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Continuous production process of retroviral vector for adoptive T- cell therapy

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Abstract

Adoptive T-Cell therapy is being considered as a promising method for cancer treatment. In this approach, patient's T cells are isolated, modified, expanded, and administered back to the patient. Modifications may include adding specific T cell receptors (TCR) or chimeric antigen receptors (CAR) to the isolated cells by using retroviral vectors. PG13 cells, derivatives of NIH3T3 mouse fibroblasts, are being used to stably produce retroviral vectors that transduce the T cells. PG13 cells are anchorage-dependent cells that grow in roller bottles or cell factories and lately also in fixed bed bioreactors to produce the needed viral vector. To scale up viral vector production, PG13 cells were propagated on microcarriers in a stirred tank bioreactor utilizing an alternating tangential flow perfusion system. Microcarriers are 10 μm – 0.5 mm beads that support the attachment of cells and are suspended in the bioreactor that provides controlled growth conditions. As a result, growth parameters, such as dissolved oxygen concentration, pH, and nutrients are monitored and continuously controlled. There were no detrimental effects on the specific viral vector titer or on the efficacy of the vector in transducing the T cells of several patients. Viral vector titer increased throughout the 11 days perfusion period, a total of 4.8×10^{11} transducing units (TU) were obtained with an average titer of 4.4×10^7 TU/mL and average specific productivity of 10.3 (TU) per cell, suggesting that this method can be an efficient way to produce large quantities of active vector suitable for clinical use.

Keywords

PG13 cells; Retroviral vector; perfusion; microcarrier; bioreactor; T Cell therapy

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Conflict of interest

The authors declare no financial or commercial conflict of interest

1 Introduction

Adoptive T Cell Therapy is a rapidly growing field that uses the patient's immune system to battle cancer cells [1]. The patient's own T cells are modified by genetic engineering to enhance their interaction with the cancer cells and improve their capability to attack them [2]. One of the approaches for T cell modification is to add tumor-specific T cell receptors (TCR) or chimeric antigen receptors (CAR) to the patient T cells by transducing cells collected from the patient with the retroviral vector [3]. Following the transduction, the modified cells are administrated back to the patient [4].

Retroviral vector can be produced in PG13 packaging cell line derived from NIH3T3 mouse cells stably expressing the Moloney murine leukemia virus gag-pol proteins and the Gibbon ape leukemia virus envelope protein [5]. These cells are stably transfected with gammaretroviral backbone encoding TCR or CAR for constitutive production of secreted retroviral vector. In 1994, von Kalle et al. published an article describing the use of PG13-derived retroviral vector for the transduction of CD34+ cell [6]. In 1995, Bunnell et al. described the use of PG13-derived retroviral vector for transduction of human peripheral blood lymphocytes [7]. In 1997, Bunnell et al. used PG13-derived vector to assess persistence of gene-marked cells in non-human primate model [8]. In 2005, Cornetta et al. published the National Gene Vector Laboratory's (Indiana University) collective PG13 vector production experience [9], and the first clinical data reported was in 2006 by Morgan et al [10]. Since 2006, many groups have published clinical results describing the introduction of T cell receptors and chimeric antigen receptors using PG13-derived vector products [11, 12]. The PG13 cells are anchorage-dependent cells traditionally propagated in dishes, T flasks, roller bottles and cell factories [5, 13] where the media can be harvested several times in a batch mode for viral vector production. Recently, these cells have been propagated in a packed-bed bioreactor which allows continuous media replacement and vector harvest increasing production efficiency [14].

A promising alternative production approach is the use of microcarriers support for growing anchorage-dependent PG13 cells. Microcarriers, first described in the 1960s by van Wezel [15], are small, approximately 10 μm – 0.5 mm, charged, coated or porous beads that provide a surface for anchorage dependent cells suspended in a culture medium, have been utilized effectively for propagation of anchorage-dependent cells in bioreactors for production of different biologicals [16]. There has been significant amount of work associated with improving capabilities of cell culture using microcarriers [17] [18]. Microcarriers have been utilized for cells and virus production for vaccines such as polio virus, as well as for antibodies and recombinant proteins [19]. Recently, attention has been directed towards utilizing microcarriers for growth and expansion of mesenchymal and pluripotent stem cells [20], [21].

Microcarriers provide support for anchorage-dependent cell growth in bioreactor and, therefore, like other anchorage-dependent cell methodologies, the surface area is finite, e.g. 3 g/L of Cytodex 1 provide surface area of 13.2 cm^2/mL . Since microcarriers are kept in suspension, it is possible to replace the media while maintaining the cells in the bioreactor

without disrupting their growth, practically simulating suspension culture conditions, and, therefore, extending the production period [22].

This report proposes a procedure for continuous large-scale production of retroviral vector by using microcarriers in a bioreactor equipped with alternating tangential flow perfusion system.

2 Materials and Methods

2.1 PG13 Cells

A PG13 stable packaging clone was previously generated, constitutively expressing a gamma retroviral vector containing T cell receptor using PG13 gibbon ape leukemia virus packaging cell line (ATCC CRL-10686) and the human ecotropic packaging cell line Phoenix ECO (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA) as previously described [13]. Cells were maintained in tissue culture flasks in a humidified incubator set at 5% carbon dioxide (CO₂) and 37°C. The PG13 cells were grown in Dulbecco's Modified Eagle Medium (Thermo Fisher, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (Atlanta Biologics, Flowery Branch, GA), Penicillin-Streptomycin (Thermo Fisher, Grand Island, NY) and 6mM final concentration glutamine (Thermo Fisher, Grand Island, NY) abbreviated as DMEM10, both in the tissue culture flasks and the bioreactor.

2.2 Microcarriers

Cytodex 1 microcarriers (GE Healthcare Life Sciences, Uppsala, Sweden) were used at a concentration of 3 g/L of culture plus 10% to account for transfer losses. The microcarriers were rehydrated in 75 mL/g Phosphate Buffered Saline (PBS, Lonza, Rockland, ME) for at least 3 hours at room temperature in a siliconized glass bottle while being agitated on a rocking platform. The microcarriers were allowed to settle and the PBS removed. The microcarriers were then washed with 40 mL/g of PBS and resuspended in 40 mL/g PBS for autoclaving, with the bioreactor. After autoclaving, 30 mL/g of DMEM10 was used to wash the microcarriers. The microcarriers were then transferred to the bioreactor with some of the initial 500 mL DMEM10 described in section 2.3.

2.3 Bioreactor

A one liter working volume univesel bioreactor with marine blades (Sartorius, Goettingen, Germany) equipped with 16 cm dissolved oxygen (Hamilton) and pH (Hamilton) electrodes, configured as shown in Figure 1, was connected to a DCU touch controller (Sartorius, Goettingen, Germany). The growth was initiated at 37°C, pH 7.5 and air flow of 0.3 L/min with 3.3 g of Cytodex 1 microcarriers, in 500 mL of DMEM10 and approximately 1.7×10^8 cells. For the first 4 hours, the agitation was set at 100 rpm for 2 min followed by 5 rpm for 20 min to allow for cell attachment. After 4 hours, the agitation was set at 100 rpm and additional 500 mL of DMEM10 was added. Dissolved oxygen, pH and temperature were continuously monitored and controlled (see next paragraph) Daily samples were collected for measurements of cell count nutrients, metabolite levels, and pH (Figure 1).

Agitation and airflow increased as the dissolved oxygen decreased, and oxygen was added by cascade control at 1 L/min when the dissolved oxygen concentration reached 50%. Agitation was also increased to 110 rpm when the microcarriers started settling. When the glucose level reached 2 g/L and/or lactate increased to 2 g/L, the media was harvested and fresh DMEM10 was added. The harvest medium was used to measure viral vector titer as described in the next section.

2.4 Perfusion

An alternating tangential flow (ATF) unit (Repligen, Waltham, MA) specific for microcarriers culture (ATF2 MC) equipped with microcarrier screen filter module (73 μ m pore, 162 cm²) operated by C24 controller was set up as shown in Figure 1. On day two, the ATF was turned on with pressure setting of 0.9, and exhaust setting of 0.3 to prime the system for perfusion. The bioreactor was run as batch culture until day four when the feed and harvest pumps were turned on. The feed and harvest flow rates were set at 0.69 mL/min for a total bioreactor volume change in 24 hours. The harvest was collected at 24 hr intervals for measurements and samples were kept in the -80°C freezer until further use.

2.5 Cell count and viability measurements

Samples were collected as described in section 2.3. To measure the cell count and viability from the microcarriers, 1 mL of culture with microcarriers was allowed to settle in a 1.5 mL Eppendorf tube. The supernatant was removed and strained into a cell strainer tube. The cells were washed with 1mL of PBS which was removed and strained in to the tube and 1 mL of trypsin (Thermo Fisher, Grand Island, NY) was added to the cells. After a 7-min incubation at 37°C, this was then also strained into the mixture with media and PBS of which 300 μ L was counted using the trypan blue exclusion method with the Cedex cell counter (Roche, Basel, Switzerland).

2.6 Nutrient and metabolite measurements

Daily samples were measured for nutrient and metabolite concentrations. Glucose and lactate concentrations were measured using YSI 2700 biochemistry analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). Osmolality was measured with Vapro vapor pressure osmometer (Wescor, Logan, UT). Glutamine, glutamate, and ammonia concentrations were measured using the Cedex bioanalyzer (Roche, Basel, Switzerland). These measurements were in accordance with manufacturer instructions.

2.7 Viral Titer and cytokine release assay

Viral titer was determined by transducing PBLs (Peripheral blood lymphocyte), from each of three patients, with the supernatant from the perfusion culture at dilutions of 1:1, 1:9 and 1:99. 2×10^6 cells/mL. The three dilutions results and the three patients were averaged together to determine an overall viral titer. PBLs were stimulated with 300 IU/mL interleukin 2 (Prometheus Laboratories, San Diego, CA) and 50 ng/mL OKT3 (Miltenyi Biotec, Auburn, CA) on day zero. A 24-well non-tissue culture treated plate was coated with 10 μ g/mL (0.5mL/well) retronectin (Takara Bio, Shiga, Japan) on day one and stored overnight at 4°C. After blocking with 5% Human Serum Albumin, (HSA, Valley

Biomedical, Winchester, VA), serially diluted supernatants were added (1mL/well) on day two, centrifuged for 2h at $2,000 \times g$, followed by the addition of PBL, 0.25×10^6 /mL. The cells were then centrifuged for 10 min at $1,000 \times g$ and incubated at 37°C and 5% CO_2 . On day eight, the T cells were analyzed by FACS using fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies directed against CD3 or CD8 (BD Biosciences, San Jose, CA) and FITC-conjugated MART-1₂₇₋₃₅/HLA-A*02 tetramers (Beckman-Coulter, Brea, CA). The relative fluorescence of live cells was assessed using the FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and data analysis using the Flowjo software (Flowjo LLC., Ashland, OR). The viral vector titer was calculated as (Total cells)(% TCR⁺) (Dilution Factor)/supernatant volume.

Two groups of melanoma cell lines each with two cell lines, mel526 and mel624 (HLA-A2+/MART-1+) and mel888 and mel938 (HLA-A2-) were isolated from surgically resected metastases as previously described [23] and were cultured in R10 medium consisting of RPMI 1640 medium (ThermoFisher, Grand Island, NY) containing 10% fetal bovine serum. Functional assessment of TCR-transduced PBLs was carried out by co-culture of 10^5 transduced T cells with 10^5 tumor target cells from each of four melanoma cell lines, and incubated in 200 μL for 18 hours at 37°C as previously described [13]. After the co-culture, specific recognition of tumor targets was assessed by the secretion of IFN γ as measured by enzyme-linked immunosorbent assay (ELISA).

3 Results

3.1 Cell growth on microcarriers in a perfused bioreactor

To determine if continuous perfusion mode is a feasible alternative production method for retroviral vector from PG13 cells one-liter bioreactor, equipped with an alternating tangential filtration device specifically design for microcarrier culture, was set up. After initial seeding and growth period, the bioreactor was operated continuously for 10 days in a perfusion mode replacing one volume per day; the growth parameters are summarized in Figure 2 (A, B). As was expected from this growth strategy cell concentration increased from 1.7×10^5 cells/mL to approximately $1-3 \times 10^6$ cells/mL, a 6 to 17-fold expansion. As the culture grew, it became difficult to obtain accurate viable cell density values due to cell aggregation, both on the microcarriers (supplemental Figure 1) and accumulation in the filtration device. However, the metabolite profile indicated that the cells continued to grow uninterrupted (Figure 2B). Glucose and glutamine were maintained around 2 g/L and 2 mmol/L respectively, and lactate, glutamate and ammonia were maintained at around 2 g/L, 1 mmol/L, and 3 mmol/L respectively. The osmolarity was kept constant at about 330 mmol/kg when the bioreactor was in perfusion mode. In addition, cell viability improved from approximately 80% initially to 95% following the medium perfusion process (Figure 2A).

3.2 Vector production

Viral vector titer increased throughout the perfusion period (Figure 3A) with an average specific productivity of 10.3 transducing units (TU) per cell. Collecting one bioreactor volume per 24 hours (one liter) in the perfusion phase, a total of 4.8×10^{11} TU was obtained

in 11 liters of harvest media with an average titer of 4.4×10^7 TU/mL (Figure 3A). Vector titer was calculated by measuring the transduction efficiency of the T cells with the retroviral vector harvested from the bioreactor for each of three patients at three different dilutions. Representative titration data from a single patient and dilution is shown (Figure 3B). Complete information from the 3 patients can be found in Supplemental Figure 2.

To evaluate the quality of the viral vector produced, a cytokine release assay was performed to determine how well the transduced T cells recognize HLA-matched antigen-positive tumor cells. Representative data from a single patient in Figure 4 shows that the transduced T cells specifically recognize HLA-A2⁺/MART-1⁺ cell lines (526 and 624) and not the HLA-A2⁻ lines (888 and 938) as measured by IFN γ ELISA. Complete information for the 3 patients can be found in Supplemental Figure 3.

4 Discussion

Viral-mediated gene delivery is an efficient way to genetically modify human lymphocytes and other cells. Therefore, for cases where a single vector product can be used to engineer cells for many patients, production of an appropriate amount of retroviral vector from PG13 cells is essential for successful cell therapy studies. PG13 are anchorage-dependent cells and, therefore, the conventional stirred tank bioreactor, commonly used for large scale production of suspension mammalian cells, is not a workable method. As a result, the existing production methods for viral vector from PG13 cells are based on utilizing cell factories, roller bottles and fixed bed bioreactors [13, 14, 24]. In this report, we described a retroviral production process from PG13 cells propagating on microcarriers in a stirred tank bioreactor by utilizing continuous media replacement using perfusion. In the described process, the cells were kept in the bioreactor for a period of 15 days in a stable physiological environment, which was confirmed by stable concentration of metabolites and nutrients, and by the viral vector production titer. In the perfusion period, eleven liters were continuously collected from one-liter bioreactor, 4.8×10^{11} transducing units were collected representing average specific productivity of 10.3 TU per cell.

The PG13 cells attached to the microcarriers were found to be well-matched to the growth conditions in the bioreactor. Once attached to the microcarriers, they continued to grow to confluency while maintaining stable viral titer. However, microscopic observation showed that the cells grew in several layers on the microcarriers and when the system was inspected at the end of the production, some of the cells were found to accumulate in the retention device. Potentially these accumulations can affect the viral vector production efficiency and the cells growth parameters, but practically no adverse effect was observed; not on the vector production capability and not on the metabolic activity of the cells as was evaluated by the metabolite concentrations throughout the two weeks production process. Very likely the reason for this behavior is well mixed and aerated loosely packed cells formation, future studies therefore should include optimization of the microcarriers concentration, cell seeding density and perfusion rate.

The filtration assembly has a retention volume of approximately 350 mL and stroke volume of approximately 100 mL; when the system operates about half of the one-liter bioreactor is

contained in the filtration unit, these conditions were found to be appropriate for maintaining the growth and the vector production. Based on the performance of the tested filtration assembly we predict that the same size filtration unit will likely be suitable for perfusing larger volumes, perhaps up to 5 liters and would possibly provide more homogenous culture, but this needs to be evaluated in additional experiments. An unexpected advantage of using the retention assembly is the increased dissolved oxygen supply to the microcarrier culture of the PG13 cells, resulting from charging and discharging the culture into the retention assembly. Sparging air and oxygen directly into the microcarrier culture were found to cause aggregation of the PG13 cells around the air sparger, but surface aeration with or without oxygen, together with the tangential mixing by the retention device and the vertical mixing of the bioreactor, kept the dissolved oxygen above 50% air saturation throughout the process without difficulties as seen in Supplemental Figure 4. The process lasted 15 days but certainly could go longer continuously producing more viral vector.

Compared with existing production strategies such as stationary processes done in T flasks and roller bottles that although robust, are labor intensive and require large footprint [25, 26], the described process offer several advantages. Fixed-bed bioreactor [27] is another possible production approach that eliminates some of the hurdles of the stationary process; it requires smaller surface area and can maintain the growth parameters such as pH and DO, however, it has imperfect mixing so there is limited nutrient distribution and the cells may not have equal access to essential growth factors. We believe that the continuous process described in this work, that uses microcarriers to create suspension like perfusion culture, by utilizing alternating tangential filtration device, is an efficient way to produce the large quantities of active vector needed for clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights for review

1. Continuous production of viral vector
2. Perfused microcarrier cell culture
3. Comparison the suggested method with existing production methodologies
4. Quality of the viral vector produced

Figure 1. Bioreactor setup

Drawing (not to scale) of the perfusion bioreactor layout with the ATF filtration system adapted from Bleckwen et al. [28]. The pump flow rates are controlled individually, the ATF has a separate control unit and the growth parameters are regulated independently by the bioreactor control unit.

Figure 2. Culture performance

A) Cell growth and perfused volume. The viable cell density (diamonds), viability (circles) and total perfused volume (solid line) as a function of process time. **B) Nutrients and Metabolite Concentrations.** Glutamate (solid circles), glutamine (open circles), ammonia (solid triangles), osmolarity (open diamonds), glucose (solid squares) and lactate (open squares) concentrations as a function of process time. The arrow shows the day perfusion was initiated.

Figure 3. Vector production

A) Vector titer (closed circles) and total accumulated vector (solid line) as a function of perfusion time. Vector concentration is an average of three dilutions for each of three patients. **B) Fluorescence Activated Cell Sorting Analysis (FACS)** Data after perfusion was initiated from one of the three patients and 1:9 dilution shown. (Because the murinized MART-1 TCR expresses the murine TCR-beta constant region, transduced cells are identified as mTCR⁺.)

Figure 4. Vector activity by functional analysis of TCR-transduced PBL

A representative assay showing the IFN γ release following overnight co-culture with HLA-A2+/MART-1+ tumor targets (526, 624) and the antigen-negative, HLA mismatched controls (888, 938) for samples after perfusion was initiated. As expected, cells released no IFN γ against mismatched controls. Data from one of three patients shown.