Proliferation of sphere-forming hepatocellular carcinoma cells is suppressed in a medium without glucose and arginine, but with galactose and ornithine

MINORU TOMIZAWA[1], FUMINOBU SHINOZAKI[2], YASUFUMI MOTOYOSHI[3], TAKAO SUGIYAMA[4], SHIGENORI YAMAMOTO[5] and NAOKI ISHIGE[6]

Departments of [1]Gastroenterology, [2]Radiology, [3]Neurology, [4]Rheumatology, [5]Pediatrics and [6]Neurosurgery, National Hospital Organization, Shimoshizu Hospital, Yotsukaido, Chiba 284-0003, Japan

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Abstract. Resistance to sorafenib in hepatocellular carcinoma (HCC) cells exhibiting stemness was evaluated using a sphere formation assay. A hepatocyte selection medium (HSM) deficient in glucose and arginine was used to suppress the proliferation of cell spheres composed of HLF and PLC/PRF/5 HCC cells, which were subjected to a sphere formation assay. Cell spheres were cultured with sorafenib and subjected to a cell proliferation assay and the expression levels of cytochrome P450 (CYP3A4) were analyzed in RNA extracted from sphere-forming cells using reverse transcription-quantitative polymerase chain reaction. Sphere-forming PLC/PRF/5 cells were more resistant to sorafenib, as compared with control cells, exhibiting higher expression levels of CYP3A4. When cultured in HSM, suppressed proliferation was observed in the sphere-forming PLC/PRF/5 cells and in the control cells, with no significant variation between them. The results suggest that deprivation of glucose and arginine is a potential novel treatment for HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common cancer of the liver with a typically poor prognosis (1). The current treatment for HCC involves local ablation, surgical resection, transcatheter arterial chemoembolization and systemic administration of chemotherapeutic agents (2,3). Molecular therapy with sorafenib has also previously been established as a viable therapeutic option for HCC (4). Sorafenib is a multikinase inhibitor with a high antitumor efficacy that suppresses cell proliferation and induces apoptosis in HCC cell lines (5-7). A limitation of this approach is that HCC cells acquire resistance to sorafenib (8,9), as it is metabolized by cytochrome P450 (CYP3A4) (10). This has necessitated the development of alternative therapies for HCC.

Cancer cells have increased glycolysis levels, requiring more glucose under insufficient oxygen supply (Warburg effect), compared with surrounding normal tissues (11). Glucose is essential for cell survival (12,13). Galactose is metabolized to galactose-1-phosphate with galactokinase, and enters the glycolysis cycle (14). Arginine is an essential amino acid that is produced from ornithine by ornithine carbamoyltransferase in the urea cycle (15). Normal hepatocytes express galactokinase and ornithine carbamoyltransferase (16,17). Normal hepatocytes are expected to survive in a medium lacking glucose and arginine but supplemented with galactose and ornithine (18,19). In our previous study, a hepatocyte selection medium (HSM) was developed, which lacks glucose and arginine but contains galactose and ornithine (20). Primary human hepatocytes are able to survive in HSM; thus, this medium selects primary human hepatocytes from a co-culture with human-induced pluripotent stem cells (21).

Cancer stem cells are characterized as self-renewing, proliferative, tumorigenic (exhibiting stemness) and chemoresistant (22). The sphere formation assay was previously reported to enrich undifferentiated neural precursor cells (23,24); however, several types of human cancer cells have been demonstrated to form spheres (25,26). HCC cells (PLC/PRF/5 cells), human breast cancer cells (MCF7 cells), glioma cells (U87) and non-small lung cancer cells (A549) form spheres when cultured in serum-free conditions supplemented with epidermal growth factor, at a density of $1x10^3$ cells/ml. Notably, the expression levels of certain stem cell markers increase, including Oct3/4, CD133, and CD44 (26). These reports suggest that cancer cells with stem cell character are enriched in a sphere formation assay.

In the present study, HCC cells were cultured for a sphere formation assay to obtain stem cell character in HCC cells. The resultant cells were cultured in HSM to investigate the potential application to treatment of HCC cells with stem cell character.

Correspondence to: Dr Minoru Tomizawa, Department of Gastroenterology, National Hospital Organization, Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido, Chiba 284-0003, Japan
E-mail: nihminor-cib@umin.ac.jp

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Materials and methods

Cell culture. HLF and PLC/PRF/5 human HCC cells were purchased from the Riken Cell Bank (RIKEN BioResource Center, Tsukuba, Japan). HLF cells and PLC/PRF/5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell lines were cultured in 10-cm dishes (Asahi Techno Glass; Funabashi, Japan) with 5% CO_2 at 37˚C in a humidified chamber.

Sphere formation assay. HLF and PLC/PRF/5 cells were trypsinized and cultured in DMEM-F12 (Sigma-Aldrich; Merck Millipore) supplemented with epidermal growth factor (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 20 ng/ml, basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd.) at 20 ng/ml, 1% B27 supplement (Thermo Fisher Scientific, Inc.) and 1% methylcellulose 25 (Wako Pure Chemical Industries, Ltd.) in Ultra-Low Attachment 6-well plates (Corning Incorporated, Corning, NY, USA). The cells were imaged under a microscope (Olympus Corporation, Tokyo, Japan) after 7 and 14 days of incubation.

Preparation of HSM. HSM was prepared from amino acid powders using the formulation of Leibovitz L-15 medium (Thermo Fisher Scientific, Inc.), omitting arginine, tyrosine, glucose and sodium pyruvate, but adding galactose (900 mg/l; Wako Pure Chemical Industries, Ltd.), ornithine (1 mM; Wako Pure Chemical Industries, Ltd.), glycerol (5 mM; Wako Pure Chemical Industries, Ltd.) and proline (260 mM; Wako Pure Chemical Industries, Ltd.). Proline (30 mg/l) was included in the medium as it is necessary for DNA synthesis (27). Knockout serum replacement (Thermo Fisher Scientific, Inc.) at a final concentration of 10% was used in place of FBS to establish xeno-free conditions. PRL/PRF/5 cells were subjected to a sphere formation assay. The spheres were transferred to a tube, trypsinized, and seeded onto 96-well flat-bottom plates (Asahi Techno Glass) at the density of 1,000 cells/well. After a 24-h culture in DMEM, HSM was applied, and subjected to a cell proliferation assay. PRL/PRF/5 cells cultured in DMEM in conventional 6-well plates (Asahi Techno Glass) were used as a control.

Application of sorafenib. PRL/PRF/5 cells were analyzed to determine whether cell proliferation was suppressed by sorafenib. Following sphere formation, the cells were seeded to 96-well flat-bottom plates at a density of 1,000 cells/well and incubated for 24 h in DMEM supplemented with 10% FBS. Sorafenib (JS Research Chemicals Trading e.Kfm, Wedel, Germany) was added to the media at concentrations of 0, 1, 3, 10 and 30 μM. PRL/PRF/5 cells cultured in DMEM in conventional 6-well plates (Asahi Techno Glass) were used as a control.

Cell proliferation analysis. The cells were cultured for 72 h and subjected to an MTS assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The cells reduce MTS to a colored formazan product that has a maximum absorbance at 490 nm. The absorbance was measured using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (5 mg) was isolated using Isogen (Nippon Gene, Co, Ltd., Tokyo, Japan) and used for the first-strand cDNA synthesis with SuperScript III and oligo-dT (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. RT-qPCR was performed with Fast SYBR Green Master Mix (Thermo Fisher Scientific, Inc.), and the results were analyzed using the MiniOpticon system (Bio-Rad Laboratories, Inc.). RT-qPCR was performed for a total of 40 cycles, with 5 sec of denaturation and 5 sec of annealing-extension. The RT-qPCR primer pairs for ribosomal protein L19 (RPL19) and CYP3A4 were 5'-CGAATGCCAGAGAAG GTCAC-3' (forward) and 5'-CCATGAGAATCCGCTTGT TT-3' (reverse) (GenBank, BC000530; expected product size, 157 bp), 5'-TGAGAAATCTGAGGCGGGAAGC-3' (forward) and 5'-CGATGTTCACTCCAAATGATGTGC-3' (reverse) (GenBank, J04449; expected product size, 111 bp), respectively. RPL19 was used as an internal control, as the target gene was a constitutively expressed housekeeping gene (28). The primer sequences were obtained with MacVector 14.0.6 (MacVector Inc., Apex, NC, USA).

Statistical analysis. The data were presented as the mean ± standard deviation. The data were analyzed by one-way analysis of variance using JMP 10.0.2 software (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant result.

Results

Sphere formation. In order to investigate cell sphere formation, HLF cells and PLC/PRF/5 cells were cultured for sphere formation for 7 or 14 days. HLF cells did not form spheres after 7 days (Fig. 1A) or 14 days (Fig. 1B) of incubation; however, sphere formation was observed in PLC/PRF/5 cells after 7 days (Fig. 1C) and 14 days (Fig. 1D) of incubation. PRL/PRF/5 cells were used for further investigation.

Sorafenib. In order to analyze the suppression of proliferation of PRL/PRF/5 cells after sphere formation, the cells were subjected to cell proliferation assay (Fig. 2). PRL/PRF/5 cells cultured in conventional 6-well plates were used for control. There was a tendency for sorafenib to be more tolerated by the PRL/PRF/5 cells following sphere formation than the control cells. At 10 μM, cell proliferation of sphere-formed cells and control cells were 137±24 and 59±17%, respectively ($P<0.0036$). The results suggest the PLC/PRF/5 cells that form spheres are more resistant to sorafenib compared with cells cultured in 6-well plates, which did not form spheres.

Expression levels of CYP3A4. In order to analyze changes in the expression levels of CYP3A4, the sphere-forming PLC/PRF/5 cells were subjected to RT-qPCR (Fig. 3). The expression levels of CYP3A4 were significantly increased after 7 ($P<0.0001$) and 14 days ($P<0.001$) of incubation, as compared with day 0.

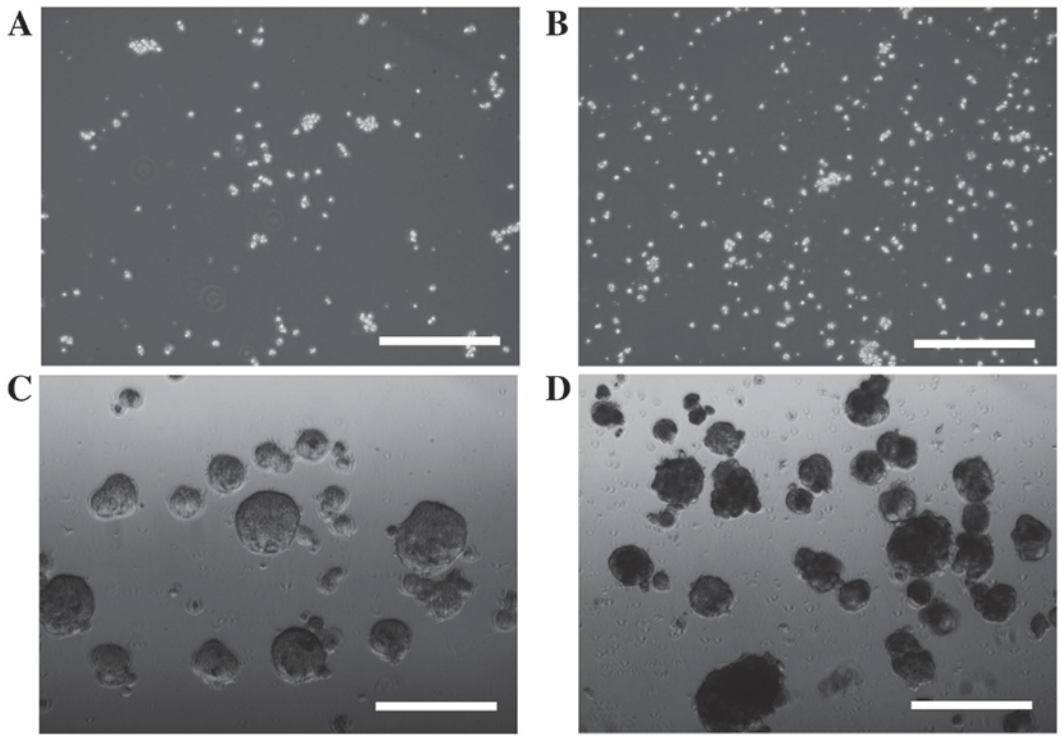

Figure 1. Spheroid formation assay. (A and B) HLF cells and (C and D) PLC/PRF/5 cells were cultured on 6-well plates or as cell spheres. The cells were imaged under a microscope after (A and C) 7 days and (B and D) 14 days of incubation. The experiments were repeated three times. Scale bar, 100 μm.

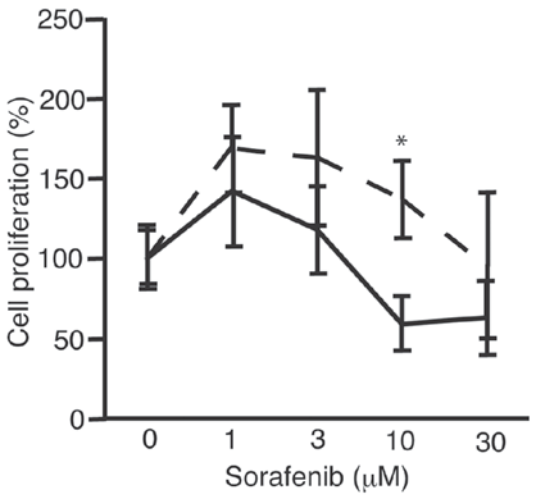

Figure 2. Cell proliferation assay. PLC/PRF/5 cells were cultured in 6-well plates (solid line) or as cell spheres (dash line), plated onto 96-well plates, with or without the addition of sorafenib (1, 3, 10 and 30 μM) to the media and subjected to an MTS assay. The cell proliferation rate of sphere-formed cells and control cells was 137±24 and 59±17%, respectively (P<0.0036), at 10 μM. Error bars, standard error; *P<0.05, vs. cells cultured in 6-well plates; n=4.

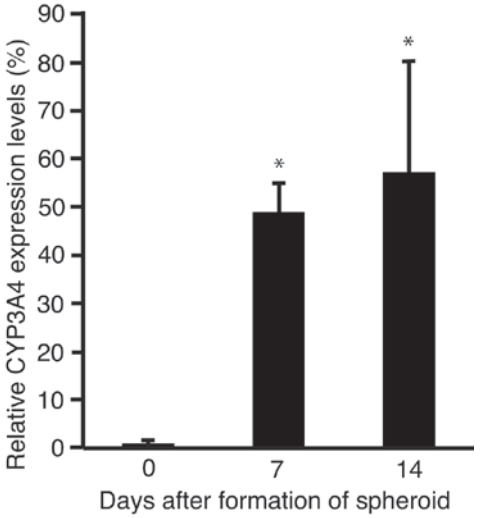

Figure 3. Expression levels of CYP3A4. To analyze the expression levels of CYP3A4, total RNA was isolated for the RT-qPCR of CYP3A4 at 7 and 14 days following the spheroid formation assay with PLC/PRF/5 cells. The expression levels of CYP3A4 were increased after 7 (P<0.0001) and 14 days (P<0.001) of incubation, as compared with day 0 with statistical significance. Error bars, standard deviation; *P<0.05, vs. day 0; n=3.

Suppression of cell proliferation with HSM. In order to compare the suppression of cell proliferation cells between after sphere formation and conventional culture, PRL/PRF/5 cells were seeded onto 96-well plates. The medium was changed to HSM, and subjected to cell proliferation assay after 72 h culture (Fig. 4). For cells cultured using a conventional method, cell proliferation decreased to 25±11% in HSM, as compared with DMEM (P<0.0001). For cells analyzed following sphere formation, it was observed that cell proliferation decreased to 32±28% in HSM, as compared with DMEM (P=0.0011). There was no statistically significant difference in cell proliferation between cells analyzed following sphere formation and those cultured in HSM (P=0.7100). These results suggest that HSM suppressed cell proliferation of PRL/PRF/5 cells following sphere formation at the same levels as conventional culture. The HSM created in the present study, which did not contain

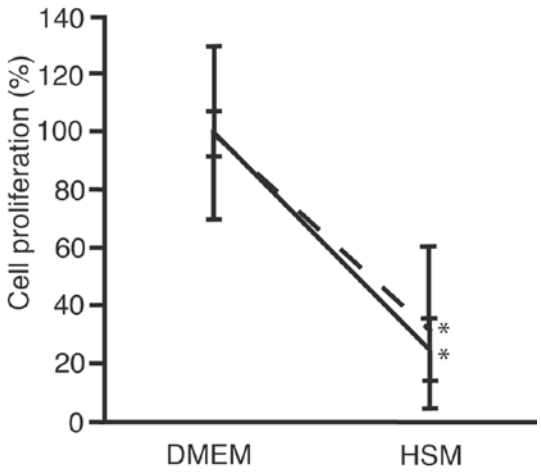

Figure 4. Cell proliferation assay. PLC/PRF/5 cells were cultured in 6-well plates (solid line) or as cell spheres (dash line), for 14 days. The cells were seeded onto 96-well plates and cultured in DMEM supplemented with 10% fetal bovine serum, or in HSM for 3 days. The cultured cells were subjected to an MTS assay. Data are presented as the mean ± standard deviation. Error bars, standard deviation; n=4. Experiments were carried out in triplicate, DMEM, Dulbecco's modified Eagle's medium; HSM, hepatocyte selection medium, *P<0.05, as compared with DMEM.

glucose or arginine, was demonstrated to suppress proliferation equally in PLC/PRF/5 cell spheres and in control cells.

Discussion

PLC/PRF/5 cell spheres exhibit stemness and resistance to fluorouracil, mitomycin C, and sorafenib (26). In the present study, sphere-forming PLC/PRF/5 cells were seeded onto 96-well plates to evaluate their sensitivity to sorafenib using a proliferation assay. Suppression of cell proliferation with sorafenib was decreased in the cells following sphere formation, as compared with control cells. These results were consistent with those of a previous report (26). The previous results and the data of the present study demonstrated that PLC/PRF/5 cells were more resistance to sorafenib due to their stemness following sphere formation. The mechanism of resistance was subsequently investigated.

Sorafenib is metabolized by CYP3A4 (10). In the present study, the expression levels of CYP3A4 were observed to increase during sphere formation. The results indicate that PLC/PRF/5 cells acquired resistance to sorafenib due to the increased expression levels of CYP3A4. Patient survival would be poor with cancer stem cells due to the resistance to chemotherapeutic agents, such as sorafenib (29). Cancer stem cells are, therefore, one of the targets of treatment (30).

Cancer cells require more glucose than normal cells due to the Warburg effect (11). Therefore, glucose metabolism is a potential therapeutic target for the treatment of HCC. The results suggest that the sphere-forming PLC/PRF/5 cells did not acquire resistance to glucose deprivation. Glucose metabolism, therefore, represents a potential target for the treatment of HCC, with a low risk of acquired resistance.

2-Deoxyglucose (2DG) is an analog of glucose that recapitulates the effect of glucose-deprivation on cancer cells (31). The anticancer effects of 2DG have been reported as limited (32); however, the administration of 2DG in combination with other anticancer agents may be a potentially effective strategy for the treatment of certain types of cancer, including HCC. The results suggest that the deprivation of glucose and arginine may be an effective approach for the treatment of HCC. Furthermore, the efficacy of arginase, which metabolizes arginine, has been previously demonstrated in HCC (33). The combination of 2DG and arginase must be investigated in further studies as a potential strategy for the treatment of HCC.

In conclusion, PLC/PRF/5 cells acquired resistance to sorafenib following sphere formation, suggesting stemness. Cell proliferation of PRL/PRF/5 cells was suppressed with HSM. Therefore, the deprivation of glucose and arginine may serve as an effective potential treatment for HCC.

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