Development of a serum-free suspension process for the production of a conditionally replicating adenovirus using A549 cells

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Abstract Conditionally replicating adenoviruses (CRAVs) are a group of recombinant human adenoviruses genetically engineered to replicate in selected tissues, such as tumors. These viruses could potentially offer significant medicinal benefits, since the restrictive replication of these viral vectors leads to the lysis of target cells without harm to the surrounding tissues. The *in vitro* propagation and amplification of the CRAV vectors often requires special host cells with deregulated growth control pathways. In order to develop an efficient cell culture process for the scaleable production of a CRAV vector, A549 cells, a human lung carcinoma cell line normally cultured in adherent culture, were adapted to suspension culture. CRAV production was demonstrated with the suspension-adapted A549 cells and a baseline production process was developed in shake flasks. The ability to scale-up virus production was confirmed in stirred tank bioreactors. Molecular characterization of the suspension-adapted A549 cells indicates no significant changes in cellular mechanisms related to adenovirus infection.

Keywords Adenovirus · Recombinant adenovirus · Gene therapy · A549 cells · Serum-free · Suspension · Conditionally replicating adenoviruses (CRAVs)

Introduction

Virus infection is the most efficient method for the delivery of genetic materials to target cells. Among the many different types of viral vectors utilized by researchers, adenovirus has the advantages of being easy to produce in large quantities at high titer, possessing the ability to infect non-dividing as well as dividing cells, having the capacity to accommodate large pieces of foreign DNA, and the non-integration nature of its genome. For these reasons, recombinant adenoviruses have been widely used in clinical applications and as research tools in both *in vitro* and *in vivo* studies (Katayose and Seth 1999; McConnell and Imperiale 2004).

Recently oncolytic therapy with specially designed adenoviral vectors has emerged as a novel approach for the treatment of malignant diseases (Nettelbeck 2003; Wildner 2003; Dobbstein 2004). Advances in molecular biology and a better understanding of tumor biology have led to the design of genetically modified adenoviruses that replicate only in tumor cells (Conditionally replicating adenoviruses or CRAV) and are replication defective in

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normal tissue. Restricted replication of CRAV in malignant cells leads to the eventual lysis of the tumor cells without any detrimental effects on the surrounding tissue(s).

One of the CRAVs, 01/PEME, was developed based on the dysfunctional p53 pathway in many tumors (Ramachandra et al. 2001). This virus carries a deletion in E1a that abolishes binding of E1A with the p53 transcriptional co-activator p300/CBP. It also expresses, driven by a p53-responsive promoter, an E2F-Rb fusion protein containing the DNA-binding domain of E2F and transrepression domain of Rb. Furthermore, it overexpresses E3-11.6K protein to enhance cell lysis. In normal cells with functional p53, 01/PEME expresses E2F-Rb that antagonizes the function of E2F to inhibit E1 expression and the entry to S phase. In tumor cells, however, the 01/PEME replicates as efficiently as wild type virus (Ramachandra et al. 2001).

The human lung carcinoma cell line A549 was chosen for the amplification of 01/PEME due to its defect in the p53 pathway (Ramachandra et al. 2001). This cell line is normally propagated in adherent culture with medium containing serum. Scaling up of anchorage-dependent cells as a monolayer culture presents a major challenge during process development. Also, viral inoculation and harvesting of virus particles associated with the cell at the end of the production cycle could further complicate operation of a process with attached cells. The establishment of a suspension cell line is one of the choices to overcome these operational hurdles. In addition, removal of serum from the process reduces cost, increases product quality and consistency, and simplifies downstream processes.

In this report, we describe the adaptation of the A549 cell line into suspension culture by stepwise weaning from serum. The suspension-adapted A549 cells were continually propagated in an animal material-free medium. The production of the CRAV 01/PEME was demonstrated in small-scale research batches. Extended culturing of the A549 cells showed no apparent changes in growth or virus production, suggesting that the cell line is stable in suspension culture. Results from further process optimization and characterization of the A549 cells in suspension culture are also presented.

Materials and methods

Cell line and suspension adaptation

A549 cells were originally obtained from ATCC (CCL-185) and cultured in DMEM medium (Biowittaker, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT). The attached A549 culture was adapted to serum-free suspension by cultivating the cells in sequential 1:1 dilutions of the serum-containing medium with the serum-free medium. Serum-free medium (denoted as A549-4-06) was prepared by supplementing IS 293-V (Irvine Scientific, Santa Ana, CA) with 0.1% pluronic F-68 (GIBCO, Grand Island, NY), 15 mM Tris pH 7.4 (Cambrex, Rockland, ME), 1 × Trace Elements A, B and C (Mediatech, Herndon, VA), 1 × FeNaEDTA (Irvine Scientific), 1:1000 dilution of Chemically Defined Lipid Concentrate (GIBCO) and 8 mM L-glutamine (GIBCO). The resulting medium mixtures contained 5, 2.5, 1.25 and 0.6% FBS, respectively. Cells were allowed to adapt at each serum level for 2 to 3 passages. The attached culture with 0.6% FBS was trypsinized and transferred into an Erlenmeyer flask with vent cap (Corning, Corning, NY). Serum weaning was then resumed in shake flasks until the growth rate stabilized. Shake flasks were agitated at 110 rpm on an orbital shaker (Model 4530, Forma). All cell cultures were maintained in a 37°C tissue culture incubator supplied with 5% CO₂. Cell banks were prepared and stored in liquid nitrogen with freezing media (80% IS 293-V, 10% DMSO (Sigma, St. Louis, MO) and 10% FBS).

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 were obtained by a YSI 2700 Select

Biochemistry Analyzer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH).

CRAV (01/PEME) production in shake flasks

A549 suspension cells were typically allowed to grow to approximately $1.8\text{--}2.0 \times 10^6$ cell ml^{-1} prior to infection in A549-4-06 in batch mode. Before virus inoculation, a medium exchange of approximately 90% of the original culture volume was performed with fresh medium by centrifugation at 850 rpm for 10 min (Model Allegra 6R, Beckman, Palo Alto, CA). Virus was inoculated at a final concentration of 1×10^8 virus particles (vp) ml^{-1} . That is equivalent to approximately a 50:1 ratio of virus particles to cells. Immediately after infection, the agitation rate of the shaker was lowered to 105 rpm from 110 rpm. Approximately 20 h post infection, another 90% medium change with fresh medium was performed by centrifugation. Three ml of culture samples were collected at 24, 48 and 72 h post infection for the quantification of virus produced.

CRAV (01/PEME) production in bioreactors

The scale-up development of virus production was initiated in Braun Biostat 2 l 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 inch pitch blade impeller (B. Braun Biotech). The culture temperature was maintained at 37°C with a heating blanket (B. Braun Biotech). 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 in A549-4-06 medium. The total working volume was 2 l. The agitation rate was maintained at 120 rpm during the subsequent cell growth and virus production. Perfusions with fresh A549-4-06 were performed during growth phase. Virus was inoculated at a final concentration of 1×10^8 vp ml^{-1} . Approximately 20 h post virus inoculation, another perfusion with 2 l of A549-4-06 medium was conducted. The pH was kept above 6.8 during the entire culture period with 1 M Na_2CO_3 solution (Sigma).

Sample preparation and analysis of viral production

Collected culture samples were subjected to three freeze/thaw cycles in a dry ice – ethanol bath and a 37°C water bath, followed by centrifugation at 3000 rpm for 20 min in a bench-top centrifuge (Model GS-6R, Beckman). The supernatant was then treated with 50 units ml^{-1} Benzonase (EM Industries, 1016979 M) 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).

Immunofluorescent studies

For examination of infectivity, adherent A549 cells in T-25 culture flasks were infected with a replication-deficient adenovirus expressing GFP at 1 or 4×10^8 vp ml^{-1} . At 24, 48 and 72 h post-infection, cells were trypsinized and suspended in FACS buffer (1× PBS, 5% FBS, pH 7.2) for analysis. For characterization of CAR expression, cells were trypsinized, washed with FACS buffer, and sequentially incubated with a mouse monoclonal antibody directed against human CAR (a generous gift from Dr. Richard Finberg (Dana-Farber Institute) and Canji (San Diego); Hsu et al. 1988), and a fluorescein-conjugated anti-mouse antibody (Molecular Probes, Eugene, OR). All samples were examined by FACS using a FACS Calibur flow cytometer (Becton Dickinson).

Results

Adaptation of A549 cells to serum-free suspension culture

The original A549 cell line from ATCC was first propagated in adherent culture using DMEM supplemented with 10% FBS. When the cells were detached from surface and transferred into a shake flask containing this medium, large cell aggregates can be observed within a few hours after the seeding. Within a few passages, the culture can not be further propagated due to extensive cell clumping (Fig. 1a). Similarly, when the detached A549 cells were transferred directly to suspension culture using the med-

Fig. 1 A549 cells in suspension culture. **(a)** Non-adapted A549 cells propagated in DMEM with 10% FBS; **(b)** Non-adapted A549 cells propagated in serum-free A549-4-06 medium; **(c)** Suspension adapted A549 cells in serum-free

A549-4-06 medium. Top panel: Microphotograph of A549 cells in suspension culture. Bottom panel: Analysis of A549 cell aggregation in suspension culture by the measurement of particle size

ium A549-4-06, cell clumps were again observed (Fig. 1b), although a peak representing single cells can also be detected (Fig. 1b, bottom). However, the degree of cell aggregation is still too extensive to allow proper cell growth during continuous passaging or efficient virus infection. This suggests that medium optimization alone is not sufficient for culturing A549 cells as a suspension culture.

We then took a different approach by selecting A549 that grew as single cells in A549-4-06. As described in Materials and Methods, serum was eliminated from the A549 culture by serial passaging of the cells in medium mixtures of A549-4-06 and DMEM (10% FBS) with step-wise decreasing amounts of serum. Starting with a 1:1 ratio, the volume ratio of A549-4-06 to DMEM (10% FBS) was doubled at each steps of serum weaning. These cells were eventually transferred into shake flasks when serum concentration reached $\sim 0.6\%$ and were then continually passaged until sustained growth in a serum-free environment ($<0.01\%$ FBS) was achieved. During the entire course of adaptation, large cell clumps were removed by allowing them to settle down to the bottom of the shake flask. The resulting A549 suspension culture consisted of

mostly single cells or small clumps with high viability (Fig. 1c).

Production of 01/PEME in suspension adapted A549 cells

Production of a CRAV, 01/PEME, from A549 cells in suspension culture was first evaluated in shake flasks. Previous results with E1-deleted recombinant adenoviral vectors in HEK293 cells indicated that medium exchanges (or feeds with fresh medium) at or during virus infection are essential for virus production (Garnier et al. 1994; Cote et al. 1998; Tsao et al. 2001). We therefore evaluated the effect of different medium exchange procedures on 01/PEME production. A549 suspension cells were first allowed to reach 2.0×10^6 cell ml^{-1} in batch mode. The subsequent virus infection following growth phase were performed using four parallel procedures. First, the culture was maintained as batch culture; no medium exchange or fresh medium feed was performed. Second, a medium exchange was performed immediately prior to virus inoculation. Third, a medium exchange was performed approximately 20 h post viral inoculation. Fourth, two medium exchange

operations were performed, one immediately prior to inoculation and one ~20 h after inoculation. Cell culture samples were collected on a daily basis post infection for the measurement of virus particles produced. Data in Fig. 2 showed that both medium exchanges, pre- and post-infection, were required for the maximum volumetric productivity of 01/PEME in suspension A549 cells.

We then evaluated the effect of virus concentration on 01/PEME production. Efficiency of infection is one of the possible limiting steps during the early stages of virus infectious cycle. Ideally, one would like to achieve synchronized infection of all production cells with the lowest amount of virus inoculum possible. In this experiment, A549 cells were cultured as described in Fig. 2. Two medium exchanges were performed for virus infection, one immediately before infection and one ~20 h post infection. The cultures were inoculated with increasing amounts of 01/PEME, from 0.125 to 8×10^8 vp ml⁻¹ with a step-wise increment of 2 fold. Virus titer was assayed on a daily basis post infection. Results shown in Fig. 3

Fig. 2 Effect of medium exchanges on virus production. Suspension-adapted A549 cells were cultured in serum-free A549-4-06 medium and inoculated with CRAV 01/PEME. Conditioned culture medium were removed and replenished with fresh medium immediately prior to and/or 1 day after virus inoculation as indicated. Culture samples were assayed daily for measurement of virus production

indicate a dose-dependent increase in volumetric virus production with inoculation concentration up to approximately 1×10^8 vp ml⁻¹. However, no significant differences were seen in virus titer when the viral inoculum concentration was greater than 1×10^8 vp ml⁻¹. Subsequent infection experiments with 01/PEME were, therefore, carried out with inoculation concentrations at 1×10^8 vp ml⁻¹.

Effect of calcium supplementation on 01/PEME production in A549 suspension culture

The main obstacles during adaptation and propagation of adherent cells in a suspension culture are the control of cell aggregation and the prevention of attachment to the vessel surface. Many cell surface molecules involved in cell–cell or cell–matrix interactions are calcium-dependent. To facilitate the adaptation and maintenance of cells in suspension culture, calcium concentration in a medium designed for suspension culture is often dramatically reduced compared to standard culture media for adherent cultures. However since calcium is critical for cell survival, the calcium concentration in suspension media usually represents a balance between control of cell aggregation/adhesion and optimized cell growth.

Although we demonstrated that the low calcium level (~108 μM) provided by A549-4-06 supports A549 cell growth with minimal cell clumping, it was unclear whether virus production is negatively affected by the relatively lower calcium concentration (Tsao et al. 2001). To demonstrate the effect of calcium on 01/PEME production, increasing concentrations of calcium chloride were supplemented to the culture medium 2 h post infection. Results from this experiment showed a dose-response in virus production to the additional calcium (Fig. 4). Volumetric production could be increased by approximately 30% with the addition of 1 mM or more of calcium chloride, with titer levels reaching $80\text{--}100 \times 10^9$ vp ml⁻¹. Time-course experiments with calcium chloride indicates that the effect on productivity is relatively insensitive to the timing of addition, since similar dose-response curves were obtained from 1 to 6 h post virus infection (data not shown). So, in order to maximize virus production by suspension cells, calcium levels must be increased.

Fig. 3 Effect of virus concentration at inoculation on virus production. A549 cells grown in serum-free suspension culture were inoculated with CRAV 01/PEME at the concentrations indicated. Cultures were sampled daily for the measurement of

virus production. (a) Volumetric virus concentration plotted against elapsed time post infection. (b) Volumetric virus production plotted against virus concentration at inoculation

Fig. 4 Effect of calcium addition post infection on CRAV 01/PEME production by A549 cells. A549 cells grown in serum-free suspension culture were inoculated with CRAV 01/PEME at the concentration of 1×10^8 vp ml $^{-1}$. Calcium chloride was added to the culture 2 h post infection at the final concentrations indicated. Virus production on a volumetric or per cell basis was measured at day two post infection

Production of 01/PEME in 2 l stirred tank bioreactors

Difficulties in controlling and monitoring a shake flask in a tissue culture incubator limit the full exploration of the production potential of A549 cells. To address this issue, we initiated process development in 2 l stirred tank bioreactors by simulating the baseline process established in shake flasks. In the experiment described in Fig. 5, two bioreactor tanks were seeded with suspension A549 cells at two

different seeding densities, 0.5 or 1.0×10^6 cells ml $^{-1}$, respectively. Perfusions with fresh A549-4-06 medium were performed with both tanks. Tank A seeded with 1.0×10^6 cells ml $^{-1}$ were perfused twice during growth, while Tank B seeded with 0.5×10^6 cells ml $^{-1}$ were perfused only once. Perfusion operations were performed on a continuous basis with one tank volume, i.e. 2 l of fresh A549-4-06. Immediately prior to viral inoculation, another perfusion was performed on both bioreactors. This was followed by another perfusion one day after infection. Volumetric virus titer was measured post infection and was in the range of 100 – 150×10^9 vp ml $^{-1}$ (Fig. 5). It is important to point out that although the maximum cell density reached with Tank A is consistently higher than that of Tank B, the volumetric yield is lower in Tank A. The apparent lower specific productivity on a per cell basis suggests that other factors may become limiting in Tank A compared to Tank B.

Stability of A549 cells adapted into suspension culture

A production host cell line must possess two characteristics with respect to stability in order to ensure a scalable process. First, the cell culture should demonstrate robust growth over extended passage. Second, there should not be a significant drop in productivity during the same period of time.

Fig. 5 CRAV 01/PEME production by suspension-adapted A549 cells in stirred tank bioreactors. A549 cells were cultured in serum-free suspension medium A549-4-06 in 2 l stirred tank bioreactors. Perfusions with fresh medium were performed periodically during growth, at the infection and 1 day post infection. Tank A and Tank B differ in the number of perfusions during growth. Both tanks were inoculated with 1×10^8 vp ml⁻¹ of 01/PEME and supplemented with 800 mM of calcium chloride post infection

In most cases of genetically engineered cell lines, genetic stability can be sustained by the use of selection agents and the degree of stability can be

determined by monitoring molecular and cellular markers. The stability of a naturally occurring cell line such as A549, however, could be less predictable and more difficult to control. To evaluate the stability of the suspension-adapted A549 cells, we monitored changes in their growth rate and virus production (Fig. 6). Our data indicate that the A549 cells are ready to grow immediately after thawing, as shown in the first data point of the growth curve. This translates to about 40% cell growth per day (specific growth rate ~ 0.014 h⁻¹). This initial growth was followed by a gradual increase until reaching an apparent plateau at approximately generation 60. The increase in growth rate with passing is common among many cell lines when the culture is initiated from a cryogenically preserved condition.

While the culture above was continuously cultured for determining growth rate, satellite cultures were split off and infected with the 01/PEME for evaluation of virus production. The maximum virus titer (typically in the range of $100\text{--}150 \times 10^9$ vp ml⁻¹) was achieved approximately 48 h post infection in all cases. Virus productivity, both volumetric and specific productivity on a per cell basis (Fig. 6) indicates that A549 productivity in suspension culture can be maintained for more than 90 generations of cell divisions.

Fig. 6 Stability of A549 cells in serum-free suspension culture. A549 cells were passaged in serum-free suspension culture continuously. At the indicated culture age, satellite production batches were initiated. (a) Average specific growth rate was calculated from the cell mass increase during the

initial growth phase of the batch. (b) Volumetric productivity was obtained from samples of day 2 post virus inoculation; specific productivity was derived from the day 2 volumetric production divided by peak cell density reached at day 1 post infection

Molecular characterization of suspension-adapted A549 cell for adenovirus production

Productive infection of a host cell by an animal virus requires a series of highly organized virus–host interactions. The mechanisms governing the balance of the interactions are sensitive to environmental changes. Cells grown in suspension experience numerous mechanical stresses not present in static adherent cultures; therefore, adaptation to suspension growth may alter the pathways leading to virus infection and replication. To address this concern, experiments were performed to characterize infection of suspension A549 cells.

Infection efficiency upon virus inoculation could conceivably be a rate-limiting step during virus infection. One sensitive and convenient way to monitor the infection kinetics is the utilization of an indicator virus carrying an easily detectable marker. We chose a replication defective (E1-minus) adenoviral vector carrying green fluorescent protein (GFP) to track the infected cell population (Wills et al. 1994). The lack of virus replication ensures that secondary infection does not take place as a result of virus release from the primary infection, allowing us to determine the infection efficiencies at the time of virus inoculation. Using this reporter virus, we observed no significant difference between the parental adherent cells and the suspension adapted cells with respect to the percent of cells infected,

suggesting that the infection efficiency of the A549 cells is not significantly altered (Fig. 7).

To further characterize the infectivity of suspension cells, we examined the cell surface expression of the coxsackievirus and adenovirus receptor (CAR). CAR is the key molecule that mediates the attachment of adenovirus to host cells (Bergelson et al. 1997; Cohen et al. 2001; Meier and Greber 2004; Zhang and Bergelson 2005). The level of CAR expression can be affected by growth signals in the culture environment (Vincent et al. 2004). FACS analysis showed that expression of CAR on the surface of suspension-adapted A549 cells is comparable to those seen prior to adaptation (Fig. 8). This further supports the finding in Fig. 7 that mechanisms involved in early infection events were maintained during suspension-adaptation of A549 cells.

We have observed that human cell lines cultured in serum-free suspension culture tend to produce fewer adenoviral particles on a per cell basis than adherent cells (unpublished data). It was not clear, however, whether this is due to the genetic changes of the cells or to the differences in culture environment. To answer this question, we infected suspension-adapted cells in stationary culture dishes using DMEM containing 10%FBS, the medium used for attached culture. These cells showed no reduction in virus production in comparison with pre-adapted control A549 cells on a per cell basis (Fig. 9). This indicates that the apparent decrease in virus production is

Fig. 7 Effect of suspension-adaptation on infection efficiency. Suspension-adapted A549 cells were allowed to grow as adherent cultures in DMEM with 10% FBS for approximately

24 h. The culture was then infected with a replication-deficient adenovirus expressing GFP at 1 or 4×10^8 vp ml⁻¹. FACS analysis was performed for GFP-positive cells post infection

Fig. 8 Cell surface expression of coxsackievirus-adenovirus receptor (CAR). Non-adapted A549 cells cultured in DMEM with 10% FBS and A549 cells adapted in serum-free suspension culture were examined for cell surface expression of CAR. The suspension-adapted A549 cells were either maintained in serum-free suspension culture or allowed to grow as an adherent culture in DMEM with 10% FBS for approximately 24 h. Cells were first treated with primary antibodies followed by FITC-conjugated secondary antibody for FACS analysis. The mean fluorescence was calculated for the entire cell population screened

likely not due to molecular or genetic changes induced by adaptation into suspension. Further experiments will need to be performed to determine if the suspension environment plays a role.

Discussion

Suspension cell culture is often the choice for the production of biologics at large scale (Hu and Aunins 1997; Iyer et al. 1999; Chu and Robinson 2001). Propagation of production host cells in an anchorage-independent manner is relatively simple during scaling up. Production in suspension culture also simplifies the downstream process and offers the potential to reduce production costs. More importantly, culturing cells in suspension mode provides an opportunity to reduce or eliminate animal-derived components and so will improve the safety profile of the final product. However, most production cell lines were originally derived from cells grown in adherent cultures. Adapting these adherent cells to suspension

Fig. 9 Effect of suspension-adaptation on CRAV production. Suspension-adapted A549 cells were allowed to grow as an adherent culture in DMEM with 10% FBS for approximately 24 h before infection with CRAV 01/PEME. Cultures were harvested daily for virus production. To calculate virus productivity on a per cell basis, the maximum virus concentration reached at day 2 p.i and day 3 p.i., respectively and peak cell population reached at day 1 p.i. were used

culture without serum supplements can in some cases be difficult. Previously, it was shown that A549 cells can be adapted to serum-free and growth factor-free conditions and be maintained for an extended period of time (Siegfried et al. 1994). However, this experiment was carried out as a stationary culture instead of as a suspension culture. Long term propagation of A549 cells in suspension culture with mechanical agitation has not been reported. Recently, an A549 derived cell line was successfully adapted to serum-free suspension culture for the production of adeno-associated viral vectors (Farson et al. 2004). However, the cell culture medium used in this report contains human serum albumin.

One of the major challenges in culturing originally adherent cells in suspension culture is to control cell aggregation. A549 cells prior to suspension-adaptation tend to form large clumps in DMEM medium or medium designed for the suspension culture of other human cell lines (Fig. 1a, b). To overcome this problem, we took the approach of gradually weaning the serum from the culture medium. In addition, during the course of serum weaning, the degree of cell aggregation was monitored and large clumps

were removed from the cultures. The combination of gradual adaptation and population selection resulted in the full adaptation of the adherent A549 cells into suspension culture (Fig. 1c).

For the production of 01/PEME, a CRAV, a baseline process was first developed in shake flasks. Medium perfusion seems to be essential for virus production (Fig. 2). It is not completely understood why medium exchange is required to achieve high productivity. We did not observe depletion of amino acids, glucose or glutamine and supplementation with amino acids and other nutrients alone cannot substitute for medium exchange. There is no sign of significant accumulation of lactate or ammonia (Ferreira et al. 2005). Volumetric productivity reached a plateau when virus was inoculated at and higher than 1×10^8 vp ml⁻¹ (Fig. 3). Supplementation of calcium to the medium post infection increases the virus production in a dose-dependent manner (Fig. 4). The baseline line process was repeated and virus production was confirmed in stirred tank bioreactors (Fig. 5). To confirm the long term stability of the suspension-adapted A549 cells to allow scaleable production, the growth rate and productivity of the CRAV were measured at different in vitro cell ages (Fig. 6). Our data indicates that both growth and virus production are maintained during the course of extended passaging.

Maintaining proper virus–host interactions in serum-free suspension culture is one of the keys for the optimization of a virus production process. We therefore compared attached and suspension-adapted A549 cells for different properties related to infection. Using a recombinant adenoviral vector expressing GFP, we have shown that the infection efficiency was comparable with the two cell populations, suggesting that the molecular events involved in the early stage of adenovirus infection were not significantly altered as the result of the suspension adaptation. In particular, CAR showed no significant changes in cell surface expression. Finally, the production potential measured on a per cell basis was not negatively impacted by the process of suspension adaptation (Fig. 9). All evidence suggests that although the suspension adaptation may have resulted in subpopulation selection with respect to some properties, such as the degree of cell clumping, the cellular mechanisms involved in adenovirus replication were maintained.

In conclusion, we have successfully adapted the human lung carcinoma cell line A549 to growth in serum-free and animal material-free suspension culture. A baseline process for the production of CRAV was developed in shake flasks and then confirmed in stirred tank bioreactors. Although the robustness and stability of the cell line were shown to be sufficient for a scalable process, the requirement for medium perfusion could present a major challenge during large scale operation. A better understanding of virus–host interactions and further characterization of A549 physiology in suspension should lead to the development of a fed-batch process at manufacturing scale.

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References

- Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW (1997) Isolation of a common receptor for Cocksackie B viruses and adenoviruses 2 and 5. *Science* 275:1320–1323
- Chu L, Robinson DK (2001) Industrial choices for protein production by large-scale cell culture. *Curr Opin Biotechnol* 12:180–187
- Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM (2001) The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci USA* 98:15191–15196
- Cote J, Garnier A, Massie B, Kamen A (1998) Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol Bioeng* 59:567–575
- Dobbelstein M (2004) Replicating adenoviruses in cancer therapy. *Curr Top Microbiol Immunol* 273:291–334
- Farson D, Harding TC, Tao L, Liu J, Powell S, Vimal V, Yendluri S, Koprivnikar K, Ho K, Twitty C, Husak P, Lin A, Snyder RO, Donahue BA (2004) Development and characterization of a cell line for large-scale, serum-free production of recombinant adeno-associated viral vectors. *J Gene Med* 6:1369–1381
- Ferreira TB, Ferreira AL, Carrondo MJT and Alves PM (2005) Effect of re-feed strategies and non-ammonogenic medium on adenovirus production at high cell densities. *J Biotechnol* 119(3):272–280
- Garnier A, Cote J, Nadeau I, Kamen A, Massie B (1994) Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytotechnology* 15:145–155
- Hsu KH, Lonberg-Holm K, Alstein B, Cromwell RL (1988). A monoclonal antibody specific for the cellular receptor for the group B coxsackieviruses. *J Virol* 62:1647–1652

- Hu WS, Aunins JG (1997) Large-scale mammalian cell culture. *Curr Opin Biotechnol* 8:148–153
- Iyer P, Ostrove JM, Vacante D (1999) Comparison of manufacturing techniques for adenovirus production. *Cytotechnology* 30:169–172
- Katayose D, Seth P (1999) Chapter 10: development of adenoviral vectors for gene therapy. In: Seth P (ed) *Adenoviruses: basic biology to gene therapy*. R.G. Landers Company, Austin, TX, pp 91–101
- McConnell MJ, Imperiale MJ (2004) Biology of adenovirus and its use as a vector for gene therapy. *Human Gene Therapy* 15:1022–1033
- Meier O, Greber UF (2004) Adenovirus endocytosis. *J Gene Med Suppl* 1:S152–S163
- Nettelbeck DM (2003) Virotherapeutics: conditionally replicative adenoviruses for viral oncolysis. *Anticancer Drugs* 14:577–584
- Ramachandra M, Rahman A, Zou A, Vaillancourt M, Howe JA, Antelman D, Sugarman B, Demers GW, Engler H, Johnson D, Shabram P (2001) Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy. *Nat Biotechnol* 19:1035–1041
- Siegfried JM, Han YH, DeMichele MA, Hunt JD, Gaither AL, Cuttitta F (1994) Production of gastrin-releasing peptide by a non-small cell lung carcinoma cell line adapted to serum-free and growth factor-free conditions. *J Biol Chem* 269:8596–8603
- Tsao YS, Condon RG, Schaefer EJ, Lindsay DA, Liu Z (2000) Biomass and aggregation analysis of human embryonic kidney 293 suspension cell cultures by particle size measurement. *Biotechnol Prog* 16:809–814
- Tsao YS, Condon RG, Schaefer EJ, Lio P, Liu Z (2001) Development and improvement of a serum-free suspension process for the production of recombinant adenoviral vectors using HEK293 cells. *Cytotechnology* 37:189–198
- Vincent T, Pettersson RF, Crystal RG, Leopold PL (2004) Cytokine-mediated downregulation of coxsackievirus-adenovirus receptor in endothelial cells. *J Virol* 78:8047–8058
- Wildner O (2003) Comparison of replication-selective, oncolytic viruses for the treatment of human cancers. *Curr Opin Mol Ther* 5:351–361
- Wills KN, Maneval DC, Menzel P, Harris MP, Sutjipto S, Vaillancourt MT, Huang WM, Johnson DE, Anderson SC, Wen SF, Bookstein R, Shepard HM, Gregory RJ (1994) Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. *Hum Gene Ther* 5:1079–1088
- Zhang Y, Bergelson JM (2005) Adenovirus receptors. *J Virol* 79:12125–12131