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A 3D collagen microsphere culture system for GDNF-secreting HEK293 cells with enhanced protein productivity

Hoi-Ling Wong[a], Ming-Xi Wang[b], Pik-To Cheung[b], Kwok-Ming Yao[c], Barbara Pui Chan[a,*]

[a]*Medical Engineering Program, Department of Mechanical Engineering, Faculty of Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong*
[b]*Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam Road, Hong Kong*
[c]*Department of Biochemistry, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam Road, Hong Kong*

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Abstract

Mammalian cell culture technology has been used for decades in mass production of therapeutic proteins. However, unrestricted cell proliferation usually results in low-protein productivity. Controlled proliferation technologies such as metabolism intervention and genetic manipulation are therefore applied to enhance the productivity. Nevertheless, these strategies induced growth arrest with reduced viability and increased apoptosis. In this study, we report a new controlled proliferation technology by encapsulating human embryonic kidney (HEK) 293 cells over-expressing glial-derived neurotrophic factor (GDNF) in 3D collagen microspheres for extended culture. We investigated the viability, proliferation, cell cycle and GDNF productivity of HEK293 cells in microspheres as compared to monolayer culture. This system provides a physiologically relevant tissue-like environment for cells to grow and exerts proliferation control throughout the culture period without compromising the viability. A significant increase in the production rate of GDNF was found in the 3D microsphere system comparing with the monolayer culture. GDNF productivity was also significantly affected by the initial cell number and the serum concentration. The secreted GDNF was still bioactive as it induced neurite extension in PC12 cells. In summary, the 3D collagen microsphere system presents a cost-effective controlled growth technology for protein production in pharmaceutical manufacturing.

Keywords: 3D culture; Biopharmaceutical manufacturing; GDNF-secreting HEK293 cells; Microsphere; Collagen; Microencapsulation

1. Introduction

Mammalian cell culture technology in production of recombinant therapeutic proteins has been developed over years [1]. Technologies for scaling up the cell culture such as 3D microcarriers have been used for decades [2,3] because it is generally accepted that 3D culture provides significantly increased surface area for cell binding and rapid and unrestricted proliferation [4]. Many generations of microcarriers ranging from dextran [5] and poly-lactic-glycolic acid beads [6], to those employing natural matrix materials such as gelatin [7,8] and collagen beads [9], have been developed over years. However, the productivity of actively and unrestrictedly proliferating cells is usually low. This is because, firstly, these cells may not synthesize proteins at maximal rate outside their tissue-specific microenvironment and, secondly, in actively proliferating cells, most of the metabolic energy is devoted to reproduction rather than synthetic activities [10].

Controlled proliferation technologies are employed to enhance the protein productivity of cells in biopharmaceutical manufacturing [11–13]. These include starvation of cells for essential nutrients or addition of DNA synthesis inhibitors [14], temperature-shift-induced growth arrest [15] and genetic manipulation with growth cycle controlling genes such as over-expressing tumor suppressor genes p53 [16], p21 [17] and p27 [18]. However, these strategies lead to significantly reduced cell viability [19] and increased apoptosis [20]. Co-expressing the cell cycle controlling tumor suppressor genes with anti-apoptotic genes such as bcl-2 has been used to improve cell viability [11], but this

*Corresponding author. Tel.: +852 2859 2632; fax: +852 2858 5415.
E-mail address: bpchan@hkucc.hku.hk (B.P. Chan).

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system still genetically interferes with the cell metabolism. Recently, 3D micro-tissue cultures using hanging-drop method has been shown with enhanced protein productivity [10]. This suggests that mimicking the physiologically relevant 3D tissue-like microenvironment may regulate cell proliferation and growth kinetics and hence their protein productivity.

Microencapsulation is a process entrapping cells within the confines of a semi-permeable membrane forming a microcapsule or within a homologous solid mass forming a microsphere. This technique has been used for many years to aid immunoisolation during allogenic or xenogenic cell transplantation [21,22]. Sodium alginate is the most commonly used material while other materials such as agarose [23] and polyethylene glycol [24] are also used. Microencapsulation of cells in these materials can be used for 3D culture, but they usually do not support cell attachment and growth that results in low cell viability and growth [24–26]. Collagen must be supplemented for improvement in cell viability [23,25]. Collagen is a naturally occurring, biocompatible and biodegradable material [27] and can be reconstituted into fibrous structures simulating the native extracellular matrix in tissues. We recently established a microencapsulation system immobilizing bone marrow mesenchymal stem cells (MSCs) within reconstituted collagen microspheres [28] and demonstrated that these microspheres are able to support cell growth, migration [29] and differentiation [30].

Here we report a 3D culture system by encapsulating glial-derived neurotrophic factor (GDNF)-secreting human embryonic kidney (HEK) 293 cells in collagen microspheres. Whether this 3D microsphere culture system controls the proliferation and affects the viability, cell cycle and productivity of these cells as compared to traditional monolayer culture was investigated. Parameters affecting the productivity including the initial cell number and the serum concentration, and the bioactivity of the secreted GDNF were also studied.

2. Methods

2.1. HEK293 cell culture

HEK293 cells over-expressing GDNF were kindly provided by Dr. P.T. Cheung [31]. Briefly, a 633 bp wild-type (wt) murine GDNF cDNA was cloned into an expression plasmid vector pHM6 (Roche Diagnostics Corp.) encoding the GDNF short transcript. HEK293 in the exponential growth phase were then transduced with this pHM6-633wtGDNF plasmid using Lipofectamine^{TM} 2000 Transfection Reagent (Invitrogen) at 4 μg DNA/10 μl. Cells were cultured in Dulbecco's modified Eagle'smedium—high glucose (DMEM-HG), supplemented with 10% fetal bovine serum (FBS) and 1% PS at 37 °C with 5% CO_2. G418 sulfate at 500 μg/ml was supplemented for selection and maintenance of positive clones. Fresh medium and G418 were replaced every 2 days. Cells at passage 4 were encapsulated for 3D culture and monolayer culture.

2.2. Microencapsulation

HEK293 cells were trypsinized using 0.25% Trypsin-EDTA. Rat-tail collagen solution type I was neutralized by 1 N NaOH and diluted to 4 mg/ml in the presence of HEK293 cells in DMEM. The cell mixture was dispensed as 5 μl droplets onto a collection platform with non-adherent surface. The microdroplets were incubated at 37 °C for 1 h to allow for reconstitution into solid microspheres, which were then collected into 35 mm Petri dish supplemented with medium.

2.3. Characterization of the microspheres

The cell-matrix microspheres were viewed under an inverted microscope at different time points up to day 12. The diameter of 10% of the microspheres was measured using an eye-piece micrometer at 40× magnification. The microspheres were cultured and G418 sulfate exchanged every 2 days. The conditioned medium was collected and stored in −20 °C freezer for subsequent analyses. In separate experiment, HEK293 cells–collagen microspheres were cultured for 10 days, fixed in 0.25% glutaraldehyde at 4 °C overnight, rinsed and dehydrated in gradients of ethanol before critical point drying and sputtering of gold for SEM analysis (Leo 1503).

2.4. Parameters affecting cell number, viability and GDNF productivity

Duration of culture, initial cell number and serum concentration, and their effects on cell number, viability, total GDNF and secretion rate of GDNF were investigated. In brief, HEK293 cells were encapsulated at a density of 500 cell/microsphere and 50 microspheres were cultured per 35 mm Petri dish in quadruplicates. Aliquots of the same cell number (2.5×10^4) were cultured in monolayer for comparison. On day 2, 4, 8, 10, 14, 18, 22, 26 and 30, cells were harvested for number and viability analysis. The conditioned medium was collected for GDNF analysis. In separate experiment, different initial cell numbers (2.5×10^3, 2.5×10^4 or 2.5×10^5) were either seeded onto six-well plate as monolayers, or encapsulated in 50 collagen microspheres at densities equivalent to 50, 500 or 5000 cell/microsphere, respectively, in quadruplicates. Cells in both systems were harvested on day 12 for subsequent analyses. In another experiment, aliquots of 2.5×10^4 cells were cultured for 12 days, with quadruplicates, either in monolayers or in 3D cultures with supplementation of 2%, 5% or 10% FBS.

2.5. Cell number and viability

At the end of incubation, microspheres were incubated with bacterial collagenase (C9891, Sigma) at 30 U/ml for 60–90 min to digest the collagen matrix. After pelleting the cells by centrifugation at 1500 r.p.m. for 10 min, Trypsin/EDTA (0.25%) was supplemented for 3 min to obtain single-cell suspensions. Trypan blue viability test was conducted and the numbers of live and dead cells were counted using a hemacytometer.

2.6. Cell cycle analysis

GDNF-secreting HEK293 cells were either encapsulated in 3D collagen microspheres or cultured as monolayers for 4, 8, 10, 14, 22, 26 or 30 days. At the end of each incubation period, cells were released by enzymatic digestion. One milliliter of cell suspension at 1×10^6 cell/ml was fixed with 70% ice-cold ethanol and stored at −20 °C until use. Cells were stained with propidium iodide (Calbiochem) after incubation with 1 ml of RNase (50 μg/ml) (Calbiochem) for 20 min at room temperature. The DNA content of at least 10 000 cells was measured using a flow cytometer EPICS Elite ESP high performance cell sorter (Coulter Electronics) equipped with a red pass filter at 600 nm and data analyzed using WinMDI 2.8 software.

2.7. GDNF productivity

The concentration of soluble GDNF in the conditioned medium was measured using the GDNF $E^{®}_{max}$ ImmunoAssay System (Promega).