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Enrichment of glioma stem cell-like cells on 3D porous scaffolds composed of different extracellular matrix

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ABSTRACT

Cancer stem cells (CSCs), being tumor-initiating with self-renewal capacity and heterogeneity, are most likely the cause of tumor resistance, reoccurrence and metastasis. To further investigate the role of CSCs in tumor biology, there is a need to develop an effective culture system to grow, maintain and enrich CSCs. Three-dimensional (3D) cell culture model has been widely used in tumor research and drug screening. Recently, researchers have begun to utilize 3D models to culture cancer cells for CSCs enrichment. In this study, glioma cell line was cultured with 3D porous chitosan (CS) scaffolds or chitosan-hyaluronic acid (CS-HA) scaffolds to explore the possibility of glioma stem cells (GSCs)-like cells enrichment, to study the morphology, gene expression, and *in vivo* tumorigenicity of 3D scaffolds cells, and to compare results to 2D controls. Results showed that glioma cells on both CS and CS-HA scaffolds could form tumor cell spheroids and increased the expression of GSCs biomarkers compared to conventional 2D monolayers. Furthermore, cells in CS-HA scaffolds had higher expression levels of epithelial-to-mesenchymal transition (EMT)-related gene. Specifically, the *in vivo* tumorigenicity capability of CS-HA scaffold cultured cells was greater than 2D cells or CS scaffold cultured cells. It is indicated that the chemical composition of scaffold plays an important role in the enrichment of CSCs. Our results suggest that CS-HA scaffolds have a better capability to enrich GSCs-like cells and can serve as a simple and effective way to cultivate and enrich CSCs *in vitro* to support the study of CSCs biology and development of novel anti-cancer therapies.

1. Introduction

Glioblastoma (GBM) is the most invasive and deadly primary tumor in the brain [1], even after total resection and adjuvant chemoradiotherapy, the prognosis for patients is still poor, and the median survival time was only 14 months after diagnosis [2]. GSCs are thought to be the source of recurrence and chemoradiotherapy resistance in GBM [3]. There are many strategies for GSCs targeted therapy, but the results are still not satisfactory [4]. Further study of GSCs is important for the treatment of glioma. However, CSCs account for only less than 1% of cancer cell population [5]. Therefore, how to successfully isolate and enrich CSCs *in vitro* has been a research focus. Thus far, CSCs have been isolated *in vitro* by using fluorescence-activated cell sorting or magnetic-activated cell sorting, and serum-free CSC culture medium has been used as the suspension culture of tumorspheres for enriching CSCs [5,6]. However, the application of these techniques often require specialized equipment and costly antibodies, time-consuming cell suspension culture, the yield of CSCs is very low [5]. Most importantly, suspension culture lacks the 3D environment required for cell-extracellular matrix (ECM) interactions to promote cancer heterogeneity [7]. Due to the limitations of traditional CSCs isolation and enrichment methods, researchers have enriched CSCs by seeding cancer cells on 3D porous scaffolds [8,9]. It was found that 3D microenvironment constructed by 3D porous scaffolds could not only promote the formation of tumor cell spheroids, but also the

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invasiveness and chemotherapeutic resistance of tumor cells cultured on 3D scaffolds were significantly improved compared with conventional 2D models [10,11]. At present, a variety of natural and synthetic scaffolds have been used to construct 3D porous scaffolds for the study of CSCs enrichment [10,12,13]. However, the most effective composition that enriches CSCs is still unclear.

In this study, glioma cell line U87 cells were grown on CS and CS-HA scaffolds. Cell viability, morphology and the expression of GSC biomarkers and EMT-related genes were analyzed under different culture conditions. Finally, we evaluated the *in vivo* tumorigenicity of cells derived from both scaffolds and compared with that of cells cultured under 2D conditions.

2. Materials and methods

2.1. Materials

Chitosan (practical grade, >90% deacetylated, MW = 250,000) was purchased from Haidebei (Shandong, China) and hyaluronic acid (sodium hyaluronate) was purchased from Freda (Shandong, China). Human glioma cell line U87 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) at 37 °C in humidified air with 5% CO_2.

2.2. Scaffolds synthesis

CS scaffolds and CS-HA scaffolds were prepared based on previously reported methods [14]. CS scaffolds were obtained by dissolving chitosan in dilute acetic acid and lyophilized. CS-HA scaffolds were prepared by forming a polyelectrolyte complex (PEC) between chitosan and hyaluronic acid molecules, and then froze and lyophilize the PEC solution. Briefly, for CS scaffolds, 2 wt% chitosan and 0.5 wt% acetic acid solution were stirred for 30 min to obtain a homogeneous CS solution. For CS-HA scaffolds, 4 wt% chitosan and 1 wt% acetic acid solution were stirred for 30 min to obtain a homogeneous CS solution, then 1 wt% hyaluronic acid solution dissolved in deionized water was gradually added in at an equal volume and mixed for 3 h to obtain a homogeneous CS-HA solution. Approximately 2 mL of CS solution or CS-HA solution was filled into each well of 24-well cell culture plate, froze overnight at −20 °C, 2 h at −80 °C, and lyophilized for 48 h using freeze dryer (LYOQUEST-85, TELSTAR). These scaffolds were cut into 2 mm thick discs, and then neutralized with 25v% ammonium hydroxide solution for 1 h. The residual ammonium hydroxide in scaffolds was removed by repeated washing with deionized water. The neutralized CS-HA scaffolds and CS scaffolds were again froze at −20 °C overnight and lyophilized for 48 h. Scaffold samples were sterilized by gamma irradiation.

2.3. Live/dead assay

Cell viability was evaluated by fluorescent live/dead assay kit (KeyGEN BioTECH, Nanjing, China) according to the protocol. Briefly, medium was removed from the wells and scaffolds were washed with PBS, followed by soaking in PBS solution containing 8 μM propidium iodide and 2 μM Calcein-AM. These scaffolds were cultured for 10 min at room temperature away from light, and washed with PBS. Cells were green (live cells) or red (dead cells). Images were obtained from fluorescence microscope (Olympus IX51,Tokyo, Japan). For each sample (n = 3), live and dead cells were counted at 200× in 5 random spots.

2.4. Scanning electron microscopy (SEM) analysis

Cell-free scaffolds and cell-laden scaffolds were fixed in 2.5% glutaraldehyde overnight at 4 °C. Samples were then dehydrated using a series of ethanol solutions (70%, 80%, 90%, 95%, 100%, and 100%) for 30 min in each solution, then critical point dried using carbon dioxide critical point dryer (LEICA, EM CPD300). Samples were sputter coated with platinum, and images were obtained from the ULTRA 55 Scanning Electron Microscope (ZEISS, Germany).

2.5. Quantitative RT-PCR

Cells were detached from porous scaffolds or well plates using versene and completely dissociated with Trizol (Invitrogen, 15,596−026), the total RNA was extracted following the manufacturer's protocol. Reverse transcription was performed using ImProm-IITM Reverse Transcription System (Promega, A3800). DNA transcripts were carried out using SYBR Green qPCR Super Mix. Thermo cycling was performed using ABI PRISM® 7500 Sequence Detection System and 18srRNA was used as the housekeeping gene. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

2.6. Flow cytometry analysis

2D and 3D cells were digested with trypsin at day 10 and a certain amount of single cell suspension was prepared with PBS. Cells were incubated with 10 μL anti-CD133-PE or mouse IgG-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^6 cells in dark at room temperature for 20 min. Subsequently, cells were washed with PBS and resuspended for preparation. The proportion of $CD133^+$ cells was analyzed with a BD FACSArial Flow Cytometer and results were evaluated by FlowJo software program (Treestar, Ashland, OR).

2.7. In vivo tumorigenicity analysis

Cells (1×10^4) were harvested from 2D cultures or 3D scaffolds on day 10 and resuspended in 200 μL serum-free medium with 50% Matrigel (Bedford, USA). Cells were inoculated subcutaneously into the right flank of BALB/c nude mice (4−6weeks old). All animal experiments were carried out in accordance with the approved program of the Animal Care Committee of the Second Affiliated Hospital of Soochow University. Tumorigenesis and growth were monitored three times a week. Tumor size was measured with a caliper and tumor volume (V) was calculated according to the following formula: $V = (L \times W^2)/2$, where L is length and W is width. All tumors were harvested at 6 weeks after inoculation.

2.8. Statistical analysis

Each experiment was carried out in three replicas, and all results are presented as the mean ± standard deviation. The Student's t-test was used to compare the means between two groups. Two-way analysis of variance (ANOVA) and a Bonferroni post-hoc test was used to compare multiple groups. Statistical significance was considered as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data analysis was performed using GraphPad Prism 7 software.

3. Results

3.1. Growth characteristics of glioma cell U87 cultured in 2D and 3D

Sections and macrostructure morphology of CS scaffolds and CS-HA scaffolds are shown in Fig. 1A and B. The average dimension of

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