Application of Serum Free Medium Cultured Vero Cells for the Production of Recombinant Oncolytic Herpes Simplex Virus 2

Zongyao Fang¹,², Leilan Wang¹, Zongxing Zou²,³, Siqi Zhang¹, Yin Zhao³, Sheng Hu⁴ and Binlei Liu¹,³,*

¹School of Pharmacy, Hubei University of Science and Technology, Xian Ning, China
²School of Bioengineering and Food, Hubei University of Technology, Wu Han, China
³Wuhan Binhui Biopharmaceutical Co. Ltd., Wu Han, China
⁴Department of Thoracic Oncology, Hubei Cancer Hospital, Wu Han, China

*Corresponding author: binleiliu@hbust.edu.cn

Abstract. The oncolytic virus, especially the recombinant oncolytic herpes simplex virus 2 (oHSV2), has attracted much attention because of its promising cancer treatment effect. To eliminate animal-derived substances from serum supplemented culture medium, which is an adverse factor for the safety of biological products, this study selected three typical serum free media (SFM) on the market, and successfully obtained three SFM cultured Vero cell lines which could be expanded stably. This work provided a reference for the application of SFM to the production of other biological products or vaccine.

1. Introduction

As the main force of cancer immunotherapy, the oncolytic virus (OV) has many advantages. These advantages include selective replication in tumor cells [1], triggering innate immune responses by recruiting immature dendritic cells and innate lymphocytes, correcting defects in antigen processing and presentation, and activating adaptive immune responses [2]. In addition to finding the natural cancer-cell selectivity and immunogenicity of the OVs, the researchers also modified the virus vectors by removing, inserting and replacing various genes to make them lose their original pathogenicity and ensure that they can replicate and reproduce in tumor tissues selectively [3-11].

Over the past few decades, the engineered OVs have been produced from both DNA and RNA viruses. DNA viruses have many advantages: Large genomes of DNA viruses can be edited without affecting virus replication; Large eukaryotic transgene of DNA viruses can be encoded to increase therapeutic activity or immunomodulation; DNA viruses express high-fidelity DNA polymerase to ensure the integrity and efficient replication of the virus genome [12, 13].

Using molecular cloning and DNA homologous recombination, our team modified herpes simplex virus type 2 by eliminating the neurovirulent gene ICP34.5 and immunosuppressive gene ICP47 in the viral genome, inserting the immune enhancing gene human granulocyte-macrophage colony stimulating factor (hGM-CSF), thus constructed a new attenuated recombinant oncolytic herpes simplex virus (oHSV2) with replicative capability in multiple types of tumor cells [14].
As a production cell, African Green Monkey kidney cells (Vero) have been widely recognized and approved by international organizations, including WHO and European Medicines Agency, for the manufacture of human vaccine.

Although the basic medium with a small amount of serum can meet most of the scientific research cell culture needs, but in vaccines, gene therapy drugs and other biological products, the application of serum, an animal-derived substance, undoubtedly increased the safety risks. The utilization of serum-free culture technology in biological products not only completely relieves the negative effects from serum sources, but also greatly facilitates the separation and purification of products for subsequent purposes.

Applying serum-free medium VP-SFM, Trabelsi K [15, 16] successfully cultured Vero cells in a bioreactor using microcarriers and produced a RNA enveloped rabies virus under serum-free conditions.

2. Materials and Methods

2.1. Cell culture

The Vero cells used in this study were originally obtained from ATCC (CCL-81). After resuscitation of Vero cells, the cell line was cultured in DME/F12 (Hyclone) containing 10% newborn bovine serum (NBS) (Zhejian Tianhang Biotechnology Co. Ltd.) at 37°C, 5% CO2. When the cell could be stably subcultured, the serum concentration in the medium was gradually reduced to 3% and the subculture cycle was about every 3 to 4 days per passage.

Three serum-free cultured Vero cell lines were gradually obtained via sequential adaptation to three serum free media: Virus Product (VP-SFM, Gibco), Vaccine Xpress (VX-SFM, Hyclone), OptiPRO™SFM (Gibco), according to operating instruction provided by the corresponding supplier.

2.2. Production of oHSV2

After serum-free acclimated Vero cells could be subcultured stably, the 12 well tissue culture plates were seeded at 5x105 Vero cells per well before virus infection. The oHSV2 at the 10th passages provided by Wuhan Binhui Biopharmaceutical Co., Ltd was used in this work. When cell confluence degree reached approximately 100%, the oHSV2 inoculum diluted with corresponding cell culture medium with or without serum was added at a multiplicity of infection (MOI) of 0.01 and the infected cells were cultured at 32°C, 5% CO2. On the third day after infection, as soon as the complete cytopathic effect and few syncytia were observed on the infected Vero cells in the plates, the culture media of different wells were replaced by hypertonic salt solutions gently. The virus releasing process was allowed at 32°C, 5% CO2 for 24 hours. Next, the supernatant samples containing the viruses released from the infected cells were harvested and stored in the freezer at -80°C. When all the samples were collected, the tests for virus titer will be conducted. The process is illustrated in (Figure 1).

2.3. Infective titer determination of oHSV2

CCID50 is a semi-quantification unit for infective viruses. The Vero cells were cultured in DME/F12 and supplemented with 3% NBS. The 96 well tissue culture plates were seeded at a concentration of 5x104 Vero cells per well and incubated at 37°C, 5% CO2 for one day prior to the virus infection. The virus samples were continuously diluted by a 10-fold gradient with inoculation medium (DME/F12 and supplemented with 1.5% NBS) in a dilution plate. The gradient dilution samples were inoculated onto Vero cells (degree of cell confluence ≥95%) in 96-well plates and the cells were cultured for more than 3 days. The wells containing the infected cells with the visual cytopathic effects (CPEs) or plaques under the microscope were counted and the titer expressed in CCID50/ml was calculated by using the Reed and Muench method [17]. The oHSV2 infective titers presented are the averages of three replicates.
3. Results
Three serum-free cultured Vero cell lines were successfully obtained in a sequential adaptation manner. Under the culture condition of three commercial SFM, oHSV2 can infect Vero cells well, and presented cytopathic effects and syncytia, which were ordinary shown in serum supplemented media.

The results of the determination of the infective titer of the oHSV2 harvested from different culture media showed 7.878 CCID50/ml for DME/F12 supplemented with NBS, 8.301 CCID50/ml for VP SFM, 7.945 CCID50/ml for VX SFM, 7.964 CCID50/ml for OptiPRO SFM (Figure 2). ANOVA F test results showed no significant difference in variance between four groups. The results of paired t-test analysis showed that there was no statistical difference between serum-containing medium control group and groups for VX SFM and OptiPRO SFM, while there was a significant difference (p<0.05) between the control group and VP SFM group. In terms of cultured virus yield, contrasted with serum-containing medium, three SFM were generally better than the control medium, while VP SFM is remarkably superior.

Above all, it is feasible for SFM to be used in oHSV2 production.

4. Conclusion
The SFM cultured Vero cells showed great potential for producing oHSV2. Among them, VP-SFM was shown to be better in virus yield than serum supplemented media.

Our data showed that all three SFM cultures resulted in the similar cells growth status and the equivalent or better virus production yields when compared to the serum supplemented medium. The lack of serum and animal-derived components in the medium reduces the variability of the biological product manufacture process.

VP SFM is a serum-free, ultra-low protein medium which containing no animal or human sourced proteins, peptides, or other components. It is designed for the growth of VERO cells for virus production. VP SFM is also suitable for the growth of MDCK, BHK-21, COS-7. Vaccine Xpress is serum-free, animal-derived component-free, and human origin-free. Vaccine Xpress cell culture medium is designed and developed for high-density growth and maintenance of kidney-derived cell lines for viral vaccine manufacturing. This medium has been used to producing vaccines such as influenza, Zika, and respiratory syncytial virus. OptiPRO™ SFM is a chemically defined serum-free, animal/human-origin free medium, designed for growth of several kidney-derived cell lines such as PK-15. It has also been used to grow several additional attachment dependent cell lines such as HeLa cells. Soraia Attie Calil Jorge [18] produced three kinds of Semliki Forest Virus replicon particles in serum free medium cultured BHK-21 cells.

The production of biologics as virus in serum free medium significantly simplified the downstream purification processing and reduced the risk of contamination.

Correspondence Author: Binlei Liu, School of Pharmacy, Hubei University of Science and Technology, Xian Ning, China (E-mail: 1836035949@qq.com). National “111” Center for Cellular Regulation and Molecular Pharmaceutics, Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei Provinical Cooperative Innovation Center of Industrial Fermentation, College of Bioengineering, Hubei University of Technology, Wuhan, China.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (Grants No. 81972308) and the National Major Scientific and Technological Special Project for “Significant New Drugs Development” during the Thirteenth Five-year Plan Period (Grants No.2018ZX09733002). We also want to appreciate the GE Medical Systems Trads & Development (Shanghai) Co., Ltd, and Mr. Jiefeng Long for the gift of Vaccine Xpress cell culture medium.
Figure 1. Process flow diagram for serum free sequential adaptation culture of Vero cells and production of oHSV2.

Figure 2. The infectious titer of oHSV2 samples harvested from various infection culture media. The infectious titers were quantified by CCID50. Data are representative of three independent experiments and presented as Mean ± SEM.

References
[1] Tianli Xia, Konno Hiroyasu, Barber Glen N. Barber. "Recurrent loss of STING signaling in melanoma correlates with susceptibility to viral oncolysis." Cancer research 76.22 (2016): 6747-6759.
[2] Bommareddy, Praveen K., and Howard L. Kaufman. "Unleashing the therapeutic potential of oncolytic viruses." The Journal of clinical investigation 128.4 (2018): 1258-1260.
[3] Xiao Hu, et al. "Progress in the treatment of tumor with oncolytic herpes simplex virus." Oncology progress 14.8 (2016): 730-733.
[4] Yujie Zhu. Construction of a novel oncolytic herpes simplex virus expressing pd-1 monoclonal antibody and its anti-tumor efficacy. Master's thesis. Peking union medical college,2019.
[5] Sophia T. Mundle, et al. "High-purity preparation of HSV-2 vaccine candidate ACAM529 is immunogenic and efficacious in vivo." PLoS One 8.2 (2013): e57224
[6] Wen Zhang, et al. "A novel oHSV-1 targeting telomerase reverse transcriptase-positive cancer cells via tumor-specific promoters regulating the expression of ICP4." Oncotarget 6.24 (2015): 20345.
[7] Shibata T., et al. "Development of an oncolytic HSV vector fully retargeted specifically to cellular EpCAM for virus entry and cell-to-cell spread." Gene therapy 23.6 (2016): 479-488.
[8] Wen Lei. Anti-tumor effects of oncolytic vaccinia virus harboring therapeutic genes for hematological malignancies. Doctoral dissertation. Zhejiang University, 2017.

[9] Lili Deng, et al. "Oncolytic and immunologic cancer therapy with GM-CSF-armed vaccinia virus of Tian Tan strain Guang9." Cancer letters 372.2 (2016): 251-257.

[10] Linkang Cai, et al. "The construction of a new oncolytic herpes simplex virus expressing murine interleukin-15 with gene editing technology." Journal of Medical Virology (2020).

[11] Wenjuan Cao. The Construction of a Bladder Cancer-Specific Chimeric CAR Independent Oncolytic Adenovirus and its Effect against Cancer Cells. Doctoral dissertation. Lanzhou University, 2017.

[12] Kaufman, Howard L., Frederick J. Kohlhapp, and Andrew Zloza. "Oncolytic viruses: a new class of immunotherapy drugs." Nature reviews Drug discovery 14.9 (2015): 642-662.

[13] Praveen K. Bommareddy, Megha Shettigar, and Howard L. Kaufman. "Integrating oncolytic viruses in combination cancer immunotherapy." Nature Reviews Immunology 18.8 (2018): 498.

[14] Bin-lei Liu, et al. "Construction of a new oncolytic virus oHSV2hGM-CSF and its anti-tumor effects." Chinese Journal of Oncology 34.2 (2012): 89-95.

[15] Khaled Trabelsi, et al. "Comparison of various culture modes for the production of rabies virus by Vero cells grown on microcarriers in a 2-l bioreactor." Enzyme and Microbial Technology 36.4 (2005): 514-519.

[16] Khaled Trabelsi, et al. "Optimization of virus yield as a strategy to improve rabies vaccine production by Vero cells in a bioreactor." Journal of biotechnology 121.2 (2006): 261-271.

[17] Reed, Lowell Jacob, and Hugo Muench. "A simple method of estimating fifty per cent endpoints." American journal of epidemiology 27.3 (1938): 493-497.

[18] Sandra Fernanda Suárez-Patiño, et al. "Semliki Forest Virus replicon particles production in serum-free medium BHK-21 cell cultures and their use to express different proteins." Cytotechnology 71.5 (2019): 949-962.