Adenovirus vector production using low-multiplicity infection of 293 cells

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Abstract The use of low-multiplicity infection of 293 cells in static culture with regular medium replacement was investigated for efficient large-scale production of adenovirus vectors for gene therapy applications. An adenovirus vector carrying the enhanced green fluorescent protein gene (Ad EGFP) was used to infect 293-F cells at a low multiplicity of infection (MOI) of 0.00001–0.1 transductional unit (TU) cell⁻¹. The cells, which have the ability to grow in suspension, were incubated in T-flasks and the serum-free culture medium was replaced with fresh medium via centrifugation every 2 days. Because only a small proportion of cells were initially infected at low MOIs (<1 TU cell⁻¹), uninfected cells continued to grow until they were infected by progeny adenoviruses released from previously infected cells. When 293-F cells at a relatively low density of 1×10^5 cells cm⁻² were infected with Ad EGFP at a low MOI of 0.001 TU cell⁻¹, the vector yield was 2.7-fold higher than the maximum yield obtained with high-multiplicity infection (MOI = 10 TU cell⁻¹) in

batch culture. These results indicate that efficient adenovirus vector production using low MOIs is achieved by minimization of either nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium.

Keywords Adenovirus vector · 293 cell · Viral vector production · Multiplicity of infection (MOI) · Low-multiplicity infection · Medium replacement · Gene therapy

Introduction

Gene therapy is a promising treatment for acquired diseases, including cancer and cardiovascular diseases, as well as for inherited diseases. The therapy involves gene transfer into the patient's cells; subsequent expression *in vivo* is required to obtain a therapeutic effect. In the clinical trials conducted to date, adenovirus vectors have been used most extensively as gene-delivery vehicles (Gene Therapy Clinical Trials Worldwide 2009) because they transduce various cell types with high efficiency (Volpers and Kochanek 2004). While major efforts have been directed towards development of a new generation of adenovirus vectors and gene therapy protocols (St George 2003; Volpers and Kochanek 2004), efficient large-scale production of clinical-grade adenovirus vectors is required to meet the increasing demand for

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adenovirus vectors in gene therapy applications (Kamen and Henry 2004; Nadeau and Kamen 2003).

Conventional, replication-deficient, first-generation adenovirus vectors are based on substitution of their essential E1 genes by the therapeutic gene to be delivered to the target cells. The production of replication-deficient, E1-deleted adenovirus vectors is achieved by infecting a complementary cell line, such as the human embryonic kidney 293 cell line (Graham et al. 1977), which constitutively expresses E1 genes to support the replication of E1-deficient adenoviral particles. The major disadvantage of this system, however, is that continuous vector production is virtually impossible because of the lytic nature of the viral infection process. Therefore, batch culture of 293 cells is commonly used for production of adenovirus vectors.

Adenovirus vector production in batch culture involves two steps: (1) growth of 293 cells to a high cell density; and (2) replication of the adenovirus vector to high titers in 293 cells after addition of a stock solution of the virus. The production of adenovirus vectors is affected by a number of factors, including both the multiplicity of infection (MOI) and the cell density at the time of infection. At a high MOI (>5 infectious viral particles per cell), essentially all cells will be infected upon addition of the virus stock solution, and the resulting infection will be synchronous. By contrast, at a low MOI (<1) only a small fraction of cells are infected initially (primary infection), and the uninfected cells will continue to grow until they are infected with progeny adenoviruses released from previously infected cells (secondary and tertiary infections). Adenovirus vector titer reportedly increases with increasing MOI up to approximately $\text{MOI} = 5$ and remains constant at $\text{MOI} > 5$ (Park et al. 2004; Wu et al. 2002). Hence, high-multiplicity infection has been the conventional method employed. However, there is a practical reason to use very low MOIs for large-scale production of adenovirus vectors, as a large quantity of the virus stock solution is required to ensure synchronous infection. Moreover, infection at a high cell density reduces the specific productivity, and, consequently, the volumetric productivity of the adenovirus vector. The decrease in productivity associated with infection at high cell density is called the “cell density effect” (Kamen and Henry 2004; Nadeau and Kamen 2003). It is thought to result from either nutrient

depletion and/or accumulation of inhibitory metabolites in the culture medium.

In the present study, the effects of the MOI and initial cell density on adenovirus vector production were investigated. First, optimal conditions were determined for high-multiplicity infection ($\text{MOI} \geq 1$) in serum-free static cultures of 293-F cells. The use of low MOIs (<1) was then examined in cultures with medium replacement every 2 days. When 293-F cells at a relatively low cell density were infected at low MOIs, they exhibited a significantly high vector yield, which was 2.4–2.7 times higher than the maximum yield obtained using high-multiplicity infection.

Materials and methods

Cells and media

The 293-F cell line (Invitrogen, Carlsbad, CA, USA), which was derived from the human embryonic kidney 293 cell line (Graham et al. 1977), was used for adenovirus vector production. The cells were adapted to a serum-free medium (293 SFM II, Invitrogen) that supports cell growth in suspension. The cells were maintained in 293 SFM II supplemented with 4 mM L-glutamine in T-flasks incubated statically in a humidified CO_2 incubator (37°C , 5% CO_2). Cell density was determined by counting the number of cells with a Bürker-Türk hemocytometer under a microscope; cell viability was determined using the trypan blue exclusion method.

On the other hand, the 293 cell line (Graham et al. 1977) (ATCC CRL-1573) was used for adenovirus titration. The adherent cells were maintained in a manner similar to that described above, except Dulbecco's modified Eagle medium (Invitrogen) supplemented with $1.8 \text{ g L}^{-1} \text{ NaHCO}_3$, 15 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid [HEPES], $10^5 \text{ units L}^{-1}$ penicillin, 0.1 g L^{-1} streptomycin, and 10% fetal bovine serum (FBS) (BioWest, Nuaille, France) was used for routine maintenance.

Adenovirus vector

A replication-deficient, recombinant adenovirus vector was constructed using the AdEasy vector system (He et al. 1998) (Qbiogene, Carlsbad, CA, USA). In

brief, the DNA encoding the enhanced green fluorescent protein (EGFP) gene was cloned into the transfer vector, pShuttle-CMV, such that the EGFP gene was under control of the cytomegalovirus (CMV) promoter. The resulting transfer vector was co-transformed into *Escherichia coli* BJ5183 cells together with the plasmid pAdEasy-1 containing the adenovirus serotype 5 (Ad5) genome with deletions in the E1 and E3 regions. Upon homologous recombination between the transfer vector and pAdEasy-1 in the *E. coli* cells, a recombinant plasmid was generated with the expression cassette inserted into the E1 region of the adenovirus genome. The recombinant plasmid was then transfected into 293 cells, resulting in the generation of the recombinant adenovirus, Ad EGFP, expressing the EGFP gene under the CMV promoter. Viral stocks propagated in 293 cells were purified using CsCl density-gradient centrifugation and were stored at -80°C .

Adenovirus vector production by 293-F cells infected at high MOIs

293-F cells in the exponential growth phase were suspended in fresh 293 SFM II and the resulting suspension was used to inoculate either 6-well plates or T-flasks. A viral stock solution was added to give an MOI of 1–20 transductional units (TU) cell^{-1} . The cells were then cultivated at 37°C in a CO_2 incubator. At the appropriate times, the infected cell suspension was removed from the 6-well plate or T-flask and stored at -80°C until adenovirus titration. Otherwise, cells and cell-free supernatants were separated via centrifugation and stored at -80°C until adenovirus titration.

Adenovirus vector production using low-multiplicity infection

Exponentially grown 293-F cells were suspended in fresh 293 SFM II and then seeded in T-flasks. A viral stock solution was added in amounts corresponding to MOIs of 0.00001, 0.001, or 0.1 TU cell^{-1} , and the cells were then cultivated at 37°C in a CO_2 incubator. Every 2 days, the cell suspension was centrifuged, and the resulting cell pellet was resuspended in fresh 293 SFM II, and cultivation in the CO_2 incubator was resumed. The cell-free supernatant was stored at -80°C for adenovirus titration.

Adenovirus titer determination

A biological assay was employed to measure the titer of adenovirus vector in each sample, taking into account both virus penetration and transgene expression. 293 cells (ATCC CRL-1573) were suspended in fresh Dulbecco's modified Eagle medium supplemented with 5% FBS and seeded in 6-well plates. The frozen cell-suspension and the cells frozen with fresh 293 SFM II were subjected to three freeze-thaw cycles to release the Ad EGFP from the cells, while the frozen culture supernatants were thawed once. A serial dilution of the samples in serum-free Dulbecco's modified Eagle medium was added to each well of the 6-well plates, and the plates were incubated at 37°C in a CO_2 incubator. After 24 h of incubation, the 293 cells were harvested in Dulbecco's phosphate-buffered saline. The number of fluorescent cells and total cells were determined using a flow cytometer equipped with an argon laser with excitation at 488 nm (FACSCalibur, Becton, Dickinson and Company, Sparks, MD, USA). EGFP fluorescence emission was collected using a 530/30 band-pass filter. The 24-h infection period allowed for entry of the adenovirus into the 293 cells and for expression of EGFP; however, no secondary infection occurred within 24 h post-infection. If a Poisson distribution is assumed, most of the fluorescent cells contain only one infectious virus particle when less than 50% of the total cell population is infected (Côté et al. 1997). In the present study, the viral titers were therefore calculated only for wells containing 5–30% fluorescent cells as previously described (Côté et al. 1997). The specific productivity was given by the ratio of the adenovirus yield (total Ad EGFP produced in a well or flask) to the number of viable 293-F cells in the well or flask at the time of viral stock solution addition.

Results and discussion

Adenovirus vector production at high MOIs

High-multiplicity infection of 293 cells is the conventional method by which replication-deficient adenovirus vectors are produced. In the case of infection at a high MOI (>5 TU cell^{-1}), essentially all cells are infected immediately after addition of the

Fig. 1 Effect of multiplicity of infection (MOI) on specific productivity of adenovirus vector. 293-F cells at a density of 5×10^5 cells cm^{-3} were infected with Ad EGFP at a high MOI of 1–20 transductional units (TU) cell^{-1} in 6-well plates and the specific productivity was measured 48 h post-infection. Bars represent the means of two independent experiments

virus stock solution. In the present study, high-multiplicity infection (≥ 1 TU cell^{-1}) of 293-F cells was examined first for comparison with low-multiplicity infection. Figure 1 shows the specific Ad EGFP productivity of 293-F cells infected at an MOI of 1–20 TU cell^{-1} in 6-well plates. The specific productivity increased with MOI up to 10 TU cell^{-1} and thereafter reached a plateau at approximately 2,500 TU cell^{-1} . This trend is nearly consistent with previous observations (Park et al. 2004; Wu et al. 2002).

Fig. 2 Effect of initial cell density on adenovirus vector production by 293-F cells infected at an MOI of 10 TU cell^{-1} in T75-flasks. **a** Intracellular (striped bars) and extracellular (blank bars) specific productivity 48 h after infection. **b**

Next, the effects of initial cell density on Ad EGFP production were investigated at an MOI of 10 TU cell^{-1} in T75-flasks (Fig. 2). Ad EGFP production by 293-F cells was markedly affected by initial cell density. Nearly the same specific Ad EGFP productivity was obtained at initial cell densities ranging from 1 to 5×10^5 cells cm^{-3} , but at higher initial cell densities the specific productivity decreased significantly (Fig. 2a). As a result, the optimal Ad EGFP yield (total Ad EGFP produced in the flask) of 2.4×10^{10} TU was attained at an initial cell density of 5×10^5 cells cm^{-3} (Fig. 2b). This characteristic decrease in both specific and volumetric productivity, which is often referred to as the “cell density effect” (Kamen and Henry 2004; Nadeau and Kamen 2003), results from either nutrient depletion and/or accumulation of inhibitory metabolites, including lactate and ammonium ion, in the culture medium. Figure 2a also shows the intracellular and extracellular specific Ad EGFP productivity measured in 293-F cells and cell-free culture supernatant separated by centrifugation, respectively. Even 48 h after infection, significant amounts of Ad EGFP were detected in the culture supernatant.

In the case of high-multiplicity infection, the optimal Ad EGFP yield in static culture was obtained when 293-F cells at 5×10^5 cells cm^{-3} were infected at an MOI of 10 TU cell^{-1} . Under these optimal conditions, the time courses of both viable cell density and Ad EGFP production were evaluated (Fig. 3). When the cells were infected at an MOI of

Adenovirus vector yield (total Ad EGFP produced in the flask) 48 h after infection. Bars represent the means $\pm$ SD of 4 different determinations from two independent experiments

Fig. 3 Adenovirus vector production by 293-F cells infected at an MOI of 10 TU cell^{-1} in static T-flask cultures. The time courses of viable cell density (a) and intracellular ($\square$), extracellular ($\triangle$), and total ($\blacklozenge$) adenovirus yield (b) are presented

10 TU cell^{-1} , the density of viable 293-F cells appeared to increase by approximately 30% 1 day post-infection (Fig. 3a). This result is not surprising because cells in the S phase will double once more after infection with adenovirus (Nadeau and Kamen 2003). The viable cell density began to decrease approximately 2 days post-infection. Expression of the EGFP gene was first detected in the cells approximately 1 day post-infection (Fig. 3b). The intracellular Ad EGFP yield reached its maximum approximately 2 days post-infection, and then decreased. Ad EGFP was also detected in the culture medium 28 h post-infection. Thereafter, the extracellular Ad EGFP yield increased continuously. The time-dependent increase in the extracellular Ad EGFP yield most likely resulted from the loss of membrane integrity due to the lytic nature of the adenovirus

infection process. Adenovirus vector production was almost complete 3 days post-infection, and a total yield of approximately $4.7 \times 10^{10} \text{ TU}$ was achieved.

Adenovirus vector production by low-multiplicity infection

For recombinant protein production by the baculovirus–insect cell system, the use of MOIs as low as $0.0001 \text{ plaque-forming units (pfu) cell}^{-1}$ has been investigated (Wong et al. 1996). When insect cells were infected at a suitable cell density in batch culture, low-multiplicity infections gave a high recombinant protein yield that was comparable to the optimal yield obtained using a high MOI. In the present study, low-multiplicity infection was examined for adenovirus vector production. In the case of infection at a low MOI ($<1 \text{ TU cell}^{-1}$), only a small proportion of cells are initially infected upon addition of the virus stock solution. Uninfected cells will be infected after initially infected cells begin to release progeny viruses. Therefore, for efficient adenovirus vector production by low-multiplicity infection, longer culture times are required for all cells to be infected and virus replication to be completed. In the present study, the culture medium was replaced every 2 days after 293-F cells at different initial cell densities were infected at low MOIs of 0.1, 0.001, and $0.00001 \text{ TU cell}^{-1}$ to minimize both nutrient depletion and accumulation of inhibitory metabolites in the culture medium in a long-term culture.

At all MOIs and initial cell densities examined, significant growth of 293-F cells was observed during days 2–6 post-infection, and a maximal cell density of approximately $2 \times 10^6 \text{ cells cm}^{-3}$ was attained with an MOI of $0.00001 \text{ TU cell}^{-1}$ (Fig. 4a, c, e). In static batch culture, uninfected 293-F cells also reached the stationary phase with a cell density of around $2 \times 10^6 \text{ cells cm}^{-3}$. At an MOI of 0.1 TU cell^{-1} the Ad EGFP yield apparently began to increase after 2 days, but the distinct increase in the Ad EGFP yield was delayed with decreasing MOI (Fig. 4b, d, f). When the initial cell density was $5 \times 10^5 \text{ cells cm}^{-3}$, the higher the maximum cell density attained, the lower the Ad EGFP yield (Fig. 4a, b). A similar result was also obtained at an initial cell density of $3 \times 10^5 \text{ cells cm}^{-3}$ (Fig. 4c, d). These results clearly demonstrate the so-called cell density effect. Adenovirus vector production appeared to be limited by

Fig. 4 Adenovirus vector production using low-multiplicity infection. 293-F cells were infected at an MOI of 0.1 (○), 0.001 (□), or 0.00001 (△) TU cell⁻¹. The initial cell densities were 5×10^5 (a, b), 3×10^5 (c, d), or 1×10^5 cells cm⁻³

(e, f), and the culture medium was replaced every 2 days. The cumulative yield of Ad EGFP in the recovered culture supernatant is presented and each plotted value is the mean $\pm$ SD of 3–6 different determinations (b, d, f)

either nutrient depletion and/or toxic metabolite accumulation, even though the culture medium was replaced every 2 days. However, when the cells did not grow beyond a cell density of approximately 1×10^6 cells cm⁻³, relatively high yields of Ad EGFP were obtained. An optimal Ad EGFP yield of 1.2×10^{11} TU was achieved when cells at an initial density of 3×10^5 cells cm⁻³ were infected at an MOI of 0.1 TU cell⁻¹ (Fig. 4d). An Ad EGFP yield of 1.3×10^{11} TU was also attained with an initial density of 1×10^5 cells cm⁻³ and an MOI of 0.001 TU cell⁻¹ (Fig. 4f). These results indicate that cells should be infected at a relatively low cell density when using low-multiplicity infection to avoid nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium.

Comparison of Ad EGFP production by 293-F cells infected at high and low MOIs is shown in Table 1. Under the optimal conditions for high-multiplicity infection (initial cell density, 5×10^5 cells cm⁻³; MOI, 10 TU cell⁻¹), an Ad EGFP yield of 4.7×10^{10} TU (vector concentration, 3.1×10^9 TU ml⁻¹) was achieved in batch culture (Fig. 3). On the other hand, when using low-multiplicity infection with medium replacement (MOI = 0.1 and 0.001 TU cell⁻¹), yields were increased nearly threefold at initial cell densities of 3×10^5 and 1×10^5 cells cm⁻³ (Table 1). These cultures required shorter pre-culture times to reach the initial cell density, but post-infection times were increased approximately threefold. In addition, the volume of culture medium required was increased more than threefold, but the

Table 1 Comparison of adenovirus vector production using either high- or low-multiplicity infections

MOI (TU cell ⁻¹)	Initial cell density (cells cm ⁻³)	Vector yield (TU)	Time post-infection (day)	Vector used (TU)	Medium used (ml)
10	5×10^5	4.7×10^{10}	3	7.5×10^7	15
0.1	5×10^5	6.8×10^{10}	6	7.5×10^5	45
0.1	3×10^5	1.2×10^{11}	8	4.5×10^5	60
0.001	1×10^5	1.3×10^{11}	10	1.5×10^3	75

vector concentration in the recovered culture supernatant was sufficiently high 6 and 8 days post-infection ($1.4\text{--}4.9 \times 10^9 \text{ TU ml}^{-1}$) (Fig. 4d, f). Therefore, the results obtained in the present study indicate that efficient adenovirus vector production using low MOIs can be achieved by choosing an appropriate combination of initial cell density and timing of medium replacement. It is noteworthy that the volume of the virus stock solution used for the low-multiplicity infection was less than one-hundredth that required for synchronous infection at an MOI of 10 TU cell^{-1} .

The nutritional capacity of a culture medium to sustain recombinant protein production without the limitation due to either nutrient depletion and/or accumulation of inhibitory metabolites is a useful parameter for optimization of the culture conditions of the baculovirus–insect cell system (Yamaji et al. 1999). In the present study, the nutritional capacity of the medium for adenovirus vector production can be estimated as the time integral of the viable cell density from post-infection time zero to the post-infection time at which the maximum Ad EGFP yield was attained under the optimal conditions for high-multiplicity infection in batch culture. The time integral of the viable cell density, $I(t_1, t_2)$ (cells day cm^{-3}), from time t_1 (day) to time t_2 (day) is given as follows:

$$I(t_1, t_2) = \int_{t_1}^{t_2} x \, dt$$

where x is the density of viable cells in the culture medium (cells cm^{-3}). The integration can be performed by summation of the areas under the time profiles of the viable cell density between two adjacent time points using the trapezoid rule. In the culture shown in Fig. 3, the nutritional capacity, $I(0, 3)$, was estimated to be $1.7 \times 10^6 \text{ cells day cm}^{-3}$. The maximum time integral of the viable cell density for 2 days between medium replacements was also calculated for each culture shown in Fig. 4 and the Ad EGFP yield was plotted against the maximum time integral of the viable cell density in Fig. 5. When the maximum time integral of the viable cell density exceeded the nutritional capacity of $1.7 \times 10^6 \text{ cells day cm}^{-3}$, the Ad EGFP yield significantly decreased. Therefore, nutritional capacity might be a useful parameter for determination of the

Fig. 5 Effect of the maximum time integral of viable cell density on adenovirus vector yield using low-multiplicity infection. The time integral of the viable cell density for 2 days between medium replacements was calculated for each culture shown in Fig. 4, and Ad EGFP yield is plotted against the maximum value. The initial cell densities were 5×10^5 (●), 3×10^5 (■), and $1 \times 10^5 \text{ cells cm}^{-3}$ (▲). The dashed line represents the nutritional capacity of the culture medium that was determined for the high-multiplicity infection shown in Fig. 3

optimal combination of initial cell density, MOI, and timing of medium replacement to avoid nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium, although a more accurate and critical value of the nutritional capacity remains to be determined.

When a low MOI was used, medium replacement effectively prevented the so-called cell density effect and resulted in efficient adenovirus vector production. Medium replacement requires the separation of medium from cells, which may be difficult in large-scale cultures of suspension-grown cells. In recent years, perfusion cultures of 293 cells using either an acoustic filter (Henry et al. 2004) or a tangential flow filtration device (Cortin et al. 2004) have been investigated for adenovirus vector production. Cell-medium separation could be easily achieved by cell immobilization using porous support particles, as has been demonstrated in cultures of both mouse myeloma and insect cells (Yamaji and Fukuda 1997; Yamaji et al. 2000, 2006). Application of this immobilization technique to adenovirus vector production by 293-F cells will be described elsewhere. Development of

scalable adenovirus purification technologies from culture broth, including chromatography (Kamen and Henry 2004), is also needed for large-scale production of adenovirus vectors.

Conclusion

In the present study, we investigated the use of low-multiplicity infection for adenovirus vector production. To minimize nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium, the culture medium was replaced every 2 days. When 293-F cells at relatively low densities of 3×10^5 and 1×10^5 cells cm^{-3} were infected at MOIs of 0.1 and 0.001 TU cell $^{-1}$, respectively, significantly high yields, which were 2.4–2.7 times higher than the maximum yield obtained with high-multiplicity infection in batch culture, were achieved. These results indicate that efficient adenovirus vector production with reduced virus stock utilization is possible when using an optimal combination of initial cell density, MOI, and timing of medium replacement, which prevents nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium.

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