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Enrichment of cancer stem cell-like cells by culture in alginate gel beads

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ABSTRACT

Cancer stem cells (CSCs) are most likely the reason of cancer reoccurrence and metastasis. For further elucidation of the mechanism underlying the characteristics of CSCs, it is necessary to develop efficient culture systems to culture and expand CSCs. In this study, a three-dimensional (3D) culture system based on alginate gel (ALG) beads was reported to enrich CSCs. Two cell lines derived from different histologic origins were encapsulated in ALG beads respectively and the expansion of CSCs was investigated. Compared with two-dimensional (2D) culture, the proportion of cells with CSC-like phenotypes was significantly increased in ALG beads. Expression levels of CSC-related genes were greater in ALG beads than in 2D culture. The increase of CSC proportion after being cultured within ALG beads was further confirmed by enhanced tumorigenicity in vivo. Moreover, increased metastasis ability and higher anti-cancer drug resistance were also observed in 3D-cultured cells. Furthermore, we found that it was hypoxia, through the upregulation of hypoxia-inducible factors (HIFs) that occurred in ALG beads to induce the increasing of CSC proportion. Therefore, ALG bead was an efficient culture system for CSC enrichment, which might provide a useful platform for CSC research and promote the development of new anti-cancer therapies targeting CSCs.

1. Introduction

Cancer development and progression is very complex. One of the most recent models of tumorigenesis assumptions is cancer stem cell (CSC) model. This model posits that cancer is the disease of stem cells (Mitrus et al., 2012). Organ-specific stem cells enable functioning of the whole organism and assure tissue renewal (Reya et al., 2001). Suffered with several factors, including DNA damage repair and change in tumor niche, accumulated mutations induce stem cells to change into CSCs (Li and Neaves, 2006). CSCs play an important role in tumor initiation, progression, and metastasis, and are responsible for high therapeutic failure rates. CSCs show enhanced motility, invasion, tumor-initiating ability, and resistance to chemotherapy (Visvader and Lindeman, 2008) which are related with the high mortality of cancer patients. Recently, it has been identified that CSCs exist within almost every kind of solid tumors (O'Brien et al., 2010) and are characterized as self-renew, quiescent, and low proportion (Al-Hajj et al., 2003; Lapidot et al., 1994). Identification and characterization of CSCs are crucial for facilitating the monitoring, therapy, or prevention of cancer.

The traditional two-dimensional (2D) cell culture, which is widely used in tumor studies, has been revealed to have limitations in the maintenance of CSCs in vitro (Eramo et al., 2007; Ricci-Vitiani et al., 2007). Because three-dimensional (3D) cell culture has the ability to better recapitulate the in vivo microenvironment than 2D cell culture, several attempts have successfully improved CSC properties by 3D culture with or without scaffolds. Because three-dimensional (3D) cell culture has the ability to better recapitulate the in vivo microenvironment than 2D cell culture (Ghajar and Bissell, 2010; Hirschhaeuser et al., 2010), several attempts have successfully improved CSC properties by 3D culture with or without scaffolds in several tumors originated from breast, ovary, kidney and melanocytes. When cultured within 3D, the CSC proportion increased accompanied with higher tumorigenicity and drug-resistance (Chen et al., 2012a, 2012b; Feng et al., 2013; Liu

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et al., 2012; Smith et al., 2011; Yang et al., 2013). However, since the spheroid formation without scaffolds lost several microenvironment factors that provided by biomimetic scaffolds, such as biophysical or biochemical factors, the morphology and characteristics of spheroids were differed from tumor tissues in vivo (Yang et al., 2013).

Hydrogels represent a class of materials suitable for numerous biomedical applications. They are hydrophilic and possess mechanical properties similar to those of native tissues and organs, which also makes them attractive for tissue engineering and regenerative medicine applications (Devolder and Kong, 2012). Several natural and synthetic hydrogels have been used in CSC research (Chen et al., 2012a; Smith et al., 2011; Yang et al., 2013). The goal of establishing a CSC culture system is to discover the mechanism of high drug resistance of CSCs and to serve as an anti-tumor drug screening model. To meet such needs, the culture system should be easy to reproduce, convenient to handle, and amenable for large-scale culture (Drury and Mooney, 2003). One such commonly used material is alginate gel (ALG) (Goh et al., 2012; Murua et al., 2008). As a natural polymer, alginate is suitable as a gel material due to good biocompatibility and lack of immunogenicity (Klöck et al., 1997). Although ALG beads were firstly reported in 1994 (Bugarski et al., 1994), and used widely in tissue engineering (David et al., 2004) and transplantation (de Vos et al., 2003; Lim and Sun, 1980), it has not been fully characterized and investigated in CSC research. ALG beads can be easily produced by gelation with some divalent cations without side effect on biological properties of tumor cells (Li et al., 2013). In addition, ALG beads can also be modified with cell-binding peptides, such as RGD or cadherins to investigate the cellular biological effect of the molecules on encapsulated cells (Dhoot et al., 2004; Sapir et al., 2011). And unlike other gel-based culture systems, cells cultured within ALG beads can also be harvested completely in a short time under physiological conditions for further investigation. So ALG bead culture system is still a convenient and efficient culture system for cell or tissue culture in vitro.

Moreover, our previous work showed that ALG bead provided an in vivo-like microenvironment for hepatocellular carcinoma (HCC) cells by improving the metastasis behavior in vitro (Xu et al., 2013). Higher metastatic prepotency is one of the main characteristics of CSCs (Li et al., 2007). In addition, when embryonic stem (ES) cells were cultured in alginate scaffolds, the stem potency was maintained in vitro without feeder cells or LIF (Maguire et al., 2006; Siti-Ismail et al., 2008). ES cells and CSCs use the similar signaling pathways to maintain their stemness (Visvader and Lindeman, 2008). Therefore, in this study, enrichment and functional properties of CSCs in ALG beads were investigated.

2. Materials and methods

2.1. Materials and cell lines

Sodium alginate was purchased from the Chemical Reagent Corp (Shanghai, China), whose viscosity was over 0.02 Pa s in 1% (w/v) aqueous solution at 20 °C. The human HCC cell line HCCLM3 was obtained from the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China) and human head and neck squamous cell carcinoma (HNSCC) cell line TCA8113 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

2.2. 2D and 3D cell culture

For 2D culture, HCCLM3 and TCA8113 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, UT, USA). All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. 2D cells were harvested when cultured near confluence.

For 3D culture, freshly harvested cells of the same batch of 2D cells were encapsulated in ALG beads by the method described previously with modifications (Bugarski et al., 1994). In brief, the cells (10^6 cells/ml alginate) were suspended in sterilized 1.5% (w/v) sodium alginate solution, extruded into 100 mM $CaCl_2$ using an electrostatic droplet generator and gelled for 30 min to form ALG beads. By adjusting parameters of the electrostatic droplet generator, we could obtain uniform-sized ALG beads with the average diameter of 330 ± 10 μm. And on average, 36 cells were encapsulated in an ALG bead. After culture, the 3D cell spheroids were harvested from ALG beads by simply treated with 55 mM sodium citrate solution, and then resuspended in culture medium for further testing. Single cell suspension of 3D cell spheroids was obtained by trypsinization and cell number was determined by direct cell counting using a hemocytometer.

2.3. Magnetic activated cell sorting (MACS)

Cells were magnetically sorted according to the manufacture's protocols. Certain amount of single-cell suspension from trypsinized 2D and 3D cells were prepared in PBS for labeling.

HCCLM3 cells were incubated with 20 μl anti-CD133 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 cells for 15 min at 4 °C. Subsequently, cells were washed once using washing buffer (Miltenyi Biotec), resuspended, and applied into MACS separation columns (Miltenyi Biotec). Positive (CD133+) and negative (CD133-) fractions were counted and resuspended for further experiments, respectively.

TCA8113 cells were incubated with anti-CD44-FITC (BioLegend, CA, USA) for 30 min on ice. After washing cells twice, 20 μl of anti-FITC microbeads (Miltenyi Biotec) per 10^7 cells was added for 15 min at 4 °C. Subsequently, cells were washed once, resuspended, and applied into MACS separation columns. Positive (CD44+) and negative (CD44−) fractions were resuspended in washing buffer for the second round of magnetic sorting. Cells were then magnetically labeled for the expression of ABCG2 (Santa Cruz Biotechnology, Inc., CA, USA) using MACS microbeads. By using MACS columns and separators, four groups of cell fractions were obtained according to the double labeling.

2.4. Quantitative real-time PCR

Total RNA was extracted using RNAiso Plus reagent (TaKaRa, Shiga, Japan) according to manufacturer's protocol. Reverse transcription was performed with the PrimeScript™ RT reagent Kit (TaKaRa). PCR amplifications were performed SYBR® Premix Ex Taq™ II (TaKaRa). The amplified signals were detected continuously with the Stratagene MX3000P (Agilent Technologies, CA, USA). Primers (listed in Table 1) used in this study were designed by Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). β-Actin was used as an internal control. The results were presented as the calculated comparative expression ratios of target sample to 2D cells for each sample by using C_T method ($2^{-\Delta\Delta C_T}$).

2.5. Drug sensitivity assay

The number of viable cells after drug treatment was assessed using CCK8 proliferation assay (Dojindo Laboratories, Kumamoto, Japan). Both 2D- and 3D-cultured cells were plated at a known number in 24-well plates, and the chemotherapeutic agent cisplatin (Hansoh Pharmaceutical Co., Lianyungang, China), 5-FU (Tianjin KingYork Amino Acid Co., Tianjin, China) and paclitaxel (Hansoh