infection on adenoviral vector production in serum-free suspension culture

The base formula of the medium used in this report (IS293-V from Irvine Scientific) contains a relatively low concentration of calcium ion (~108 μM) as opposed to ~ 2 mM in a typical DMEM medium. Reduction in calcium level was designed to minimize the HEK293 clumping which was shown to be calcium-dependent (Peshwa et al. 1993; Nadeau et al. 1996). In our experience, the calcium concentration can be as low as 10 μM without significant effect on cell growth or viability (data not shown). Since the calcium level is one of the major differences between the DMEM formulation and the suspension medium used in this report, we decided to explore the possibility of improving AdV vector production by supplementing additional calcium post infection. In this experiment, varying concentrations of calcium chloride were added to the culture 2 h post viral inoculation and were maintained throughout infection. Figure 4A showed a dose-dependent increase in virus production on calcium concentration. The maximum volumetric production was reached at ~ 500 μM calcium chloride. No significant increase was observed beyond this level. A time-course experiment was also performed to identify the optimal time of calcium addition. For example, when the concentration of calcium chloride post infection was maintained at 800 μM, the best time for calcium fortification ranged from 0.5 to 6 h after virus inoculation (Figure 4B). In summary, supplementing calcium chloride post infection to the HEK293 suspension culture resulted in at least one fold increase in AdV vector production.

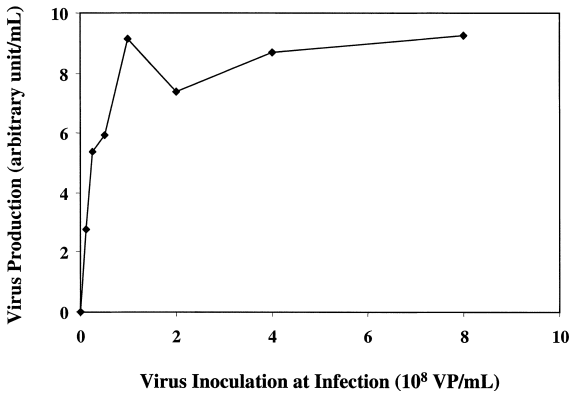

Figure 3. Effect of adenovial inoculum concentration at infection on virus production in serum-free suspension culture. HEK293 cells grown in serum-free suspension culture were inoculated with an E1-deleted recombinant adenoviral vector at the concentrations indicated. Culture samples were taken 48 h post infection and assayed for virus particle numbers by an analytical anion-exchange HPLC method. Only the arbitrary units were presented in this report.

Production of the adenoviral vector in 2L stirred tank bioreactors

Difficulties in pH control of shake flasks placed in a standard tissue culture incubator resulted in the pH reaching as low as 6.0 post infection. Thus, the potential of the serum-free suspension process could not be fully explored with ease in shake flasks. A stirred tank bioreactor was, therefore, chosen for the scale-up process development that would provide better control of process parameters. At 150 rpm with the setups described in the section of Materials and methods, the cell clumping was well controlled with high culture viability ($>$ 95%). The design for the bioreactor process resembles the baseline process in shake flasks that starts with a growth phase in batch mode with a target cell density of $2.0–2.5 \times 10^6$ cell ml^{-1} for infection. Immediately prior to virus inoculation, a perfusion was performed with 3.8 l of 293-4-06 (containing no sodium heparin) at a relatively fast rate (2.5 l h^{-1}). Calcium chloride was added to one of the bioreactors at a final concentration of 800 μM 2 h post infection. Another medium exchange was conducted 20 h post infection with 2 l of 293-4-06. The pH was maintained above 6.8 during infection. To reduce possible damage to the infected cells, the agitation rate was decreased to 120 rpm after infection. Figure 5A and 5B summarize the culture

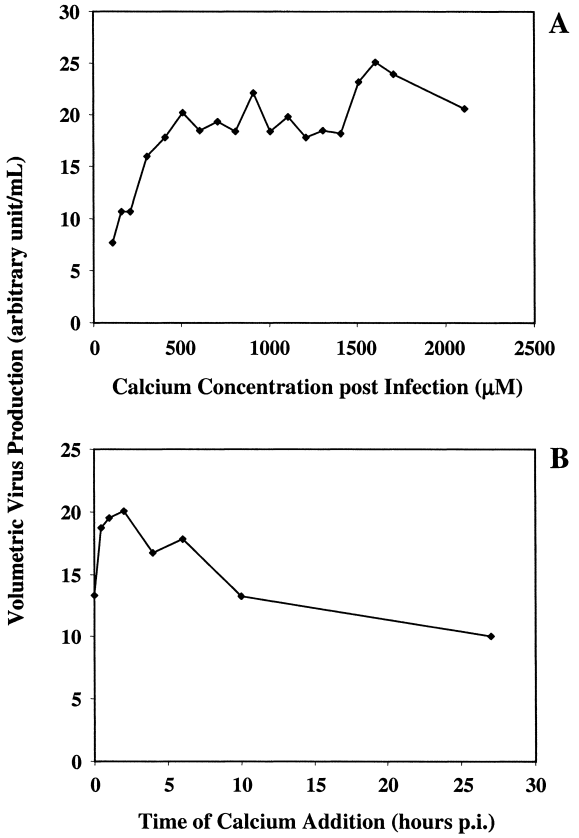

Figure 4. Effect of calcium fortification post infection on adenoviral vector production. Dose-dependence (A): Calcium chloride was added 2 h post infection and was maintained at the concentrations indicated. Time-course (B): A final concentration of 800 μM calcium chloride was added to the culture at various points relative to the time of infection. Virus production was measured 48 h post virus inoculation in both series of experiment.

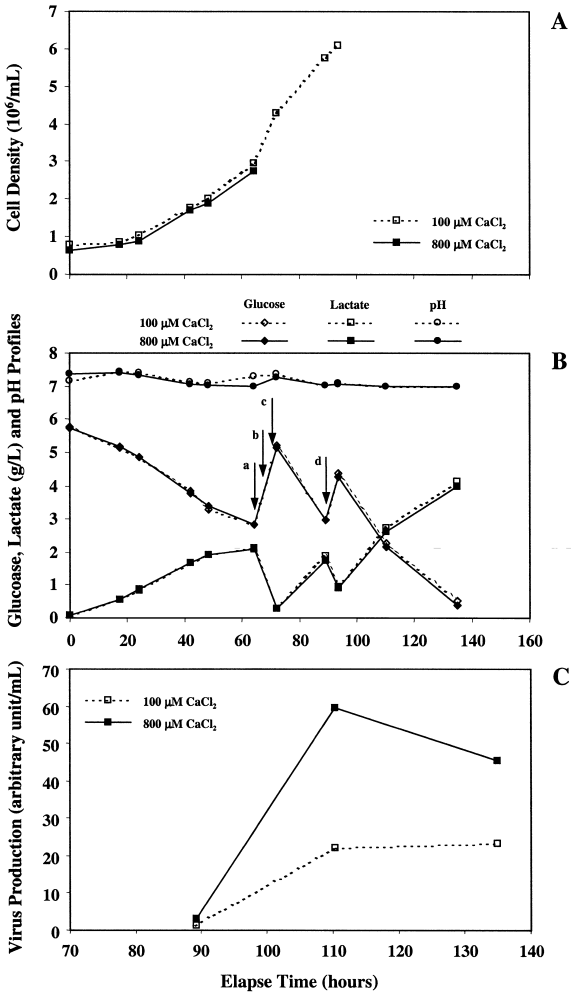

Figure 5. Adenoviral vector production by suspension HEK293 cells in stirred tank bioreactors. HEK293 cells in serum-free suspension culture were allowed to grow in batch-mode in 2-l bioreactors. Medium exchanges were performed prior to and 20 h after virus inoculation. Additional calcium chloride was added to one of the bioreactors. (A) Cell growth profile: Due to severe cell aggregation after calcium addition, reliable cell counts post infection could not be obtained by either manual cell count or particle size measurement. (B) Selected metabolic profiles. Shown here are glucose, lactate and pH profiles. The arrows indicate: a = medium perfusion prior to infection; b = Virus inoculation; c = Addition of calcium chloride; and d = medium perfusion post infection. (C) Kinetics of virus production. Virus particles produced were measured at days 1, 2 and 3 post infection.

and metabolic profiles of the bioreactor tanks. Virus production was measured at the times indicated (Figure 5C). Results from the bioreactor experiment confirmed the improvement of virus production by calcium fortification that was first observed in shake flasks.

Calcium chloride was chosen to fortify the medium during the production phase primarily because the growth medium (293-4-08) contains a much lower level of calcium ion as compared to that of most media typically used for attached culture. We speculate that insufficient amounts of calcium ion in a serum-free suspension formulation may contribute to the apparently lower specific productivity of adenoviral vectors by HEK293 cells reported in the literature and observed by us. Since higher concentrations of calcium cause increased levels of cell aggregation, which may at least in theory interfere with the virus infection, the time of calcium addition during subsequent development work was set at 2 h post infection. This gap should allow sufficient time for the completion of the entry of adenovirus into host cells (Greber et al. 1993; Nakano and Greber 2000).

Further improvement of virus production through medium development

Further increase in production output of a mammalian process can be achieved through various means of process development. One of the strategies is the inclusion of stimulation agents in the medium during the production phase. Numerous reports have been published with the use of these agents in mammalian systems to improve the production of recombinant proteins. However, production kinetics of AdV vector is more complicated due to the complexity in virus assembly as well as its cytopathic effect on host cells (Shenk 1996; Schmid and Hearing 1999). Therefore, general strategies for stimulating the production of recombinant peptides may or may not be applicable to the virus production process.

To evaluate the effectiveness of these agents on the production of AdV, we performed screening experiments in shake flasks. In these experiment, the individual agents were added two h after the fortification with calcium chloride (i.e. 4 h p.i) and maintained at the desired doses during the entire course of infection. Higher AdV titers were observed with the following medium supplements: dimethyl sulfoxide (DMSO), ethyl alcohol, sodium butyrate and N-acetyl-L-cysteine. Figure 6 showed the dose-response of these agents assayed 48 h post infection. It is worth mentioning that these compounds failed to demonstrate any additive effect among the various combinations tested (data not shown).

The pleiotropic compounds, such as DMSO, sodium butyrate and ethyl alcohol have been shown to induce differentiation and cause cell cycle arrest in many cell types (Collins et al. 1978; Tsao et al. 1983; Frank et al. 1979; Wands et al. 1979). Specifically, sodium butyrate was shown to enhance acetylation of histones that contributes to higher levels of gene expression (Gorman et al. 1983; Arts et al. 1995). Furthermore, both NAC and DMSO are potential anti-apoptotic factors due to their antioxidant effect (Malorni et al. 1993; Fiore and Degrassi 1999).

Even though the mechanisms of their action still remain largely unknown (Saito et al. 1992; Mas-

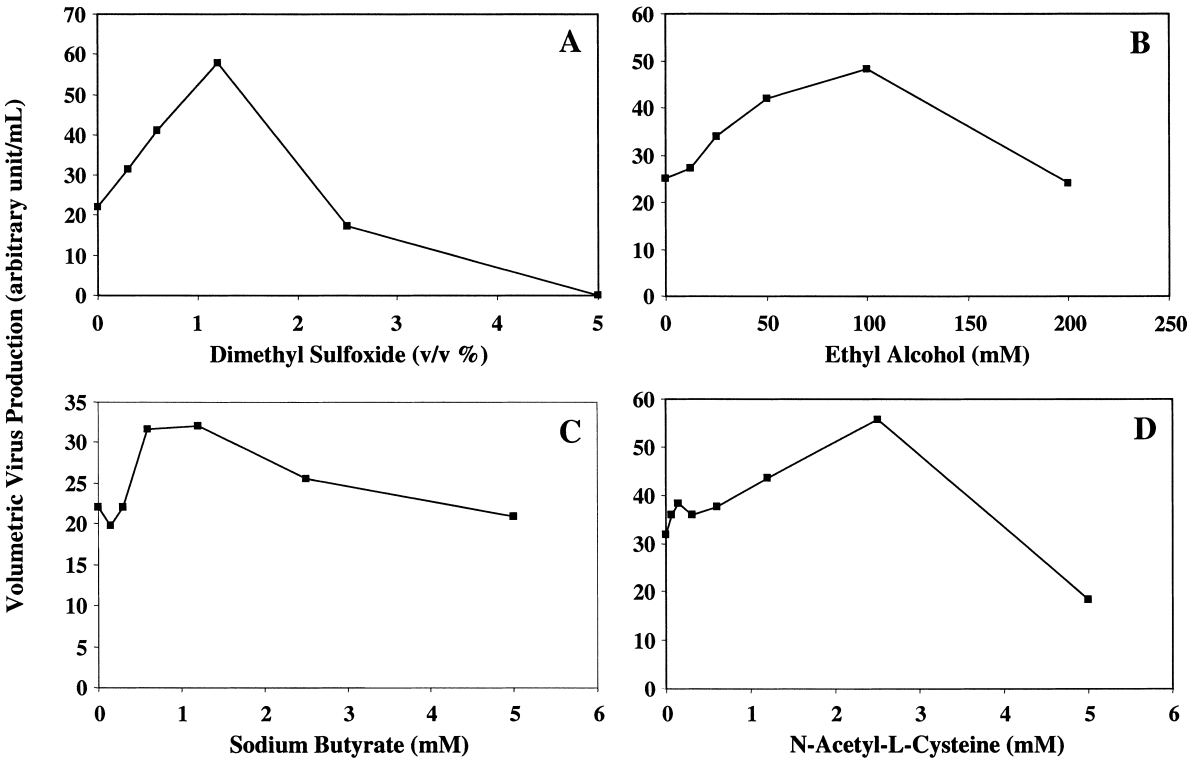

Figure 6. Stimulating agents that further increase the production of adenoviral vectors by HEK293 cells in serum-free suspension culture. These infection experiments were performed in shake flasks with calcium fortification post infection. The individual agents were added 2 h after calcium addition (4 h post infection) and were maintained at the concentrations indicated during the course of entire infection. Virus titer was measured at 48 h post infection.

trangelo et al. 1999), we did observe that DMSO displayed a growth inhibition effect on the uninfected HEK293 cells (data not shown). The identification of the cellular targets of these compounds may help to reveal the key limiting steps in virus production. It would also help to understand the virus-host interactions as a result of virus infection and transgene expression. Finally, although the virus generated with the addition of these compounds showed no significant changes in biological activities measured by viral infectivity, it remains necessary to further characterize the virus product for possible changes in virus structure, biochemical composition and long-term stability.

Addition to the results and discussion

In conclusion, anchorage-dependent HEK293 cells have been successfully adapted into serum-free suspension culture by gradually weaning from serum. Cell growth can be further optimized through reduction of cell clumping by the addition of sodium heparin to the culture medium. During the production of adenovirus vectors, fortification of calcium ion post infection significantly improves virus titer in a dose-dependent manner. Virus yield can be further increased by supplementation with selected agents including dimethyl sulfoxide, sodium butyrate, ethyl alcohol and N-acetyl-L-cysteine. These results should allow for a better understanding of virus-host interactions during infection. In addition, the baseline procedures described here will contribute to the development of a scalable serum-free suspension process for the high level production of adenoviral vectors.

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