

Analogy between sphere forming ability and stemness of human hepatoma cells

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Abstract. Increasing evidence suggests that cancers contain a small subset of cancer-initiating cells, so-called cancer stem cells (CSCs) that are capable of regenerating a tumor after chemoradiation therapy. Sphere forming ability is known to be one of properties of CSCs, but the significance remains unclear. The present study focused on sphere formation of human hepatoma cells in three-dimensional culture in order to evaluate the analogy between sphere forming ability and stemness of cancer cells *in vitro*. Under three-dimensional culture condition, HepG2, Hep3B and PLC/PRF/5 cells demonstrated the sphere formation while SK-Hep1 and Huh-7 cells did not. The population of G0/G1 phase increased in the spheres compared with the monolayer (67 vs. 38%). In spite of no significant difference in stem cell surface markers (CD44, CD90, CD133, EpCAM and ABCG2), remarkable up-regulation of p27 CDK inhibitor was observed in sphere forming cells. Immunofluorescence analysis revealed the nuclear expression of p27 in the whole of the sphere, but weak expression of p21 only at the peripheral area. The spheres acquired chemoresistance to cisplatin compared with the monolayers (58.9 vs. 16.2 μ M in IC50). This model was

useful for assessment of the role of cell-cycle quiescence in the stemness and chemoresistance of cancer cells.

Introduction

There is an emerging concept of cancer stem cells (CSCs) that cancer cells do not consist of homogeneous population but include a small subpopulation of cells having the ability of self-renewal and differentiation into multiple phenotypes (1). Several unique properties of CSCs have been identified including drug-efflux ability (side population) (2,3), maintenance of quiescence, sphere formation, high tumorigenicity, and resistance to hypoxia and chemoradiation (4). After anticancer treatment that kills most cancer cells, such drug-resistant CSCs might survive and finally generate new populations, resulting in cancer recurrence and metastasis. Detailed analysis on biological characteristics of CSCs are required to overcome the resistance of cancer.

Recent studies revealed that the sphere formation might be essential for cancer-initiating ability of CSCs (5-7), but its significance and mechanism remain unclear. The sphere-forming cells in human hepatoma were reported to associate with the expression of stemness markers such as CD90 (8,9), CD133 (10,11), EpCAM (12), ABCG2 (13) and CD44 (14). In this study, we focused on the sphere formation ability using 3D culture system to evaluate the *in vitro* analogy to the stemness phenotypes in human hepatoma cells.

Materials and methods

Cell lines. Human hepatoma cell lines, HepG2, Huh-7, PLC/PRF/5 and SK-Hep1 were analyzed (15). Additionally, we used p53-deficient hepatoma cell line Hep3B as well as Hep3B transfected with wild-type p53 gene (16); named as Hep3B-p53(-) and Hep3B-p53(+), respectively. Culture media were the recommended media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cell lines were cultivated in a humidified incubator at 37°C in 5% CO₂. Conventional monolayer culture is referred to as two-dimensional culture (2D culture) hereafter in this report.

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Abbreviations: CSCs, cancer stem cells; EpCAM, epithelial cell adhesion molecule; ABCG2, ATP-binding cassette sub-family G member 2; CDK, cyclic-dependent kinase; IC50, half-maximal inhibitory concentration; PBS, phosphate-buffered saline; PFA, paraformaldehyde; DMSO, dimethyl sulfoxide; SD, standard deviation

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Three-dimensional culture (3D culture). Sphere formations were initiated by 3D culture system using low attachment plate (NanoCulture Plate; SCIVAX, Kanagawa, Japan) (17). About 5×10^3 cells were seeded on each well of a 96-well plate. The culture media were the same as that of 2D culture.

Cell cycle analysis. For cell cycle analysis, spheres were collected by gentle centrifugation (800 rpm) and dissociated mechanically and enzymatically (for 10 min in trypsin). The single cells were fixed in 70% ethanol at 4°C for 12 h. Cells were washed twice with PBS and stained with propidium iodide at 37°C for 30 min. Cell cycle analysis was performed on FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Protein expression analysis. Total protein was extracted from 2D cultured cells and sphere forming cells at days 3 and 6 (not dissociated) using cell lysis buffer. Western blot analysis was performed using antibodies for CD44/HCAM, p27, p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (Cell Signaling Technology, Beverly, MA, USA), ABCG2/BRCP (Abcam, Cambridge, UK), CD90 (Abgent, San Diego, CA, USA), EpCAM (AbD Serotec, Oxford, UK) and anti- α -tubulin (Sigma-Aldrich, St. Louis, MO, USA). For analysis of CD133 expression, cells were labeled with anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by assays on a FACS Calibur flow cytometer (Becton-Dickinson), as previously described (15).

Immunofluorescence analysis. Spheres were cryoembedded in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned at 10 μ m thickness with microtome. The sections were fixed in 4% PFA at 4°C for 10 min and washed three times with distilled water, then stained with an automated immunostainer (BenchMark XT; Ventana Medical Systems, Tucson, AZ, USA). Fluorescence microscopy was performed using Axio Observer (Carl Zeiss, Oberkochen, Germany) and images were acquired digitally using AxioVision (Carl Zeiss).

Chemosensitivity test. For 2D culture, cells were seeded onto a 96-well plate at a density of 2×10^3 cells per well and incubated for 24 h. For 3D culture, cells were seeded on to a 96-well NanoCulture Plate (SCIVAX) at a density of 5×10^3 per well and incubated for 72 or 144 h (referred to as sphere day 3 and sphere day 6, respectively). Then, cells were exposed to DMSO control and different concentrations of cisplatin (0.01–300 μ M; provided by Bristol-Myers Squibb, Tokyo, Japan) for 72 h. As chemosensitivity test, CellTiter-GLO luminescence Cell Viability Assay (Promega, Madison, WI, USA) was adopted. CentroLB 960 (Berthold, Bad Wildbad, Germany) was used for recording luminescence.

Results

Sphere formation ability of hepatoma cell lines. It is known that cells seeded onto NanoCulture Plate grow and aggregate three-dimensionally and finally form spheres. It depends on characteristics of cell lines whether they can form spheres or grow like 2D culture. In HepG2, Hep3B-p53(-), Hep3B-p53(+) and PLC/PRF/5 cell lines, sphere formation was

observed at day 3. In contrast, sphere formation was not observed in SK-Hep1 and Huh-7 cell lines (Fig. 1). In Hep3B-p53(-) cells, sphere formation was observed at day 3, but the verge of spheres began collapsing at day 6. Some of them could not keep spherical body and finally spread two-dimensionally. HepG2 cell line was best in respect of the efficacy of sphere formation, so we determined to use HepG2 cell line in the sphere analysis.

Accumulation of quiescent cells in the spheres. We analyzed cell cycle of 2D cultured cells and sphere forming cells at days 3 and 6 (Fig. 2). The population of G0/G1 phase was accumulated in sphere forming cells (67%) compared with in 2D cultured cells (38%). At day 6 in sphere forming cells, remarkable decrease of S phase was observed. These results indicate that the cultivation of cells as spheres inhibits the transition from G1 to S phase.

Expression of cell cycle regulators. In cell cycle analysis, we found that the transition from G1 to S phase was inhibited in sphere forming cells. To search for the factors that govern G1-S phase transition, we analyzed the expression of cell cycle regulators (p27, p21 and p53) in 2D cultured cells and sphere forming cells at days 3 and 6. p27 and p21 are known to be CDK inhibitors, which regulate G1 to S phase transition by binding to CDKs, and besides, the expression of p21 is tightly controlled by p53. In addition to those cell cycle regulators, we analyzed the expression of cell surface markers specific for CSCs (ABCG2, EpCAM, CD44, CD90 and CD133) to investigate the correlation between spheres and CSCs. In Western blot analysis (Fig. 3A), up-regulation of p27 protein level was observed in sphere forming cells at day 6, but in contrast, p53 and p21 protein levels were down-regulated. No significant difference in hepatocellular carcinoma stem/progenitor cell surface markers was found between 2D cultured cells and sphere forming cells. By immunocytofluorescence of spheres (Fig. 3B), interestingly, the expression of p27 was localized in the nucleus, but the expression of p21 was localized in cells at the peripheral area of spheres.

Sphere forming cells acquired chemoresistance ability. To analyze whether the cultivation of human hepatoma cells as spheres can affect chemosensitivity, 2D cultured cells and sphere forming cells at days 3 and 6 were exposed to 0.01–300 nM cisplatin or DMSO control for 72 h (Fig. 4). The chemosensitivity test of cisplatin (0–300 μ M) did not show a significant change of the IC50 value between the cells of 2D culture and sphere at day 3. In sphere at day 6, however, the IC50 value of cisplatin was three folds higher compared with 2D cultured cells.

Discussion

The present study demonstrated that sphere formation contributes to increase of G0/G1 quiescent cells. In cell cycle analysis, the population of G0/G1 phase was accumulated in sphere forming cells (67%) compared with in 2D cultured cells (38%). We then explored factors in inducing quiescence in sphere forming cells. In sphere forming cells, up-regulation of p27 expression was observed at day 6, and the immuno-

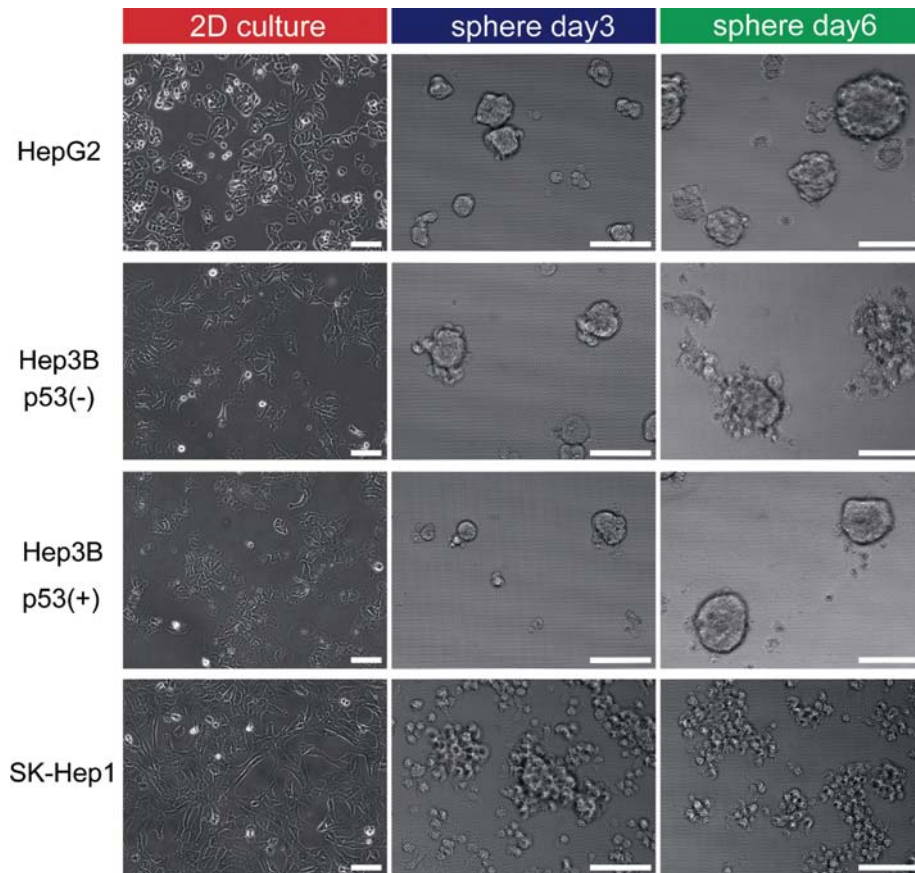


Figure 1. Sphere formation in hepatoma cell lines using 3D culture system. HepG2 cells formed spheres. Hep3B-p53(-) cells formed spheres, but they collapsed at day 6. On the other hand, Hep3B-p53(+) cells formed sphere stably even at day 6. SK-Hep1 cells did not form spheres. Scale bar, 100 μ m.

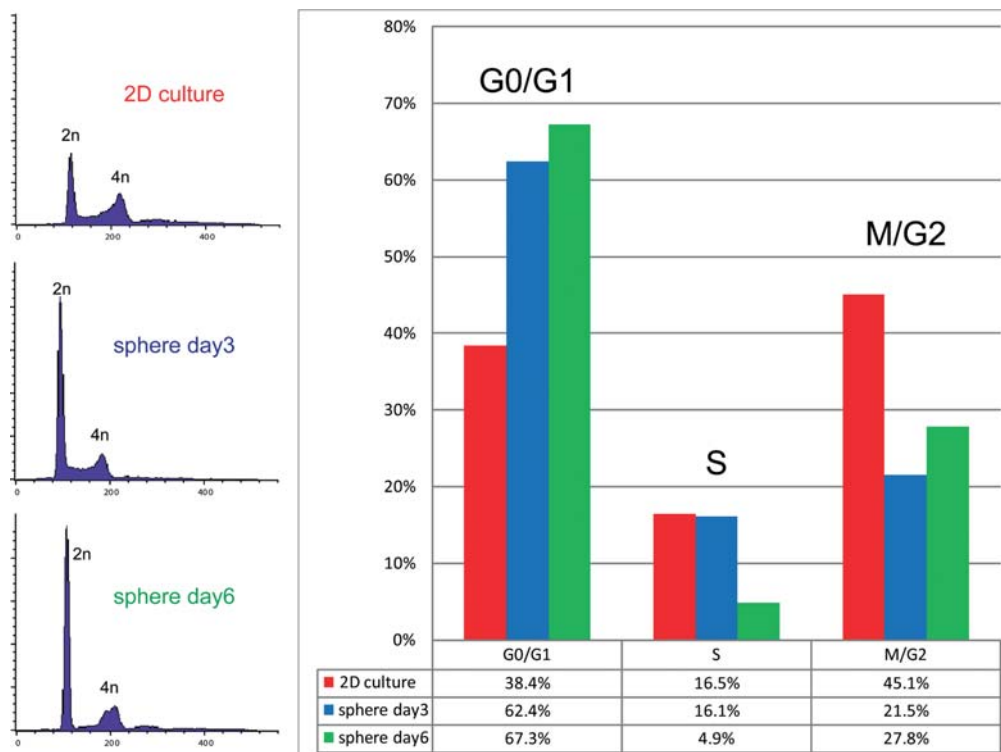


Figure 2. Analysis of HepG2 cell cycle distribution by flow cytometry. The population of G0-G1 phase was increased in sphere forming cells compared with 2D cultured cells. In sphere forming cells at day 6, a remarkable decrease of the population of S phase was observed.

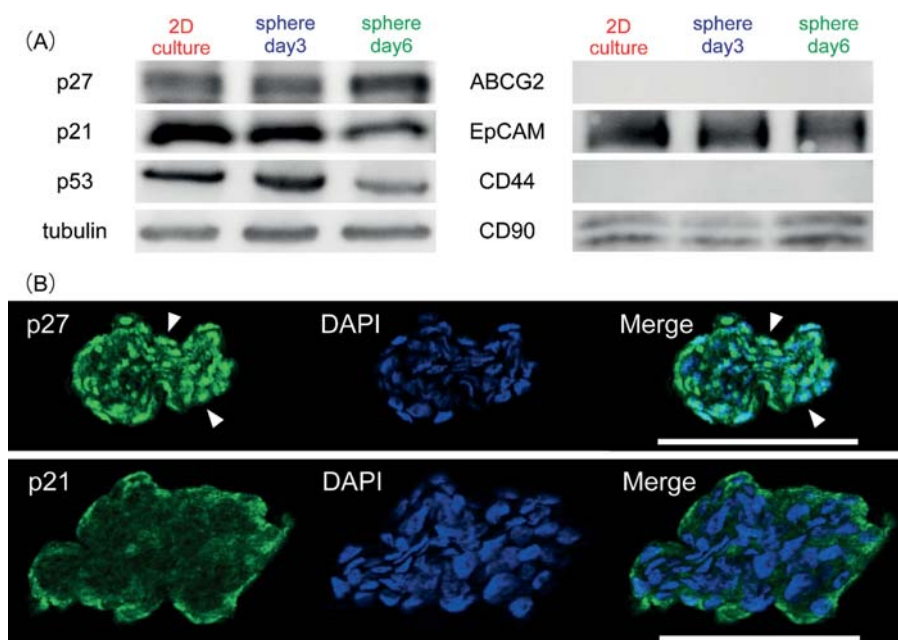


Figure 3. Expression of cell cycle regulators and hepatocellular carcinoma stem/progenitor cell surface markers in spheres of HepG2 cells. (A) Western blot analysis of cell cycle regulator (p27, p21 and p53) and hepatocellular carcinoma stem/progenitor cell surface markers (ABCG2, EpCAM, CD44 and CD90). Tubulin was measured as control. (B) p27 and p21 distribution in HepG2 sphere frozen section. p27 and p21 protein are stained green. The nuclei are stained blue. Scale bar, 100 μ m.

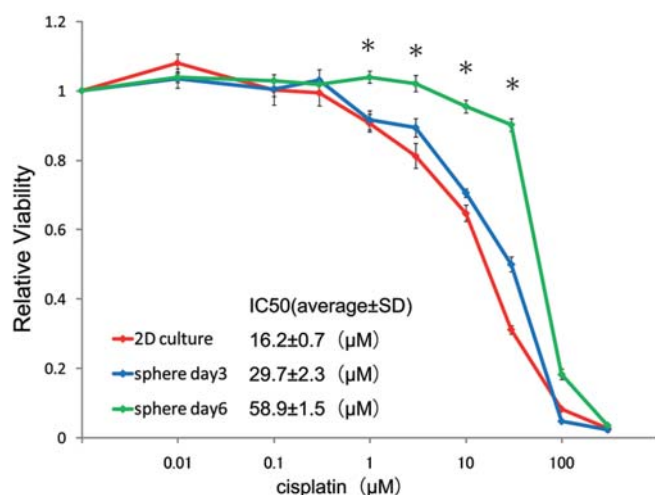


Figure 4. The dose response curve of cisplatin (0-300 μ M) in HepG2 cells of 2D culture and sphere at days 3 and 6. Cells were treated with DMSO control or 0.01-300 nM cisplatin. Data represent relative cell viability of each dose when compared with that of DMSO control. Data are representative of three independent experiments. Statistical analysis was performed using Student's t-test. * P <0.05; between 2D cultured cells and sphere at day 6.

cytofluorescence analysis revealed the nuclear localization of p27. The intranuclear p27 was reported to modulate G1-S phase transition by regulating the activity of CDKs (18,19). It is supposed that p27 in nucleus binds to cyclin E-CDK2 complex and inactivates the complex in G0/G1 phase, but once p27 is transported to cytosol and degraded, the complex is activated and it triggers the transition from G1 to S phase. Our data were consistent with this kinetics of p27, so it was

suggested that intranuclear p27 plays a central role for cell-cycle quiescence in sphere forming cells (20-22).

On the other hand, the p21 expression was down-regulated in the sphere forming cells. The CDKs inhibitor p21 functions as the key of stress response and major transcriptional target of tumor suppressor gene *p53* (23,24). Therefore, the down-regulation of p21 might apparently be inconsistent with the cellular quiescence. As suppressor of tumor proliferation, however, the function of p21 is rather ambiguous compared with p27. In fact, the poor prognosis and clinical progression of HCC were significantly associated with the lack of p27 but not p21 (25-27). By immunocytofluorescence analysis on the spheres, the expression of p21 was localized in cells at the peripheral area of spheres where comparatively active cell proliferation might occur. Considering the down-regulation of p21 protein levels, the function of p21 may be different from inhibiting cell proliferation in the spheres.

In addition, spheres of Hep3B-p53(+) cells could keep their shapes longer than those of Hep3B-p53(-) cells. Collapse at the verge of spheres was observed at day 6 in Hep3B-p53(-) cells, but spheres of Hep3B-p53(+) cells could keep spherical bodies even at day 6. It was suggested that the maintenance of sphere formation should be controlled by p53 status. Recently, the p53-p21 pathway has been reported as obstacles to generate the induced pluripotent stem cells, and this pathway is a hot issue in stem cell investigation (28,29). Our data implicated that p53-p21 pathway might play some essential roles in the sphere formation. In chemosensitivity test, the sphere forming cells acquired significant chemoresistance at day 6, while there were no significant difference of chemosensitivity between the sphere forming cells at day 3 and 2D cultured cells. It is possible that the difference of chemosensitivity was caused not only by cellular quiescence



by some other factors including p53-p21 pathway. Studies are required to clarify the functional roles of p21 and p27 in cancer stemness as well as chemo-resistance using our model of sphere formation.

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