Immobilization of 293 cells using porous support particles for adenovirus vector production

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Abstract Adenovirus vector production by anchorage-independent 293 cells immobilized using porous biomass support particles (BSPs) was investigated in static and shake-flask cultures for efficient large-scale production of adenovirus vectors for gene therapy applications. The density of cells immobilized within BSPs was evaluated by measuring their WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reduction activity. In shake-flask culture, 293-F cells, which were adapted to serum-free suspension culture, were not successfully retained within reticulated polyvinyl formal (PVF) resin BSPs (2 × 2 × 2 mm cubes) with matrices of relatively small pores (pore diameter 60 μm). When the BSPs were coated with a cationic polymer polyethyleneimine, a high cell density of more than 10⁷ cells cm⁻³-BSP was achieved in both static and shake-flask cultures with regular replacement of the culture medium. After infection with an adenovirus vector carrying the enhanced green fluorescent protein gene (Ad EGFP), the specific Ad

EGFP productivity of the immobilized cells was comparable to the maximal productivity of non-immobilized 293-F cells by maintaining favorable conditions in the culture environment.

Keywords Adenovirus vector · 293 cell · Viral vector production · Immobilization · Biomass support particles · Polyvinyl formal resin · Polyethyleneimine · Gene therapy

Introduction

Gene therapy is the use of genes as medicine and involves the transfer of therapeutic genes into the patient's cells to achieve a therapeutic effect. Expression of the transferred genes could result in the synthesis of therapeutic proteins and potentially the treatment for inherited and acquired diseases. While viral and physicochemical vehicles have been employed in the delivery of therapeutic genes to target cells, recombinant adenovirus vectors are used in the majority of gene therapy clinical trials today (Gene Therapy Clinical Trials Worldwide 2009). Adenovirus vectors offer many advantages over other virus vectors: they can infect a broad range of cells including quiescent, differentiated cells; they can transduce cells efficiently in vivo; the viral DNA does not integrate into the host cell genome; and, they can be produced in high titers (Volpers and Kochanek

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2004; Vorburger and Hunt 2002). In order to meet the growing demand for adenovirus vectors for preclinical and clinical trials, the development of efficient large-scale processes for the production of clinical-grade adenovirus vectors is desirable (Kamen and Henry 2004; Nadeau and Kamen 2003; Warnock et al. 2006).

Most adenovirus vectors have been rendered replication-deficient by substitution of their essential E1 genes with the therapeutic gene to be delivered to the target cells. Replication-deficient adenovirus vectors can be produced by infecting a complementing cell line, such as the human embryonic kidney 293 cell line (Graham et al. 1977), which constitutively expresses the E1 genes, to support the replication of E1-deficient adenoviral particles. Because of the lytic nature of the viral infection process, a batch culture of 293 cells is commonly used for the production of adenovirus vectors. When infection is performed at high cell densities in a batch culture, however, adenovirus vector production is often limited by either nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium (Kamen and Henry 2004; Nadeau and Kamen 2003). To address this problem, therefore, a fed-batch, or perfusion, culture has been studied as an attractive alternative to a batch culture (Cortin et al. 2004; Ferreira et al. 2005; Henry et al. 2004; Lee et al. 2003). A perfusion culture is preferable because it can prevent nutrient depletion as well as accumulation of toxic metabolites in the culture medium. However, culture systems require the separation of the medium from the cells, which has been achieved by the use of special filtration devices such as an acoustic filter (Henry et al. 2004) and a hollow fiber tangential flow filtration device (Cortin et al. 2004). The cell-medium separation can be more simply and easily achieved using an immobilization technique. In previous studies, we applied the “passive” immobilization technique using porous biomass support particles (BSPs) to anchorage-independent cells, which include mouse myeloma cells (Yamaji and Fukuda 1992, 1997) and Sf9 insect cells (Yamaji et al. 2000, 2006). The advantages of this immobilization technique are its simplicity and convenience. Passive immobilization can be achieved in such a way that physically entrapped cells in BSPs form aggregates within the matrices of the support material as a consequence of their growth during culture.

In the present study, the application of the immobilization technique using BSPs to adenovirus vector production by anchorage-independent 293 cells was investigated in static and shake-flask cultures. When reticulated polyvinyl formal (PVF) resin BSPs were coated with a cationic polymer polyethyleneimine, 293-F cells that were adapted to serum-free suspension culture were successfully immobilized within the BSPs at a cell density of more than 10^7 cells cm^{-3} -BSP. After infection with a recombinant adenovirus vector, cells immobilized within the BSPs showed a high specific productivity by replacing the culture medium at appropriate intervals.

Materials and methods

Cells, media, and adenovirus vector

Two kinds of human embryonic kidney 293 cell lines were used in the present study. The 293-F cell line (Invitrogen, Carlsbad, CA, USA), adapted to a serum-free medium 293 SFM II (Invitrogen) that supports cell growth in suspension, was used for adenovirus vector production. 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, while 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. This anchorage-dependent cell line was maintained in a manner similar to that described above, except for the use of Dulbecco's modified Eagle medium (Invitrogen) supplemented with 1.8 g L^{-1} NaHCO_3 , 15 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 10^5 units L^{-1} penicillin, 0.1 g L^{-1} streptomycin, and 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA).

A replication-deficient adenovirus vector containing the enhanced green fluorescent protein (EGFP) gene under the control of the cytomegalovirus (CMV) promoter, Ad EGFP (Yamada et al. 2009), was used.

Cell immobilization and culture

A reticulated PVF resin sponge sheet (Aion, Osaka; mean pore diameter 60 μm ; porosity 0.88; apparent density 0.15 g cm^{-3}) was cut into $2 \times 2 \times 2$ mm cubes for use as BSPs. Two hundred BSPs were sterilized by autoclaving with purified water in 100 mL screw-capped Erlenmeyer flasks for 20 min at 121 °C. After removal of the water, the BSPs were coated with polyethyleneimine (MW 70,000; Wako Pure Chemical Industries, Osaka, Japan) in the following method. Polyethyleneimine was dissolved at a concentration of 0.1 g L^{-1} in purified water and the resulting solution was sterilized by filtration through a 0.22 μm membrane filter. A 15 mL portion of the solution was then decanted into the Erlenmeyer flask and incubated with the BSPs for 4 h at 37 °C on a reciprocal shaker (90 oscillations min^{-1} ; amplitude 25 mm).

The 293-F cells in the exponential growth phase were collected by centrifugation and resuspended in fresh medium. After washing the BSPs with fresh medium and addition of 15 mL of the cell suspension to each Erlenmeyer flask, the cells and the BSPs were incubated for 2 days on a reciprocal shaker (90 oscillations min^{-1} ; amplitude 25 mm) in a non-humidified CO_2 incubator (37 °C, 5% CO_2) for inoculation. Following removal of the cell suspension, cells entrapped within the BSPs were cultivated using two different methods. In the first method, 15 mL of fresh medium was added to the Erlenmeyer flask and incubation on a reciprocal shaker resumed. In the second culture method, the BSPs were transferred into a T75-flask, 15 mL of fresh medium was added, and cells in the BSPs were then cultivated statically in a non-humidified CO_2 incubator (37 °C, 5% CO_2). In both methods, the culture medium was replaced completely with 15 mL of fresh medium every 2 days. At the time of medium replacement, 1–5 BSPs were sampled from each flask for assessment of the density of viable cells entrapped within them.

Ad EGFP production by immobilized 293-F cells

After growth of immobilized cells to a density of more than 10^7 cells cm^{-3} -BSP, the number of BSPs was adjusted to 100 in each of the Erlenmeyer and T-flasks. Following medium removal, 7 mL of viral

stock solution diluted with fresh medium was added to each Erlenmeyer and T-flask to give a multiplicity of infection (MOI) of 10 transductional units (TU) cell^{-1} . The time of viral solution addition was designated as post-infection time zero. For the shake-flask culture, immobilized cells were incubated with the viral solution on a reciprocal shaker (90 oscillations min^{-1} ; amplitude 25 mm) for 1 h; for the static culture, T-flasks were manually shaken slowly at appropriate intervals for 1 h. Eight milliliter of fresh medium was then added to each flask, and the shake-flask and static cultures were resumed, with the culture medium being replaced completely with 15 mL of fresh medium every 2 days. At the time of each medium replacement, one Erlenmeyer or T-flask was sampled, and the culture broth was removed and stored at -80 °C until adenovirus titration while the BSPs were stored frozen with 5 mL of Dulbecco's phosphate buffered saline (PBS) at -80 °C.

Evaluation of immobilized cell density

The density of viable cells entrapped within BSPs was evaluated by measuring WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reduction activity using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). WST-8 is a water-soluble tetrazolium salt that produces a water-soluble formazan upon reduction by living cells in the presence of an electron carrier (Ishiyama et al. 1997; Tominaga et al. 1999). For measurement of the WST-8 reduction activity of non-immobilized 293-F cells, 1 mL of cell suspension was incubated with 0.1 mL of WST-8 solution, which was supplied with the kit, in a six-well plate in a humidified CO_2 incubator (37 °C, 5% CO_2) for 2 h. After addition of 0.1 mL of 1% (w/v) sodium dodecyl sulfate (SDS) solution, the preparation was centrifuged. The absorbance of WST-8 formazan in the supernatant was measured with a spectrophotometer using a test wavelength of 450 nm and a reference wavelength of 650 nm, and the difference between the two absorbances ($A_{450} - A_{650}$) was calculated.

For measurement of the WST-8 reduction activity of immobilized cells, fresh medium was added to 1–5 BSPs in a microtube to give a total volume (BSPs + medium) of 1 mL, which was then incubated with 0.1 mL of the WST-8 solution in a

humidified CO₂ incubator for 2 h. Following addition of 0.1 mL of 1% (w/v) SDS solution and centrifugation, the absorbance of the supernatant was measured as described above.

Adenovirus titer determination

The titer of adenovirus vector in each sample was measured by a biological assay (Côté et al. 1997; Yamada et al. 2009). Anchorage-dependent 293 cells were inoculated in six-well plates with fresh Dulbecco's modified Eagle medium supplemented with 5% FBS. The frozen culture broth and the BSPs frozen with PBS were subjected to three freeze–thaw cycles to release the Ad EGFP from the cells. A serial dilution of the samples in serum-free Dulbecco's modified Eagle medium was added to each well of the six-well plates, and the plates were incubated in a humidified CO₂ incubator (37 °C, 5% CO₂). After 24 h of incubation, the 293 cells were harvested in PBS. The number of fluorescent cells and total cells were determined using a flow cytometer (FACSCalibur, Becton, Dickinson and Company, Sparks, MD, USA). In the present study, the viral titers were calculated only for wells containing 5–30% fluorescent cells, as previously described (Côté et al. 1997; Yamada et al. 2009).

Results and discussion

WST-8 reduction activity of 293-F cells

It is clearly important to quantitatively assess the growth of immobilized cells in an immobilized cell culture. In immobilized cell culture systems using porous BSPs, however, direct measurement of viable cell density by counting unstained cells under a microscope using a dye exclusion method is not applicable. In previous studies, the growth of mouse myeloma cells and Sf9 insect cells immobilized within reticulated PVF resin BSPs has been monitored indirectly by a colorimetric assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Yamaji and Fukuda 1992; Yamaji et al. 2000). MTT assay is based on the capacity of mitochondrial enzymes of viable cells to reduce MTT to water-insoluble MTT formazan, of which absorbance is measured after dissolution in an organic solvent such as 2-propanol. The use of a different

Fig. 1 Relationship between the number of viable non-immobilized 293-F cells in the exponential growth phase in static cultures and difference in absorbance at 450 nm and at 650 nm ($A_{450} - A_{650}$) in the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) reduction assay

tetrazolium salt, WST-8, allows a convenient assay without a solubilization step, because WST-8 produces a water-soluble formazan dye (Ishiyama et al. 1997; Tominaga et al. 1999). In the present study, the application of a colorimetric assay using WST-8 to 293-F cells immobilized within reticulated PVF resin BSPs was examined.

Figure 1 shows the relationship between the number of viable non-immobilized 293-F cells in the exponential growth phase in static cultures and production of WST-8 formazan. The absorbance of WST-8 formazan increased linearly with the number of cells, indicating that the specific activity of WST-8 reduction by non-immobilized 293-F cells in the exponential growth phase is almost constant. In the present study, the density of 293-F cells immobilized within BSPs was, therefore, estimated from the absorbance of WST-8 formazan produced by immobilized cells, based on the linear correlation determined with non-immobilized cells (Fig. 1).

Cell immobilization and growth

After 293-F cells were shaken for 2 days with reticulated PVF resin BSPs with and without polyethyleneimine treatment, the cells inoculated into the BSPs were incubated either statically in T-flasks or in Erlenmeyer flasks on a reciprocal shaker, and the

Fig. 2 Growth of 293-F cells immobilized within reticulated polyvinyl formal (PVF) resin biomass support particles (BSPs) without (a) and with (b) polyethyleneimine treatment in static

(circle) and shake-flask (filled rectangle) cultures. Cell density in the untreated BSPs in shake-flask culture is not shown

culture medium was completely replaced every 2 days. The time course of changes in the density of viable cells entrapped within the BSPs is shown in Fig. 2. When the BSPs without polyethyleneimine treatment were used, an initial density of 293-F cells physically entrapped in the BSPs was around 1×10^5 cells cm^{-3} -BSP (Fig. 2a). Although the BSPs comprised of reticulated PVF resin matrices with relatively small pores (mean pore diameter 60 μm) were used, the density of cells retained within the untreated BSPs did not increase in the shake-flask culture (data not shown in Fig. 2a). By contrast, entrapped cells grew exponentially in the initial period of static T-flask culture, and the immobilized cell density reached 2×10^7 cells cm^{-3} -BSP on the 12th day (Fig. 2a), which was almost 10-fold higher than the maximum cell density obtained in a static culture of non-immobilized 293-F cells.

When the reticulated PVF resin BSPs were coated with polyethyleneimine, a relatively higher initial cell density was obtained and the immobilized cell density reached 2×10^7 cells cm^{-3} -BSP on day 8 in the static culture (Fig. 2b). Interestingly, a comparably high density of cells entrapped within polyethyleneimine-treated BSPs was achieved on the 14th day in the shake-flask culture, though the apparent growth rate of entrapped cells was faster in the static culture than in the shake-flask culture. These results clearly show that the treatment of reticulated PVF resin surface with polyethyleneimine is effective in promoting the immobilization of 293-F

cells within BSPs. In mammalian cell culture, coating the plastic culture surface with a positively charged polymer such as poly-lysine and polyethyleneimine has been employed to facilitate the attachment and spreading of many types of anchorage-dependent cells (McKeehan and Ham 1976; Rüegg and Hefti 1984; Vancha et al. 2004). Reportedly a macroporous cellulose carrier treated with polyethyleneimine and stabilized by cross-linking has been successfully used for high-density culture of hybridoma cells (Ong et al. 1994) and recombinant L-929 cells (Terashima et al. 1994). In reticulated PVF resin BSPs, *Escherichia coli* cells can be efficiently immobilized by simply coating their surface with positively charged polymers such as poly-L-lysine and polyethyleneimine (Huang et al. 2007). It appears that anchorage-independent 293-F cells can be efficiently immobilized in reticulated PVF resin BSPs by electrostatic interaction between the negatively charged ions on the cell surface and the positively charged polyethyleneimine adsorbed onto the BSP surface. After the immobilized cells reached the stationary phase, viable 293-F cells leaked from the BSPs were barely observable in the culture medium.

Adenovirus vector production by immobilized cells

After immobilized cell density was more than 1×10^7 cells cm^{-3} -BSP, the 293-F cells immobilized within polyethyleneimine-coated BSPs were infected with Ad EGFP at an MOI of 10 TU cell⁻¹,

Fig. 3 Adenovirus vector production by 293-F cells immobilized within polyethyleneimine-treated BSPs in static (**a**) and shake-flask (**b**) cultures. Immobilized cells at an initial density of 2.1×10^7 cells cm^{-3} -BSP in static culture and 1.1×10^7 cells cm^{-3} -BSP in shake-flask culture were respectively infected with Ad EGFP at a multiplicity of infection (MOI)

of 10 transductional units (TU) cell^{-1} . The culture medium was replaced completely with 15 mL of fresh medium every 2 days. The time courses of Ad EGFP yield in the BSPs (*open rectangle*), cumulative vector yield in the culture broth (*open triangle*), and total yield (*filled diamond*) are presented. Bars represent the means $\pm$ SD of 3–5 different determinations

and adenovirus vector production by the immobilized cells was investigated in static and shake-flask cultures. In the immobilized cell cultures, the culture medium was replaced completely with fresh medium every 2 days to minimize nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium. Figure 3 shows the time course of Ad EGFP production by immobilized 293-F cells. The Ad EGFP yield in BSP-immobilized cells reached its maximum 2 days post-infection in both static and shake-flask cultures, and then decreased. In contrast, the cumulative Ad EGFP yield in the culture broth increased continuously in both cultures. Similar profiles of intracellular and extracellular Ad EGFP yield were also observed with non-immobilized 293-F cells in static culture (Yamada et al. 2009). The time-dependent increase in the Ad EGFP yield in the culture broth most likely resulted from the loss of membrane integrity due to the lytic nature of the adenovirus infection process. The total Ad EGFP yield (total Ad EGFP produced in the flask) reached a plateau approximately 4 days post-infection. Adenovirus vector production by the immobilized cells may be delayed even by using high multiplicity infection because cells in an inner part of the BSPs would have remained initially uninfected and then been infected with progeny adenoviruses released from initially infected cells in an outer shell of the BSPs.

A total Ad EGFP yield of approximately 4.0×10^{10} TU was achieved 6 days post-infection in shake-flask culture, while the yield was 3.0×10^{10} TU on day 4 in static culture (Fig. 3). The vector yield in the static culture remained only three-fourths that of the shake-flask culture, whereas the initial density of immobilized cells was 1.9-fold higher in the static culture than it was in the shake-flask culture. In addition, the specific productivity on post-infection day 2 was calculated to be approximately 4,300 TU cell^{-1} in the shake flask culture, but only 1,900 TU cell^{-1} in the static culture. The decrease in specific productivity, and thereby in the vector yield, in the static culture most likely corresponded to the so-called “cell density effect” (Kamen and Henry 2004; Nadeau and Kamen 2003), which results from either nutrient depletion and/or accumulation of inhibitory metabolites, including lactate and ammonium ion, in the culture medium. However, the specific productivity of immobilized cells in the shake-flask culture was roughly comparable to the maximal specific productivity obtained with non-immobilized 293-F cells (Yamada et al. 2009). The above results indicate that even in an immobilized cell culture with a cell density as high as 1×10^7 cells cm^{-3} -BSP, the efficient production of adenovirus vectors with a high specific productivity can be achieved by replacing the culture medium at appropriate intervals to minimize either

nutrient depletion and/or accumulation of inhibitory metabolites in the culture medium. For large-scale production of adenovirus vectors, further studies are needed concerning the use of low-multiplicity infection of 293 cells (Yamada et al. 2009) in immobilized cell culture and the development of a suitable bioreactor system for BSP-immobilized cells.

Conclusion

Anchorage-independent 293-F cells can be immobilized at a high density within reticulated PVF resin BSPs by simply coating the particle surface with polyethyleneimine. The passive immobilization facilitates the separation of the cells from the culture medium. When the culture medium was replaced with fresh medium at appropriate intervals, 293-F cells immobilized within the BSPs at a density of around 1×10^7 cells cm^{-3} -BSP showed a specific productivity comparable to the maximal productivity obtained with non-immobilized cells. These results indicate that efficient adenovirus vector production in immobilized 293 cell culture is possible by maintaining favorable conditions in the culture environment. Since the immobilization technique described herein should be readily available for scale-up to bioreactor systems, it offers a novel approach for the efficient large-scale production of adenovirus vectors.

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