Justification of continuous packed-bed reactor for retroviral vector production from amphotropic Ψ CRIP murine producer cell

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Abstract

To identify a plausible large-scale production system for retroviral vector, three culture systems, i.e., batch culture with medium exchange, microcarrier culture, and packed-bed reactor culture were compared. In batch cultures with medium exchange, high cell concentrations were maintained for about a month, and the harvested retroviral titer remained constant. In microcarrier cultures, although cell growth was rapid, the retroviral titer was unexpectedly low, suggesting that the low titer was due either to serious damage to the retroviral vector or to a reduction in the production rate of retroviral vector, caused by mechanical shear forces. Although the retroviral titer (maximum titer, 1.56×10^6) in the packed-bed reactor was a little bit lower than that obtained in the batch culture with medium exchange (maximum titer, 1.91×10^6), continuous production made it possible to increase the cumulative titer up to 16-fold of that from the batch culture with medium exchange. Moreover, as the packed-bed reactor system requires less labor and shows excellent volumetric productivity in comparison to batch cultures with medium exchanges, it will be an appropriate production system for retroviral vector in large quantities.

Introduction

The theory behind gene therapy is rather simple. However, many hurdles exist to making it work in practice because of difficulty in delivering transgenes efficiently and obtaining sustained expression of these genes. Among various gene delivery systems, retroviral vectors are the most widely used vehicles for stable gene delivery and expression in the target cell (Miller, 1992), despite the limited application of the retrovirus to dividing cells. The wide use of retrovirus can be attributed to stable gene transfer into a broad range of host cells (Jolly, 1994). As the number of clinical trials has increased drastically since the first trial in 1990, a large amount of retroviral vectors is needed for gene therapy to be conducted on a large scale. To meet the burgeoning demand, the large-scale culture of producer cell is necessary. Although many studies have focused on the construction of high-producer cell

lines and tissue-specific viral vectors (Ory et al., 1996; Parente and Wolfe, 1996), studies on downstream processing, such as the production process, are scarce (Andreadis et al., 1999). Only limited information is available on the factors affecting retroviral production (Lee et al., 1996; Lee et al., 1998; Shen et al., 1996). Short half-lives of retroviral vectors at 37 °C, i.e., 2–8 hr (Lee et al., 1998; Shen et al., 1996; Le Doux et al., 1999), is one of the problems to consider for large-scale production. In addition, since the producer cells are anchorage-dependent, a high surface-to-volume ratio of the culture vessel is desirable for batch cultures as well as for continuous or semi-continuous cultures. For this reason, we are interested in comparing microcarrier culture systems, packed-bed reactor systems, and hollow fiber reactor systems.

The microcarrier culture system has been widely used because of a large surface area-to-volume ratio and the homogeneous environment (Varani et al.,

1983; Wiedell et al., 1984), although it exhibits shear stress as well as slow initial cell attachment rates at high agitation speeds (Croughan et al., 1987; Hu et al., 1985). On the other hand, the packed-bed reactor system has several advantages, such as yielding high cell densities and productivities of target protein, protection from mechanical shear, easy medium exchange and product separation, continuous removal of inhibitory metabolites and easy scale-up potential. Moreover, it is not impossible to overcome the limitations of the packed-bed reactor system, such as poor oxygen transfer, excessive biomass build-up, and difficulties in recovering biomass from the bed (Griffiths and Looby, 1991). Therefore, many researchers are attracted by this system for anchorage-dependent cells (Chiou et al., 1991; Looby and Griffiths, 1988, Park and Stephanopoulos, 1993). The Hollow fiber reactor system also allows us to obtain very high cell densities (Oh et al., 1994). One attractive feature of the hollow fiber reactor system is the easy set-up of simultaneous separation of the target product, especially when a serum-free medium is used. However, hollow fiber reactor systems are more frequently used for suspension cells such as hybridoma cells than for anchorage-dependent cells, largely because fiber walls are not conducive to cell attachment (Hu and Peshwa, 1991; Strand and Quarles, 1984). For this reason, the hollow fiber reactor system is excluded in this comparison.

In this paper we compared the three culture systems to determine which system is the most conducive to large-scale production of retroviral vectors. We show that the packed-bed reactor system is the most promising approach for continuous retroviral vector production.

Materials and methods

Cell lines and culture conditions

The retroviral vector MFG-lacZ and Ψ CRIP/MFG-lacZ producer cell have been described elsewhere (Dranoff et al., 1993; Ohashi et al., 1992). NIH3T3 cells were used to determine MFG-lacZ retroviral vector titers. Both Ψ CRIP/MFG-lacZ and NIH3T3 cells were cultured in Delbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated calf serum in a humidified incubator with 5% CO₂ in gas phase at 37 °C.

Batch culture with medium exchange

The producer cells ($1.0 \pm 0.1 \times 10^5$ cells) were inoculated into six-well culture plates by using 2 ml of DMEM containing 10% calf serum per well. After three days of cultivation, the culture temperature was reduced to 34 °C, and the media were exchanged over the culture period as follows: (1) daily total replacement of the medium with the fresh medium (2 ml day⁻¹); (2) daily one-half replacement of the medium with fresh medium (1 ml day⁻¹); and (3) one-half replacement of the medium with fresh medium every 12 hr (1 ml 0.5 day⁻¹). After trypsinization of the cells, the viable cell concentrations were determined using a hemocytometer by the trypan dye exclusion method (Kaltenbach et al., 1958).

Microcarrier culture

The producer cells were cultured on cytodex-3 beads in a spinner flask (Bellco, Vineland, NJ) in 100 ml of culture volume under 5% CO₂ humidified atmosphere at 37 °C. The concentration of the beads was 0.3 g 100 ml⁻¹, corresponding to 810 cm² of surface area. The agitation speed of the impeller in the spinner flask was 25~30 rpm. To prevent the microcarriers from sticking to the vessel walls, the spinner flask was siliconized prior to use. Microcarriers were hydrated and washed with Ca²⁺ and Mg²⁺ free phosphate buffered saline (PBS) and autoclaved before use. Sterile microcarriers were washed with the culture medium before they were transferred to the culture vessels. The producer cells ($1.0 \pm 0.1 \times 10^7$ cells) were inoculated into the culture medium containing cytodex-3 with 2 min, intermittent agitation (40 rpm) every 30 min. during the initial 6 hr. The agitation speed was then kept at 25~30 rpm. As for the batch culture with medium exchange, the medium was replaced with fresh medium after two days of cultivation. The medium exchange rate was one-half of the culture volume per day. The concentrations of attached cells were determined by the nuclei count method using a hemocytometer, after incubation of the samples for 1 hr at 37 °C with 0.1% (v/v) crystal violet solution in PBS containing 0.1M citric acid. The viable cell concentrations were determined taking into account the viable cells measured by trypan dye exclusion method after trypsinization of the cells.

Packed-bed reactor culture

A mini packed-bed reactor was designed for this work (Figure 1). All the vessels were connected with gas permeable silicon tubing (MR-96400, Cole-Parmer

Figure 1. Schematic diagram of the mini packed-bed reactor.

Instrument Co., Vernon Hills, IL, USA). Peristaltic pumps (Miniplus3, Gilson, Middleton, WI, USA) were used for medium transport. 0.7 g of polyester disks, corresponding to 810 cm² surface area (Fibra-Cel, New Brunswick Scientific Co., Edison, NJ, USA) were stacked in a glass column (I.D. 2.2 cm, height 3 cm) with a working volume of 10 ml. The bioreactor containing the Fibra-Cel (810 cm²) was autoclaved in PBS and incubated overnight with 100% serum before use. The cell suspension ($1.0 \pm 0.1 \times 10^7$ cells) from a 175 cm² T-flask was injected into the column. The cells gradually attached to the surface of the disks as the medium flows through the circulation loop. During the period of cell attachment, the flow rate was maintained at 70~80 ml h⁻¹. The cell attachment was evaluated by counting the cells from the effluent samples using a hemocytometer. After 3 hr of circulation, no cells were detected any more in the effluent. Then, the circulation loop was closed, and the medium feeding was started at 3 ml h⁻¹ of flow rate. The cell concentration could not be determined accurately because of incomplete detachment of

cells from the Fibra-Cel matrix. Although retrovirus titer still increased after three days' cultivation, the cell concentration determined by both trypan blue and nuclei count methods rarely exceeded the initial inoculum concentration. Therefore all the experimental data were compared by using retroviral titers.

Retroviral vector titration

NIH3T3 cells ($3 \pm 0.1 \times 10^4$ cells) were incubated in 24-well culture plates with 0.5 ml of the growth medium. After a cultivation of 24 h, the spent medium was replaced by retroviral vector supernatant serially diluted with fresh medium containing polybrene (8 µg ml⁻¹). After 16 hr incubation at 37 °C, the infection medium was replaced with fresh medium, and the cells were allowed to grow for another day. The confluent target cells were stained with X-gal staining solution as described previously (Saines et al., 1986). After a subsequent incubation of the culture plates at 37 °C for 8 to 12 h, the stained colonies were counted by using a microscope and the titers of retroviral vectors were

Figure 2. Effect of medium exchange methods on (a) cell growth, (b) retroviral vector titer, and (c) cumulative retroviral vector titers in batch cultures with medium exchange. All cultures were done in six-well plates. The closed circle, open circle and closed triangle represent 2 ml/24 h, 1 ml/24 h and 1 ml/12 h, respectively.

calculated as colony forming units (cfu) per milliliter of retroviral vectors culture supernatant used.

Results and discussion

Batch culture with medium exchange

By performing the three sets of the medium exchange experiments in six well plates, the effects of different medium exchange volumes (2 ml/day vs. 1

ml/day) and medium exchange frequencies (1 ml/day vs. 1 ml/0.5 day) on cell growth and retroviral vector production were examined. Figure 2a shows the growth curves of the producer cells. When the larger volumes of the medium were exchanged more frequently, higher cell concentrations were maintained. Surprisingly, the production rate of the retroviral vector was consistent for about one month (Figure 2b). Because the exchange rate of the medium was different in each run, retroviral vector production was compared via the cumulative titers as shown in Figure 2c. As with the cell growth (Figure 2a), more frequent medium exchange using larger volumes yielded higher titers, suggesting that higher cell densities are positively correlated with the obtaining of high retroviral vector titers. Depletion of nutrients or accumulation of inhibitory metabolites (Shen et al., 1996) usually causes the decline of cell concentration. By performing medium exchanges, inhibitory metabolites and limiting nutrients can be removed and supplied, respectively. Furthermore, a very short half-life of retroviral vector at 37 °C (Lee et al., 1998; Shen et al., 1996), i.e. 2–8 hr, rules out the possibility that the accumulation of the retroviral vectors in the media significantly affects the production rate of the new retrovirus, hence the retrovirus titer. In consequence, these results show that frequent medium exchanges help in maintaining high cell concentrations as well as high daily vector productivities, indicating the potential advantages of using continuous process for the retroviral vector production.

Microcarrier culture

A microcarrier culture is commonly used to achieve high production yields of various biologicals from anchorage-dependent mammalian cells. In comparison with batch cultures, microcarrier cultures offer certain advantages, such as yielding high cell densities (Thilly, 1986) and no limitations of mass transport between the cells and medium (Croughan et al., 1987). Microcarrier culture systems are also easy to scale up (Reuveny, 1990). Therefore, we attempted to compare the microcarrier system with the batch culture system with medium exchange operated under static conditions. Figure 3a shows the viable cell growth profile in the microcarrier system. Although the producer cells grow well, and high cell densities were obtained and could be maintained, the retroviral vector titer was about 10-fold lower in the microcarrier culture than that of the batch culture (Figure 3b). The low titer is at-

Figure 3. Comparison of (a) cell growth and (b) retroviral vector titers between the microcarrier culture and the static batch culture with medium exchange. In the both cases, after three days of cultivation, half of the culture volume was daily replaced with the fresh medium.

tributed to the reduction in the vector production itself and/or instability of the produced retroviral vector under the culture conditions used. Considering the good cell growth and the low glucose and glutamate consumption in the media (data not shown), the reduction in the vector production is less likely. However, from a practical point of view, it is not easy to verify the reduction in the vector production because the retroviral titer is determined by the dynamic interplay between the vector production and deactivation rates (Lee et al., 1998). Therefore, the possibility of the instability of the produced retroviral vector was examined. Because of collisions between viruses, producer cells, and microcarriers, and adsorption to microcarriers, impeller, spinner flask wall etc., the retroviral vector present in the media may degrade rapidly or lose its infectivity. It is possible that the agitation speed of 25~30 rpm may be high enough to lower the integrity of the retroviral vector. In order to investigate the agitation effect on retroviral stability, the following experiments were carried out. The harvested retroviral vector supernatants were (1) stored statically in a culture dish, (2) stirred in a spinner flask without

microcarrier at 25~30 rpm agitation, and (3) stirred in a spinner flask with microcarrier at 25~30 rpm agitation. The samples from these settings were collected and assayed.

Figure 4 clearly shows that an agitation rate 25–30 rpm had an adverse effect; in the case that the virus supernatant was stirred in the presence of microcarriers, the effective virus titer was almost 10% less than that without microcarriers. Although the mechanism for the retroviral deactivation is not yet well understood, the infectivity of retroviral vector appears to be sensitive to shear stress. This result suggests an important point in that although slight mixing during retrovirus infection is helpful to eliminate the diffusion limitation of retrovirus (Chuck and Palsson, 1996), even slight mixing is harmful for the retrovirus production. As a result, despite many intrinsic advantages of the microcarrier culture system, it would not be suitable for large-scale retroviral vector production. However, this result does not eliminate the feasibility of using microcarrier systems in a perfusion mode (Reuveny, 1990).

Packed-bed reactor culture

To overcome the disadvantage of the microcarrier system for retroviral vector production without losing good cell growth, the packed-bed reactor system is an easy choice and a high surface area can be achieved when appropriate packing material is selected. Operation of the packed-bed reactor system in a perfusion mode, which is in any case the recommended mode, can also overcome limitations in oxygen, nutrients, mechanical shear stress and build-up of toxic waste products.

To increase the surface area as well as biocompatibility, several packing materials were selected. Among them, Siran (Schott, Mainz, Germany) is an open-pore sintered glass bead, MIF-5 (EYELA, Tokyo, Japan) is a porous sponge square, and Fibra-Cel (New Brunswick Scientific Co., Edison, NJ, USA) is a round disk made of charged polyester fiber. When the cultures were started with the same inoculum size, Fibra-Cel showed the highest retroviral titer (Figure 5a). Therefore, all subsequent packed-bed reactor cultures were done and optimized using Fibra-Cel.

In order to identify the right inoculation method, three methods, i.e. static, intermittent, and continuous, were compared. As shown in Figure 5b, the continuous inoculation methods appeared to be best one in terms of retroviral titers, suggesting that the

Figure 4. Relative stability of retroviral vectors produced in spinner flasks. The initial retroviral titer was set to 100%.

Table 1. The comparison of three culture systems^a

	Batch culture with medium exchange ^b	Microcarrier culture	Packed-bed reactor culture ^c
maximum			
viable cell concentration (cell/ml)	4.79×10^5	6.9×10^5	not determined
maximum titer (cfu/ml)	1.91×10^6	1.56×10^5	1.56×10^6
cumulative titer (cfu)	6.29×10^7	2.03×10^7	1.03×10^9
volumetric productivity			
(cfu/day/reactor volume)	5.03×10^5	2.71×10^4	8.24×10^6
surface area/reactor volume (cm ² /ml)	2.32	4.22	32.4

^a All culture systems were operated for 5 days.

^b For the batch culture with medium exchange, the producer cells were plated at 6×10^5 cells (i.e. 10^4 cells/cm²) per 100 mm dish containing 10 ml of medium. After three days of cultivation, the medium was totally replaced with the fresh medium once a day.

^c Flow rate was 8 ml/h.

major factor to attach the cells to Fibra-Cels is not the time to attach, but the frequent collision of the cells with Fibra-Cel as the cells become trapped in the fiber system.

In animal cell cultures using continuous packed-bed reactor, common major operational parameters are dissolved oxygen levels, substrate (carbon or nitrogen source) concentration, pH and dilution rate. Since we have not detected any depletion of O₂, nor noticeable changes in pH, cell growth, nor depletion or low levels

of glucose and amino-acids in the media (data not shown), the only adjustable parameter significantly affecting retroviral titers and productivity was the flow rate of the medium. As the half-life of the retroviral vector is rather short, and 99% of the produced vector particles are inactivated within 24 h at 37 °C (Lee et al., 1998; Shen et al., 1996), an optimal flow rate for high retroviral yield and productivity should exist. In the case that the cell growth rate on Fibra-Cel is almost zero and specific retrovirus production rate is constant

Figure 5. Comparison of retroviral titers obtained by using different (a) support matrixes, (b) inoculum methods, and (c) flow rates. In (b), 'static' means that the inoculated cells were incubated with the support matrixes for 3 h. 'Intermittent' means that the inoculated cells were incubated with the support matrixes for 20 min and then circulated for 10 min, the process was repeated for 3 h. 'Continuous' means that the inoculated cells were circulated for 3 h, by changing the direction of the circulation every 20 min.

at steady state, the theoretical optimal flow rate can also be easily calculated. In order to determine the optimal flow rate, various flow rates were compared (Figure 5c). During 48 h cultivation, the flow rate was initially set to 3 ml/h and then was changed to 3

ml/h, 5ml/h, 8 ml/h, 12 ml/h, respectively. In the mini packed-bed reactor with 10 ml of working volume, the optimum flow rate was 8 ml/h, as shown in Figure 5c. This flow rate almost corresponds to the calculated theoretical optimum flow rate of ca. 10 ml/h (data not shown). This result suggests that the dilution rate of the packed-bed reactor is a very important parameter for maximizing the retroviral vector production. If this reactor is scaled-up, the neglected parameters such as pH, nutrient concentration and column dimension certainly become important parameters.

Conclusion

The maximum and cumulative titers and volumetric productivities of the three tested culture systems are compared in Table 1. In general, in spite of good cell growth and high titers, the batch culture with medium exchange showed poor volumetric productivity due to a low surface-to-volume ratio for the cell growth. Although maximal cell concentrations were achieved in the microcarrier culture, the unexpected low retrovirus titer makes the microcarrier culture unsuitable for the retroviral production. As low titers were also observed with other microcarriers, this looks like a general problem when using microcarriers (data not shown). The instability of the produced retroviral vector offsets the many advantages of microcarrier culture such as homogeneous environments and high surface-to-volume ratio. The packed-bed reactor culture yielded ca. one order of magnitude higher volumetric productivity than the batch culture with medium exchange. This result appears to be mainly caused by the high surface to volume ratio owing to the packed beads. The 14-fold increase of the surface to volume ratio was paralleled by a 16-fold increase in the volumetric productivity. An approximate linear relationship was obtained between the surface to volume ratio and the volumetric productivity. Moreover, the continuous product harvest resulted in the highest cumulative titer among the three systems. It is probable that a continuous supply of the fresh media and a concomitant removal of toxic wastes are likely to reduce the retrovirus deactivation. Further studies have to verify this.

Our results confirm that suspension culture systems are not appropriate for retroviral vector production, suggesting that the choice of the cell culture system is somewhat restricted. Hence, hollow fiber type membrane reactors and continuous packed-bed

reactors seem to be the best candidates for production systems. We prefer continuous packed-bed reactors to hollow fiber systems because hollow fiber type reactors are affected by the drawback that a uniform initial cell attachment in the case of anchorage-dependent cells is difficult to achieve. Our results clearly demonstrate that continuous packed-bed systems are superior to batch cultures with medium exchange (i.e., repeated batch culture). Consequently, more accurate and detailed investigation of identifying operating parameters of packed-bed culture system and their optimization is required for the retrovirus production.

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