SK-Hep1: not hepatocellular carcinoma cells but a cell model for liver sinusoidal endothelial cells

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Received February 1, 2018; Accepted March 18, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: SK-Hep1 cells serve as a cell model of hepatocellular carcinoma and hepatocyte biology. However, SK-Hep1 cells are markedly different from normal hepatocytes and other hepatocellular carcinoma cells in their gene expression and protein levels. Furthermore, endothelial-specific markers and morphological characteristics are found in SK-Hep1 cells, indicating an endothelial origin. To confirm their cell phenotype, we investigated and compared the surface ultrastructure, endothelial function, and molecular markers of SK-Hep1 cells in vitro and in vivo. The results revealed that SK-Hep1 cells expressed endothelial-specific makers and exhibited the endothelial function of endocytosis and tubular formation. Capillary-like structures with CD31 expression were also observed in SK-Hep1 allografts in nude mice. Moreover, SK-Hep1 cells possessed fenestrae without diaphragms, consistent with liver sinusoidal endothelial cells, as seen by electron microscopy. In conclusion, SK-Hep1 cells would be better considered a cell model for liver sinusoidal endothelial cells instead of hepatocellular carcinoma cells.

Keywords: Hepatocellular carcinoma, liver sinusoidal endothelial cell, fenestration, endocytosis, tubular formation

Introduction

SK-Hep1 is a permanent cell line derived from the ascitic fluid of a 52-year-old male Caucasian with hepatic adenocarcinoma, according to the American Type Culture Collection (ATCC). It has widely served as a cell model of hepatocellular carcinoma (HCC) and hepatocyte biology since its establishment in 1971 [1]. However, no hepatic-specific genes, including albumin and α- and γ-fibrinogen, were expressed in SK-Hep1 cells [2]. In addition, SK-Hep1 cells were markedly different from normal hepatocytes and other HCC cells by proteomic analysis [3, 4]. Impressively, SK-Hep1 cells showed positive expression for endothelial markers, including von Willibrand factor (vWF) and endothelial leukocyte adhesion molecule-1 (ELAM-1). Additionally, the ultrastructure of pinocytotic vesicles and organelles consistent with Weibel-Palade bodies under transmission electron microscopy (TEM) implied the endothelial origin of SK-Hep1 cells [2].

Although they were known to be of endothelial rather than HCC origin as early as 1992, the identification phenotype of SK-Hep1 cells remains undetermined due to their limited availability and outdated studies. Therefore, a comprehensive and systematic exploration of the SK-Hep1 phenotype is necessary. Considering the biological distinctions between endothelial cells and epithelial (HCC) cells, conclusions from previous studies using SK-Hep1 cells as an HCC cell model are doubted. To eliminate misinterpretations, the differences between SK-Hep1 cells and conventional HCC cells in structural and functional properties also need further investigation.

Liver sinusoidal endothelial cells (LSECs) form the barrier of the liver sinusoids and play a crucial role in substance exchanging between hepatocytes and sinusoids [5]. Research on LSECs has helped to understand the complex network of sinusoidal cellular interactions in both physiological and pathophysiological sta-
LSEC phenotypes of SK-Hep1

tes [6-8]. Primary LSECs isolated from rodents undergo capillarization and lose their endothelial characteristics during culture [9]. Conventional endothelial cell lines (e.g., human umbilical vein endothelial cells, HUVECs) cannot accurately imitate LSECs biology in vitro due to the lack of fenestrae. Therefore, the identification of an immortal endothelial cell line with fenestrae would contribute substantially to the research of LSECs. Interestingly, numerous pores consistent with fenestrae were found on the membrane of SK-Hep1 cells using scanning electron microscopy (SEM) [10]. Their ultrastructural similarity makes them a potential cell model for LSECs biology in vitro. Nevertheless, whether the membrane fenestrae is an unique feature of SK-Hep1 cells or a general feature of immortal endotheliocytes or HCC cells deserves further clarification.

To confirm the cell phenotype of SK-Hep1, we investigated the surface ultrastructure, endothelial function and molecular markers of SK-Hep1 cells in vitro and in vivo. In addition, the ultrastructural and functional characteristics of conventional endothelial cells and HCC cells were also compared in the study.

Materials and methods

Cell culture

SK-Hep1 cells were obtained from the Procell Life Science and Technology Co., Ltd. (Wuhan, China). Their identity as SK-Hep1 cells was verified by short tandem repeat (STR) locus analysis. HUVECs and human HCC cells Bel7402 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SK-Hep1, HUVECs and Bel7402 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Cromwell, CT, USA), 100
U/ml penicillin, and 100 μg/ml streptomycin (HyClone) in a humidified atmosphere at 37°C with 5% CO₂ in air.

**Scanning electron microscopy**

SK-Hep1, HUVECs, and Bel7402 cells were grown on coverslips coated with Matrigel (BD Biosciences, San Jose, CA, USA) at the bottom of 24-well plates. Once the cells were adhered to the coverslips, they were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer with 1% sucrose. Then, cells on coverslips were dehydrated in an ethanol gradient. After the critical point drying, the coverslips were coated with platinum and examined by SEM (JSM-5900LV, JEOL, Tokyo, Japan).

**Immunofluorescence staining**

SK-Hep1 cells were cultured on coverslips at the bottom of 24-well plates. The cells were fixed with 4% paraformaldehyde before permeabilization with 0.1% Triton X-100. After being blocked with 10% goat serum, the cells were incubated with primary antibodies against endothelial nitric oxide synthase (eNOS, 1:100, SAB, Baltimore, MD, USA), vascular endothelial growth factor (VEGF, 1:50, Proteintech, Wuhan, China), VEGF receptor 2 (VEGFR2, 1:100, Proteintech, Wuhan, China), vWF (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CD31 (1:50, Santa Cruz Biotechnology) overnight at 4°C followed by incubation with TRITC-conjugated secondary antibodies (1:200, ZSGB-BIO, Beijing, China) at room temperature for 45 minutes. Cell nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, Roche, Basel, Switzerland). A negative control, in which the primary antibodies were omitted, was included in all assays. Finally, the coverslips were mounted with anti-fading medium and visualized with a fluorescence microscope (BX41, Olympus, Tokyo, Japan).

**Endocytosis of Dil-Ac-LDL and FITC-albumin (FITC-ALB)**

For endocytosis evaluation, SK-Hep1 cells were cultured on coverslips at the bottom of 24-well plates. Once the cells were 60-70% confluent, Dil-Ac-LDL (Yiyuan Biotechnology, Guangzhou, China) and FITC-ALB (Bioss, Beijing, China) were added at a concentration of 20 μg/ml each, and the cells were incubated at 37°C for 4 hours in the dark. Cells were then washed with PBS and fixed with 4% paraformaldehyde. After cell nuclei were stained with DAPI, the coverslips were mounted with anti-fading medium and examined with the fluorescence microscope.

**Tubular formation assay**

SK-Hep1, HUVECs, and Bel7402 cells were seeded at a density of 5×10⁴-10⁵ cells per well in Matrigel coated 24-well plates. After culturing for 4 hours, the cells were visualized, and photographs were taken under the inverted phase-contrast microscope (IX71-22PH, Olympus).

**SK-Hep1 xenografts in nude mice**

Six healthy male BALB/c nude mice, weighting 18-23 g, were obtained from the Experimental Animal Center of Sichuan University (Chengdu, China). The mice were kept under 12-hour light-dark cycles at a constant temperature and humidity with free access to chow and water. Briefly, 5×10⁶ of SK-Hep1 cells dissolved in normal saline were subcutaneously injected into the back of nude mice. All mice were sacrificed under anesthesia, and xenograft tumors were dissected after 2 weeks. Tumor tissues were fixed in 4% paraformaldehyde for histopathologic and immunohistochemical examinations. The animal experiment was approved by the Animal Use and Care Committee of West China Hospital, Sichuan University, and was conducted according to the regulations.

**Histopathologic and immunohistochemical (IHC) analysis**

Tumor tissues were processed for paraffin embedding and 3 μm of sections. Sections were deparaffinized in xylene, rehydrated with graded ethanol dilutions, and then stained with hematoxylin-eosin. For IHC staining, antigen retrieval was performed at high temperature under high pressure in 10 mM sodium citrate buffer. After blocking with H₂O₂ and 10% goat serum, the sections were incubated with rabbit anti-CD31 (1:100, Santa Cruz Biotechnology) overnight at 4°C followed by incubation with biotin-streptavidin HRP detection (ZSGB-BIO) at room temperature for 30 minutes. Finally, sections were stained with a solution of 3,3’-diaminobenzidine (DAB, ZSGB-BIO) and counterstai-
had attached to the surface of Matrigel. Under high magnification, the surface of SK-Hep1 cells possessed numerous pores of various sizes, consistent with fenestrae. Some appeared to be organized into sieve plates, remarkably different from the appearance of HUVECs and Bel7402 cells. There were few pseudopodia on the surface of SK-Hep1 cells or HUVECs, whereas plenty of microvilli were found on Bel7402 cells (Figure 1).

Expression of endothelial makers in SK-Hep1 cells

On immunofluorescence analysis, SK-Hep1 cells had positive staining and cytoplasmic expression for endothelial-specific proteins, including eNOS, VEGF, VEGFR2 and vWF, with negative staining for CD31 (Figure 2).

Uptake of Dil-Ac-LDL by SK-Hep1 cells

After incubation with Dil-Ac-LDL for 4 hours, there were bright red dots in the cytoplasm of SK-Hep1 cells under fluorescence microscope, indicating extensive cytoplasmic uptake of Dil-Ac-LDL into the SK-Hep1 cells. However, no green fluorescence was observed in SK-Hep1 cells incubated with FITC-ALB (Figure 3A).

Tubular formation by SK-Hep1 cells

Consistent with HUVECs features, SK-Hep1 cells formed a tubular network on the surface of Matrigel within 4 hours of plating without reagent induction, whereas Bel7402 cells showed no traces of tubular formation (Figure 3B).

Tumorigenic capacity of SK-Hep1 cells

Two weeks after cells injection, the xenograft tumor models were successfully established in

Results

Endothelial cell morphology of SK-Hep1 cells

SK-Hep1 cells grew against the wall of the flask and approached confluence rapidly in DMEM. SK-Hep1 cells showed spindle and polygon shapes similar to those of mesenchymal cells under light microscopy after adhesion. When viewed by SEM at low magnification, SK-Hep1 cells and HUVECs were stretched and flattened with extending tentacles, whereas Bel7402 cells were ovoid or globular in shape after they

Figure 2. Expression of endothelial markers in SK-Hep1 cells. Immunofluorescence analysis showing positive staining for eNOS, VEGF, VEGFR2, and vWF (×400 magnification).
all six nude mice (Figure 4A), exhibiting the tumorigenic capacity of SK-Hep1 cells. Histological sections showed that the nucleus was flattened and the cytoplasm formed tubular structures containing lumen, consistent with capillary formation (Figure 4B). The newly formed capillaries expressed CD31 by immunohistochemical staining (Figure 4C, 4D).

Discussion

SK-Hep1 has long been recognized as a cell model for HCC and hepatocyte biology [11-13]. However, prominent differences between SK-Hep1 and other HCC cells have been found in gene expression and protein levels. In this study, we made efforts to identify the cell phenotype of SK-Hep1 comprehensively and systematically in ultrastructure, endothelial function, molecular markers, and in vivo tumorigenic capacity. Consistent with previous study [10], we found that SK-Hep1 cells had positive expression of eNOS, vWF, VEGF, and VEGFR2. Both eNOS and vWF are specific makers of endothelial cells, including but not limited to LSECs [5, 14]. In addition to endothelial cells, VEGF and VEGFR2 have been found in cultured HCC cells [15, 16], making them non-specific makers to distinguish SK-Hep1 from conventional HCC cells. CD31 is an intercellular adhesion molecule classically expressed at the surface of endothelial cells, which is absent from hepatic sinusoid and isolated LSECs.
and becomes expressed gradually in capillarized LSECs along with the disappearance of fenestrae [17]. Our study revealed that cultured SK-Hep1 cells in vitro showed negative staining for CD31. In allografts in nude mice, SK-Hep1 cells formed capillary-like structures, and CD31 expression was detected in newly formed vessels by IHC. Positive expression of endothelial markers demonstrated the endothelial origin of SK-Hep1 cells, and unique expression pattern of CD31 in vitro and in vivo was in accordance with LSECs.

A striking endocytic activity represents a functional hallmark of endothelial cells. LSECs exhibit the highest endocytic activity in the body by clearing waste from the blood and transferring molecules from the sinusoids to the Disse space. Primary LSECs endocytose Dil-Ac-LDL and FITC-labeled formaldehyde-treated serum albumin (FITC-FSA), but they partially lose their endocytic capacity during culture in vitro [9]. Dil-Ac-LDL and FITC-ALB were added as labeled biomolecules for endocytosis evaluation in our study. The results showed that there was extensive cytoplasmic uptake of Dil-Ac-LDL into the SK-Hep1 cells, indicating high and rapid endocytic activity. Endocytosis of acetylated lipoproteins was first shown in SK-Hep1 cells and was consistent with a virally transformed immortal LSEC line [18]. Although SK-Hep1 cells are able to take up FITC-FSA [10], no fluorescence signal was observed in SK-Hep1 cytoplasm incubated with FITC-ALB in this study, indicating the selective endocytosis of labeled molecules by SK-Hep1 cells.

The capacity to form tubular structures in vitro is another functional property of endothelial cells and provides the pathophysiological basis of angiogenesis during the process of liver fibrosis and portal hypertension [19]. This study compared the capacity of tubular formation in SK-Hep1, HUVECs, and Bel7402 cells. Given an abundant and complex extracellular matrix substrate for growth, SK-Hep1 cells were able to produce capillary-like structures, similar to the morphology of immortal HUVECs (which are endothelial) [20]. In contrast, the conventional HCC cell line Bel7402 failed to form tubular structures on the surface of Matrigel within the same time frame. The newly formed capillaries with CD31 expression in SK-Hep1 allografts in nude mice confirmed the capacity of tubular formation in vivo. Combined with the tumorigenic capacity in nude mice, SK-Hep1 cells would be a valuable model for studying angiogenesis in vivo and in vitro. The typical endothelial function of endocytosis and tubular formation provided additional evidence that SK-Hep1 is of endothelial origin.

Fenestrae in the ultrastructure, which perforate in continuous cytoplasm and provide the structural basis for substance exchange, is the only specific feature of LSECs distinguishing them from conventional endothelial cells (e.g. HUVECs). In this study, a LSEC-specific ultrastructure was confirmed in SK-Hep1 cells. When viewed by SEM, both SK-Hep1 cells and HUVECs presented stretched and flattened with extending tentacles, compared to the globular shape with abundant microvilli of Bel7402 cells. In addition, numerous pores of various sizes were organized in sieve plates, consistent with fenestrae, were observed on the surface of SK-Hep1 cells, whereas HUVECs and Bel7402 cells presented with consecutive cytoplasm. The fenestrae of LSECs rapidly disappear once isolated and cultured, making it impossible to maintain their innate biology in vitro [9]. However, our study revealed that cultured SK-Hep1 cells maintained their fenestration persistently without reagent induction. Based on these findings, SK-Hep1 cells could potentially be a cell model for LSECs in vitro, since they possess membrane fenestrae with endothelial origin, which are akin to LSECs features.

Lacking suitable cell models, primary dissociated cells cultured from rats and mice have been the major materials in which LSECs biology has been studied, though with great difficulty and low yield. The capillarization and short survival of these cells restrict their application in LSECs studies [9]. To overcome the disadvantage of culturing primary LSECs, considerable efforts have been made to develop human and murine immortalized LSEC lines. Transfection with lentivirus (SV40 and hTERT) [18, 21] or large tumor antigen [22] has successfully induced an immortal phenotype of human LSECs. However, the fenestrae disappear during the immortalization process in majority of the established cell lines. An immortalized LSEC cell line named M1LEC, induced by virus antigen transduction, revealed fenestrae
under SEM, but it failed to maintain fenestration unless co-cultured with other cells [23, 24]. Encouragingly, the SK-Hep1 cell line inherently possesses fenestrae and can maintain fenestrae persistently without reagent induction, making it a desirable cell model for LSECs.

According to the above, SK-Hep1 cells were remarkably different from conventional HCC cells in ultrastructure, endothelial function and molecular markers. In contrast, SK-Hep1 cells expressed endothelial-specific makers and exhibited the endothelial function of endocytosis and tubular formation. Moreover, SK-Hep1 cells possessed fenestrae consistent with LSECs. They should be considered an immortal human LSECs line instead of HCC cell line.

Acknowledgements

This work was supported by National Natural Science Foundation of China (U1702281, 81670551), Chinesisch-Deutsches Zentrum für Wissenschaftsförderung (GZ1065), the Science and Technology Support Program of Sichuan province (2016SZ0041), and National Key R&D Program of China (2017YFA0205400).

Disclosure of conflict of interest

None.

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