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Original Articles

Establishment of a novel system for the culture and expansion of hepatic stem-like cancer cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is a major primary liver malignancy in adults. Despite the progress made, the outcome of the treatment to this disease is less than satisfactory as the post therapy tumor recurrence is almost inevitable. Accumulating pieces of evidence have suggested that the recurrence is due to the existence of a subpopulation of the HCC cells that possess the properties of stem cells and are resistant to radiation and chemotherapy. It is therefore important to understand the characteristics of this subpopulation of HCC cells, and which requires the establishment of an *in vitro* system to study these stem-like cancer cells. However, despite extensive efforts, the progress in establishing such an *in vitro* system has been slow largely due to the lack of definitive biomarkers in the isolation and expansion of these cells. In order to successfully maintain and expand HCC CSCs, we first optimized the culture system. We establish a novel medium system that allows the culture and enrichment of these hepatic stem-like cancer cells from both hepatoma cells and human primary HCC cells. These cells exhibited typical stem cell properties, such as enhanced stem cell markers, gain of EMT properties and drug resistance, and more importantly, stronger tumor-initiating capabilities. The medium may help to establish an *in vitro* model for hepatic cancer stem cell (HCSC) studies, which may contribute to the development of novel cell therapies and new drugs for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy in adults. It is also the fifth most common solid cancer worldwide and the third leading cause of cancer related death [1,2]. Currently, surgical resection is the main option for the treatment of HCC, but unfortunately, due to resistance to both radiation and chemotherapy, postoperative tumor recurrence is almost inevitable. There are two theories that attempt to explain the formation of HCC: the classical clonal evolution model and the cancer stem cell (CSC) or tumor-initiating cell (TIC) model. The foundation of the TIC model of HCC formation is that HCC is a disease of adult stem cells. The model postulates the existence of a subpopulation of tumor cells exhibiting the stem cell properties of self-renewal and differentiation but also showing an innate resistance to radiation and chemotherapy. These cells are often referred as hepatic cancer stem cells (HCSCs) and they are defined by their ability to initiate tumor when transplanted into immune-compromised mice (transplantability) and by their capacity to recapitulate the heterogeneity of original cells in the primary lesions where they are derived from. The TIC model has recently received wide attention because it provides an explanation for resistance to therapeutic intervention due to the quiescent or slow cycling HCSCs that may survive the treatment such as radiation and/or chemotherapy and result in recurrence. This model is also supported by clinical observations and experimental evidence, as a subpopulation of HCC cells possesses two functional properties that are widely accepted to be associated with CSCs: resistance to radiation and chemotherapy and efficient transplant ability [3].

To better understand the etiology of HCC and develop effective anticancer approaches, there is a clear need of *in vitro* model systems for the study of the HCSCs. In the general CSC field, three types of *in vitro* models have been established [4]: (a) subpopulations selected from existing tumor lines; (b) cell lines created from tumor or normal cells by genetic manipulation; and (c) CSCs selected from tumors or sorted tumor cells using defined serum-free conditions.

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There have been ongoing efforts in identifying and characterizing these cells. In the last decade, several promising specific cell surface markers for putative HCSCs have been identified including CD133 [5], CD90 [6] and EpCAM [7]. These markers have been used to negatively or positively select for a subpopulation of HCC cells that are capable of initiating tumor in immune-comprised mice, but still possess the resistance to radiation and cytotoxic agents. However, these markers showed different expression patterns in different subsets of tumor-initiating HCC cells, suggesting the lack of definitive markers for HCSC selection and the heterogeneity of these HCSCs, which may be activated by different signaling pathways in normal stem/progenitor cells where these HCSCs may originate. Another method of HCSC isolation is based on the differential efflux of fluorescent dyes, such as rhodamine 123 or Hoechst 33342. However, as Hoechst 33342 is cytotoxic, the enrichment of tumor-initiation abilities of side population (SP) cells is most likely due to an artifact of Hoechst 33342 toxicity, because these SP cells are protected by their membrane transport properties; whereas non-SP cells are unprotected and unable to grow due to the toxicity. Thus, the enrichment of tumor-initiating SP cells is unlikely due to intrinsic stem-cell properties. Recently, the physical properties of the HCC cells were applied to select HCSCs, in which these cells were reported to be enriched by density gradient centrifugation [8]. However, the quantity of the cells obtained is limited and the cells cannot be further enriched with this method.

The selection of CSCs by using defined serum-free culture conditions is a powerful approach to create *in vitro* models for CSC expansion and differentiation [9–11]. The strength of this method is the ability to define the correct environment (niche) required to maintain the stemness of the CSCs. This method has been successfully used to select and expand cell populations with CSC characteristics from patients with colon and lung cancers [12] and gliomas [13]. In this study, we developed a defined serum-free medium that is able to enrich and expand HCSCs, maintain their stem cell traits but are still capable of tumor initiation. After extensive screening, we selected a formula that allows us to culture HCSCs from both hepatoma cells and primary human HCC cells. These cultured cells exhibited up-regulated HCSC biomarkers such as CD90, enhanced epithelial–mesenchymal transition (EMT) properties, invasive potentials and resistance to chemotherapy drugs. More importantly, the tumor initiation capability of these cells was significantly increased. Taken together, the medium developed in this study allows us to effectively enrich the population of HCSCs from both hepatoma and human primary HCC cells, which may facilitate the study on HCSCs.

Materials and methods

Primary tumor cell culture

The protocol was approved by the Institutional Review Board of the Eastern Hepatobiliary Hospital, Second Military Medical University, Shanghai, China. The HCC biopsies were provided by the Department of Liver Surgery, Eastern Hepatobiliary Surgical Hospital, Shanghai, China. Written consents were obtained from patients prior to sample acquisition. The tumor tissues were washed and minced into fragments of $1 \times 1 \times 1$ mm^3 before being digested in 0.5 mg/ml type-I collagenase at 37 °C for 20–30 min to make a single cell suspension. Cells were then washed with D. Hanks solution twice, and 1×10^5/ml cells were seeded in petri dishes with 2–3 ml DMEM supplemented with 10% fetal bovine serum (FBS) or defined serum-free media, and cultured in a 5% CO_2 incubator at 37 °C.

Cell line culture, sphere formation and passage

Human hepatoma cell lines Hep3B and Huh7 were obtained from the American Type Culture Collection (ATCC). MHCC97-L, MHCC97-H [14], and HCCLM3 [15] were generous gifts from Shanghai Zhongshan Hospital (Shanghai, China). All of the cells were maintained in DMEM with 10% FBS, 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. For suspension sphere culture, cells were first washed with PBS to remove serum, and then suspended in seven defined serum-free media with formulas summarized in Table S1. Tumor cells at different passages were plated at ultralow attachment 6-well plates (Corning Inc., Corning, NY, USA) at a density of 5000 cells/well. The formation of tumor-spheres was observed under an inverted light microscope at 100× and 200× magnifications. The spheres were collected by gentle centrifugation, then dissociated with trypsin–EDTA and mechanically disrupted with a pipette. The resulting single cell suspension was then centrifuged to remove the trypsin–EDTA and re-suspended in serum-free medium to allow the re-forming of spheres. The spheres were passaged every 5–8 days before they reached a diameter of ~100 μm.

Cell cycle assays

Cell cycle analysis was performed after fluorescence labeling of the cellular DNA with propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA). After two rinses with cold PBS, HCC cells were harvested during the exponential growth phase, fixed with cold 70% ethanol, and then incubated at 4 °C for 4 hours. After centrifugation at 1000 rpm for 3 minutes and being rinsed with cold PBS, cells were resuspended in 1 ml PI stain (50 mM PI, 100 mg/ml RNase) and incubated at room temperature for 30 min. The cell cycle of the cells was then analyzed by FACS (BD, US).

Invasion assay

The invasive potentials of HCC and sphere-forming cells were compared by using BioCoat Matrigel invasion chambers (BD, Franklin Lakes, NJ) with 8 μm pore membrane filters in a 24-well culture plate by following the manufacturer's instruction. HCC cells were resuspended at a final concentration of 1×10^4 cells/ml in 0.1 ml DMEM containing 1% FBS in the upper chamber. The lower chambers were loaded with 0.6 ml DMEM containing 10% FBS. After 24 h or 48 h, cells that migrated through the Matrigel and attached to the bottom of the membrane were fixed and stained with crystal violet. Migrated cells in three different fields were counted. Three replicated experiments were performed and error bars represented the standard error of the mean.

In vivo *tumorigenesis assay*

The protocols for *in vivo* mouse xenograft model were approved by Medical Experimental Animal Care Unit of the Second Military Medical University. Six to eight week-old BALB/c nude mice were provided by Shanghai SLAC Laboratory Animal Co. Ltd of Chinese Academy of Sciences, and the mice were maintained in a pathogen-free condition. The defined number of parental and sphere-forming cancer cells mixed with 0.1 ml serum-free DMEM/Matrigel (1:1) was inoculated in the left or right flank of mice. Tumor growth was monitored every 3 days starting on the third week after the inoculation. The mice were sacrificed at day 90 or when the tumors grew to a maximum of 1000 mm^3. Tumor xenograft volume in mm^3 was calculated by the formula: Volume = $(\text{width})^2 \times \text{length}/2$.

Chemotherapy sensitivity assays

The sensitivities of the Hep3B and HuH7 parental and sphere-forming cells to chemotherapeutic drugs were measured by Cell Counting Kit-8 (CCK-8) assay using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium. Briefly, 4000 cells/well were seeded in 96-well plates, and various concentrations of doxorubicin (Sigma-Aldrich) were added at the beginning. After 72 h incubation, viable cells were measured by CCK-8 assay by following the manufacturer's instruction.

Quantitative reverse transcription PCR (qRT-PCR)

RNA samples were extracted from both the parental and the sphere cells, cDNA were synthesized using the Superscript III first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). qRT-PCR was carried out using SYBR Green PCR Master Mix. The PCR protocol was: 95 °C for 10 min, followed by 40 cycles of 95 °C 15 s, 60 °C 60 sec and 72°C 45 sec. The following primers were used: Wnt-1, 5′-TAAGCAGGTTCGTGGAGGAG-3′ (forward) and 5′-GGTTTCTGCTACGCTGCTG-3′ (reverse); CD90, 5′-TGAAGGT CCTCTACTTATCCGC-3′ (forward) and 5′-GCACTGTGACGTTCTGGGA-3′ (reverse). EpCAM, 5′-GCTGGGGGAGGGGAGCCTAC-3′ (forward) and 5′-ACTGCTCACCTCTGGCCGCT-3′ (reverse). NOTCH1, 5′-GTTGGGGTCCTGGCATC-3′ (forward) and 5′-GGTGAGA CCTGCCTGAATG-3′ (reverse). NOTCH2, 5′-CACAGGGTTCATAGCCATCTC-3′ (forward) and 5′-GGAGGCGACCGAGAAGAT-3′ (reverse). NOTCH3, 5′-CCTGAGTGACAGGGGTCCT-3′ (forward) and 5′-TGTGCAAATGGAGGTCGTT-3′ (reverse). Oct4, 5′-GAACCATACT CGAACCACATCC-3′ (forward), 5′-CGTTCTCTTTGGAAAGGTGTTC-3′ (reverse). Nanog, 5′-TTTGGAAGCTGCTGGGGAAG-3′ (forward), 5′-GATGGGAGGAGGGGAGAGGA-3′ (reverse). CD133, 5′-AGAGCTTGCACCAACAAAGTACAC-3′ (forward), 5′-AAGCA CAGAGGGTCATTGAGAGA-3′(reverse). CD44, 5′-TGCCGCTTTGCAGGTGTAT-3′ (forward), 5′-GGCCTCCGTCCGAGAGA-3′ (reverse). Beta-Actin, 5′-ATCTGGCACCACACCTTCTACAA-3′ (forward), 5′-GTACATGGCTGGGGTGTTGAAG-3′ (reverse). Relative gene-expression quantification method was used to calculate the fold change of mRNA expression according to the comparative C_t method using β-actin as an endogenous control. Final results were determined as: $2^{-(\Delta Ct\ \text{sample}-\Delta Ct\ \text{control})}$. Data were represented as ratio or fold changes to parental cells.