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Coaxial extrusion bioprinted shell-core hydrogel microfibers mimic glioma microenvironment and enhance the drug resistance of cancer cells

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ABSTRACT

Glioblastoma (GBM) is the most common primary malignant central nervous system tumor. The current treatment is mainly surgical resection combined with radiotherapy, chemotherapy and other comprehensive treatment methods. However, the treatment effect is unsatisfactory, the resistance of cancer cells to alkylating agent is the major reason for the recurrence of GBM. It is necessary to develop an ideal *in vitro* model to investigate the drug resistance of glioma cells. In this study, shell-glioma stem cell GSC23/core-glioma cell line U118 (G/U) hydrogel microfibers with high cell viability were constructed by coaxial extrusion bioprinting. It was found that core-U118 cells gradually proliferated to form fiber-like cell aggregates and the interactions between cell-cell and cell-extracellular matrix (ECM) were increased. Furthermore, compared with shell/core-U118 (U) hydrogel microfibers, the expressions of matrix metalloproteinase-2 (MMP2), MMP9, vascular endothelial growth factor receptor-2 (VEGFR2) and O6-methylguanine-DNA methyltransferase (MGMT) which are related to tumor invasion and drug resistance were significantly enhanced in G/U hydrogel microfibers. Moreover, U118 cells derived from G/U microfibers had greater drug resistance *in vitro* and the level of MGMT promoter methylation in G/U cultured U118 cells was significantly lower than that of U cultured cells. In summary, coaxial extrusion bioprinted G/U hydrogel microfiber is a preferable platform for mimicking glioma microenvironment, as well as for drug development and screening.

1. Introduction

Although chemotherapy can further kill the residual tumor cells after glioblastoma (GBM) surgery, the drug resistance of tumor cells causes recurrence of GBM [1,2]. Achieving a desired tumor model for drug evaluation remains a challenge, because of the inability of the current models to mimic *in vivo* tumor microenvironment. In the past few decades, traditional two-dimensional (2D) cell culture system has been widely used for cancer research, as well as drug evaluation [3]. However, 2D culture system cannot mimic three-dimensional (3D) tissue structure *in vivo*, which makes cell functions such as cell morphology, viability, proliferation, differentiation, gene and protein expression, and drug response limited or lost [4–6]. It may be the reason that a variety of anticancer drugs have been proven to be effective *in vitro*, but shown an unfavorable therapeutic effect in clinical trials [7]. In addition, due to the difference in species, the results of drug test from animal models can not accurately predict its effect of human application [8]. Therefore, it is imperative to construct a model that best mimics the *in vivo* tumor microenvironment. Such models should well represent the biological behavior of cancer cells and can be used to develop highly effective anticancer drugs [9]. To address these issues, several attempts have developed numerous 3D cell culture models in recent years for different applications. An increasing number of evidence demonstrates that 3D cell culture can establish physiological cell-cell and cell-ECM interactions, which can mimic *in vivo* tissue specificity, and its physiological relevance is significantly stronger than

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traditional 2D cell culture [10]. This is especially important in anticancer drug development and screening.

Various 3D tumor models have been established and applied to cancer research. 3D porous scaffolds which composed of different natural or synthetic polymers promoted the formation of cell spheroids and increased the drug resistance of tumor cells compared to 2D culture [11–13]. However, the distribution of cells in 3D porous scaffolds cannot be accurately controlled and cells could only be seeded on the surface of these models. Therefore, the amount of cells cultured in these scaffolds may be limited. Tumor models formed by gel or matrix embedding cells have advantages in maintaining 3D structure, but are limited in diffusion of nutrients and mimicking the drug concentration gradient [14]. With the development and more extended exploration of 3D bioprinting technology, 3D bioprinted tumor model has been widely used to investigate the biological behavior of cancer cells [15]. Demirci's group first reported the use of 3D bioprinting technology to construct tumor model containing human ovarian cancer (OVCRA-5) cells and MRC-5 fibroblasts [16]. The presented approach provided a promising platform for cancer research and high-throughput drug screening. Sun's group investigated bioprinting of HeLa cells to fabricate cervical tumor model and found that the MMP protein expression and chemoresistance of cells in 3D bioprinted tumor model were higher than those of 2D control [17]. Organovo demonstrated scaffold-free breast tumor model using the NovoGen Bioprinting^{TM} platform, where the drug resistance of tumor cells was assessed and results showed that cancer cells cultured in 2D were more susceptible to chemotherapeutic drug than cells in 3D model *in vitro* [18]. Although multiple cell types have been used to bioprint tumor models, direct cell-cell interactions are limited by the biomaterials surrounding cells in these established tumor models. Moreover, there is little information on the interaction and effect of different cell types in co-culture 3D tumor model. As we know, tumor microenvironment consists of cancer cells, cancer stem cells and mesenchymal cells, among which cancer stem cells play a key role in tumorigenesis, progression, recurrence and drug resistance. Cancer stem cells interact with tumor microenvironment to promote the development of tumor [19]. Up to this date, glioma models constructed with bioprinting platform either contain only glioma cells or glioma stem cells (GSCs) [20,21]. Constructing a model consisting of glioma cells and GSCs is beneficial for mimicking glioma microenvironment and studying of biological behavior of glioma cells, as well as drug resistance.

In this study, shell-GSC23/core-U118 (G/U) hydrogel microfibers were fabricated by coaxial extrusion bioprinting. The unique shell-core structure can not only well mimic the glioma microenvironment, but also the shell structure can be removed when necessary to facilitate the analysis of biological effects of cells in shell on cells in core under co-culture condition. Here, cell viability and morphology of shell-core hydrogel microfibers were analyzed. Furthermore, the expression of drug resistance-related genes of U118 cultured in G/U microfiber was evaluated and compared to that of U microfiber cultured cells. Moreover, the *in vitro* sensitivity of U118 cells cultured in different conditions to chemotherapeutic drug temozolomide (TMZ) was investigated. Finally, we explored the mechanism of increased drug resistance in U118 cells derived from G/U hydrogel microfibers.

2. Materials and methods

2.1. Cell culture

Human glioma cell line U118 was bought from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco). Human glioma stem cells GSC23 were kindly donated by the MD Anderson Cancer Center, University of Texas [22]. Cells were maintained in DMEM/F12 medium with 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, B27 supplement (50X) (all from Gibco, Carlsbad, CA, USA). All cells were cultured at 37 °C and 5% CO_2 in a fully humidified environment. The culture medium was changed every 2–3 days.

2.2. Preparation of printing materials

Sodium alginate powder (A0682) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and sterilized by gamma ray radiation (15 Gy). A 3% (w/v) sodium alginate solution was obtained by dissolving sodium alginate powder in 0.9% sodium chloride solution (w/v). GSC23 cells (1×10^5/mL) were centrifuged and mixed with 3% sodium alginate solution to obtain a solution for shell stream. U118 cells (1×10^7/mL) were resuspended in medium for core stream.

2.3. Coaxial bioprinting

In order to more clearly show the shell-cells and core-cells, GSC23 and U118 were stained with two different fluorescent dyes before coaxial bioprinting. PKH 67 green fluorescent cell linker kits and PKH 26 red fluorescent cell linker kits were purchased from Sigmae Aldrich (St. Louis, MO) and used to stain shell-GSC23 cells and core-U118 cells, respectively, following the manufacturer's instruction. Briefly, cells were digested and washed with serum-free medium. After centrifugation at 400 × g for 5 min, cells were then resuspended in diluent C (provided in the staining kit) and 2×10^{-6} M PKH 67 or PKH 26 solution was added at a concentration of 1×10^7/mL, and incubated for 5 min at room temperature. The staining reaction was stopped by addition of an equal volume of serum, and cells were washed twice with complete medium to remove unbound dye.

Shell-core hydrogel microfibers were bioprinted by previously reported method with a custom-made coaxial extrusion bioprinting device [20]. The printing device was mainly composed of a sheath/core coaxial nozzle which allowed easy installation and disassembly and the print head was made of a pair of 21 G and 16 G needles. For shell-core hydrogel microfibers fabrication, an alginate solution with or without GSC23 cells was used for shell stream, and a cell suspension containing U118 was used for core stream. The extrusion rate of shell stream was 20 mL/h and 5 mL/h for core stream. A petri dish containing 3% $CaCl_2$ solution crosslinked with sodium alginate was used as printing receiving platform. After bioprinting, shell-core hydrogel microfibers were gently washed with phosphate buffer solution (PBS) to remove excessive cross linker before culturing. Subsequently, cell-laden shell-core hydrogel microfibers were maintained in stem cell medium supplemented with 10% FBS at 37 °C and 5% CO_2.

2.4. Cell viability analysis

Cell viability after bioprinting was analyzed by fluorescent live/dead assay kit (KeyGEN BioTECH, Nanjing, China) according to the protocol. Briefly, the shell-core hydrogel microfibers were stained by incubation with a mixed solution of 8 μM propidium iodide and 2 μM Calcein-AM for 10 min at room temperature in dark. Then samples were washed three times with PBS. Images were obtained by a fluorescence microscope (Olympus IX51, Tokyo, Japan) and live cells shown in green and dead cells in red. Cell viability was calculated following the formula: Survival rate (%) = (number of green stained cells/number of total cells) ×100. Different longitudinal sections along the center axis were obtained and used to quantify the total cell viability of shell/core structure. The live and dead cells of each sample (n = 3) were counted in 5 random fields at 200× magnification.

2.5. Cell proliferation analysis

Cell proliferation of cell-laden shell-core hydrogel microfibers was analyzed with Alamar Blue Kit (MAIBIO, Shanghai, China) according to