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Proliferation and motility of hepatocellular, pancreatic and gastric cancer cells grown in a medium without glucose and arginine, but with galactose and ornithine

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Abstract. Human primary hepatocytes are able to survive in a medium without glucose and arginine, but supplemented with galactose and ornithine (hepatocyte selection medium; HSM). To address the possibility of the application of HSM in cancer therapy, hepatocellular carcinoma cells, pancreatic cancer cells and gastric cancer cells were cultured in HSM. Cell proliferation was analyzed using an MTS assay. Morphological changes were analyzed using hematoxylin and eosin staining. Apoptosis was analyzed using a TUNEL assay and cell motility was assessed with a scratch assay. Cell proliferation was significantly suppressed in cell lines grown in HSM ($P<0.01$ in all the cell lines). Hematoxylin and eosin staining revealed pyknotic nuclei, suggesting that these cells had undergone apoptosis. The number of TUNEL-positive cells was significantly increased in HSM. In the scratch assay, the distance between the growing edge and the scratched edge was significantly lower ($P<0.01$ in all the cell lines) in cells cultured in HSM, compared with those grown in Dulbecco's modified Eagle's medium or RPMI-1640. Therefore, the proliferation and motility of hepatocellular carcinoma cells, pancreatic cancer cells and gastric cancer cells was suppressed, and these cells subsequently underwent apoptosis in a medium without glucose and arginine, but containing galactose and ornithine.

Introduction

The liver is a target organ implicated in a number of primary and metastatic types of cancer. Primary liver cancer types include hepatocellular carcinoma and cholangiocarcinoma; the most frequent original sites of metastasis in liver cancer are the stomach, pancreas and colon (1). Surgery is the most effective curative treatment for primary and metastatic liver cancer (2,3); however, if surgery is not a viable option, chemotherapy or molecular target therapy are considered for further treatment (4-6). The agents used in chemotherapy and molecular targeted therapy frequently cause hepatotoxicity, which limits the efficacy of this treatment (7,8). Therefore, the development of therapeutic approaches with a lower risk of hepatotoxicity is required.

Metabolism in cancer cells is characterized by increased aerobic glycolysis and lactate production, even under a sufficient oxygen supply (the Warburg effect), and cancer cells require more glucose compared with the surrounding normal tissues (9). This phenomenon is applied to positron emission tomography, in which 18-fluorodeoxyglucose, an analogue of glucose, is taken up by cancer cells (10). Unlike glucose, 18-fluorodeoxyglucose is not metabolized and accumulates in cancer cells; therefore, a positive signal may be detected from cancerous tissues (11). Glucose is an important source of energy that is essential for cell survival (12,13). Galactose enters glycolysis as a substrate for the enzyme galactokinase (GALK), which is expressed in the liver and kidney (14,15). Arginine is an essential amino acid produced from ornithine by ornithine carbamoyltransferase (OTC) in the urea cycle, which is exclusive to hepatocytes (16). Normal cells produce arginine *de novo*, whereas cancer cells take up arginine from extracellular tissues (17).

Hepatocytes express GALK and OTC, and therefore, may be expected to survive in a medium lacking glucose and arginine, but supplemented with galactose and ornithine (18,19). Our previous study developed a hepatocyte selection medium (HSM), which lacks glucose and arginine but contains galactose and ornithine (20). Primary human hepatocytes are able to survive in HSM, and this medium purifies primary human hepatocytes from co-culture with human-induced pluripotent stem cells (21).

Therefore, the present study analyzed the suppression of the proliferation and motility of hepatocellular carcinoma cells, pancreatic cancer cells and gastric cancer cells in HSM.

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Materials and methods

Cell culture. Human hepatocellular carcinoma HLF and PLC/PRF/5 cells, human pancreatic cancer MIA-Paca2 and PANC-1 cells and human gastric cancer MKN45 and MKN74 cells were purchased from the Riken Cell Bank (Cell Engineering Division, Riken Biosource Center, Tsukuba, Japan). HLF cells, PLC/PRF/5 cells and MIA-Paca2 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). PANC-1, MKN45 and MKN74 cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck Millipore) supplemented with 10% FBS. Cell lines were cultured with 5% carbon dioxide at 37˚C in a humidified chamber, and passaged twice a week.

HSM. HSM was prepared from amino acid powders, using the formulation method of Leibovitz-15 medium (Thermo Fisher Scientific, Inc.), but omitting arginine, tyrosine, glucose and sodium pyruvate, and adding galactose (900 mg/l; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 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 is necessary for DNA synthesis, and therefore, it was included in the medium (30 mg/l) (22). Knockout serum replacement (KSR; Thermo Fisher Scientific, Inc.) was used at a final concentration of 10% in place of FBS in order to establish defined xeno-free conditions in HSM.

Cell proliferation analysis. HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells were trypsinized, harvested and seeded onto 96-well flat-bottomed plates at a density of 1,000 cells/well, then incubated at 37˚C for 24 h in DMEM or RPMI-1640 supplemented with 10% FBS. Subsequent to changing the medium to HSM, the cells were cultured at 37˚C for a further 72 h, and subjected to an MTS assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. MTS is reduced by the cells into a colored formazan product that reduces the absorbance at 490 nm; the absorbance at 490 nm was evaluated using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Hematoxylin and eosin staining. HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells were cultured in chamber slides (Matsunami, Kishiwada, Japan) in a 5% CO_2 atmosphere at 37˚C in a humidified chamber, and subjected to hematoxylin and eosin staining (Muto Glass, Tokyo, Japan). The cells were fixed in 10% formalin at room temperature for 10 min. The cells were stained with hematoxylin, and incubated at room temperature for 4 min. The slides were incubated in water at 25˚C for 30 min. The cells were dehydrated in 100% ethanol for 10 min three times, and were stained with eosin for 4 min. The cells were dehydrated in 100% ethanol for 10 min three times, and mounted under cover slips. The slides were observed under x200 magnification using an AX80 microscope (Olympus Corporation, Tokyo, Japan). Five different fields of view were observed.

TUNEL staining. Cells were cultured in chamber slides with 5% carbon dioxide at 37˚C in a humidified chamber and apoptotic cells were detected using the Wako Apoptosis *In Situ* Detection kit (Wako Pure Chemical Industries, Ltd.), following the manufacturer's protocol. Apoptotic cells were analyzed using a TUNEL assay, which consists of the addition of TdT to the 3'-terminus of apoptotically fragmented DNA, followed by immunochemical detection using an anti-fluorescein antibody conjugated with horseradish peroxidase and diaminobenzidine (DAB) as the substrate, following the manufacturer's protocol. The stained slides were observed under x100 magnification using an AX80 microscope.

Scratch assay. HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells were plated on 4-well chamber slides, and upon reaching 100% confluency, the cell monolayer was scratched with a sterile razor, incubated at 37˚C for 48 h and stained with hematoxylin and eosin. The stained slides were observed under x200 magnification using an AX80 microscope. The distance between the scratched and growing edges of the cells was evaluated at five different fields.

Statistical analysis. Data were presented as the mean ± standard deviation. Cell proliferation, TUNEL assay and scratch assay data were analyzed by one-way analysis of variance. Statistical analysis was performed using JMP 5.0J software (SAS Institute, Inc., Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

To examine the effect of culture in HSM on cell proliferation, an MTS assay was performed following three days of culture in DMEM, RPMI-1640 or HSM (Fig. 1). Proliferation of HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells cultured in HSM decreased to 23.4±5.9, 15.7±7.0, 24.0±10.4, 19.0±10.3, 25.3±9.3 and 34.7±13.2%, respectively. The results revealed that cell proliferation was significantly suppressed in HSM for all cell lines ($P<0.01$).

To observe morphological changes, hematoxylin and eosin staining was performed following two days of culture in HSM (Fig. 2). HLF cells (Fig. 2A), PLC/PRF/5 cells (Fig. 2B), MIA-Paca2 cells (Fig. 2C), PANC-1 cells (Fig. 2D), MKN45 cells (Fig. 2E) and MKN74 cells (Fig. 2F) all exhibited pyknotic nuclei, suggesting that the cells had undergone apoptosis.

To further examine whether HSM-induced apoptosis had occurred, a TUNEL assay was performed (Fig. 3). TUNEL-positive cells were observed in all cell lines (Fig. 3A). The percentage of apoptotic cells in HLF, PLC/PRF/5, MIA-Paca2, MKN45 and MKN74 cells cultured in DMEM or RPMI-1640 was 0.1±0.0, 0.2±0.0, 1.1±0.0, 1.3±0.0, 10.9±2.1 and 0.1±0.0%, respectively. The percentage of cells in HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells cultured in HSM increased to 40.0±12.3, 11.5±3.2, 25.0±4.3, 13.7±2.3, 15.5±3.4 and 6.3±1.4%, respectively. The number of TUNEL-positive cells was significantly higher in cells cultured in HSM ($P<0.01$ in HLF, PLC/PRF/5, MIA-Paca2, PANC-1 and MKN74 cells; $P=0.02$ in MKN45 cells) (Fig. 3B).

To investigate whether HSM suppresses cell motility, a scratch assay was performed (Fig. 4). Each cell monolayer

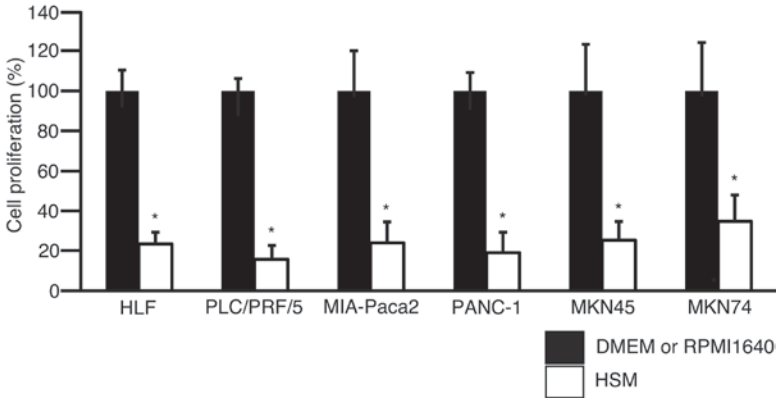

Figure 1. Cell proliferation suppressed by HSM. Each cell line was cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum or in HSM for three days. Data are presented as the mean ± standard deviation. Error bar, standard deviation. *P<0.05, compared with culture in DMEM or RPMI-1640; n=3. DMEM, Dulbecco's modified Eagle's medium; HSM, hepatocyte selection medium.

was scratched and cultured in DMEM, RPMI-1640 or HSM for two days (Fig. 4A). The distances between the growing edge and the scratched edge were $5.4\pm1.0x10^2$, $4.2\pm0.9x10^2$, $4.8\pm1.0x10^2$, $5.8\pm1.2x10^2$, $1.2\pm0.2x10^2$ and $1.8\pm0.4x10^2$ mm, respectively, in HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells cultured in DMEM or RPMI-1640. The distances between the growing edge and the scratched edge were $4.3\pm0.5x10^2$, $7.7\pm1.3x10$, $2.3\pm0.9x10^2$, $3.1\pm0.9x10$, $3.0\pm0.8x10$ and $1.5\pm0.3x10^2$ mm, respectively, in HLF, PLC/PRF/5, MIA-Paca2, PANC-1, MKN45 and MKN74 cells cultured in HSM. The distance between the growing edge and the scratched edge was observed as significantly decreased (P=0.03 in HLF cells; P<0.01 in PLC/PRF/5, MIA-Paca2, PANC1, MKN45 and MKN74 cells) in HSM-cultured cells compared with cells cultured in DMEM or RPMI-1640 (Fig. 4B).

Discussion

In the present study, cell proliferation was significantly suppressed when cells were cultured in HSM, concordant with previous findings of reduced cell proliferation in a medium lacking glucose (23). The suppression of cell proliferation occurs in part due to cell cycle arrest (23).

Morphological analysis using hematoxylin and eosin staining suggested that the all the cell cultured in HSM had undergone apoptosis in the current study, which was further supported by the results of the TUNEL assay. Cancer cells primarily depend on glycolysis for energy (9); therefore, if glucose is lacking in the medium, cancer cells are unable to proliferate and undergo apoptosis (24). HSM does not contain glucose, and therefore, it is hypothesized that cancer cells grown in HSM undergo apoptosis due to glucose deprivation (25-27). In combination with previous findings, the results of the current study suggest that glucose metabolism may present a novel target for cancer treatment (28).

Arginine deprivation has also been demonstrated to trigger apoptosis in cancer cells, and is proposed as a novel approach for cancer treatment in melanoma and cervical cancer (29). One example is the use of arginase, which degrades arginine and induces apoptosis in cancer cells (30). A previous study indicated that cancer cells are resistant to arginine deprivation

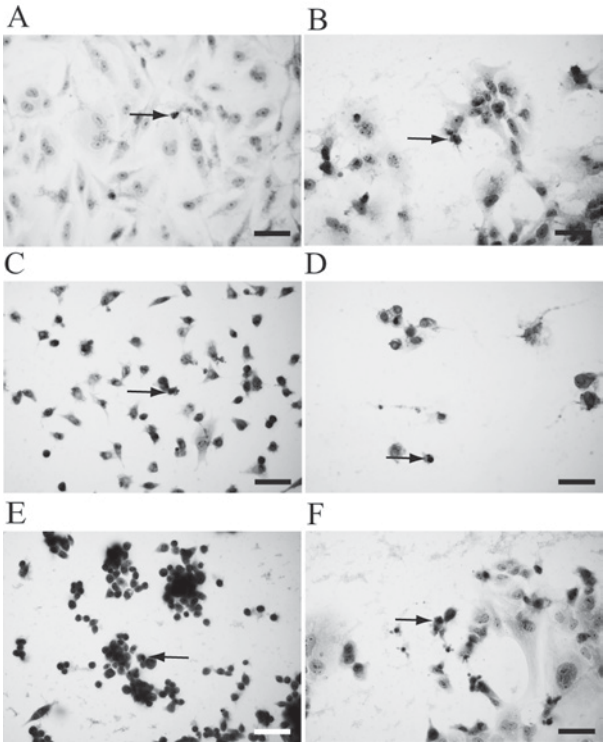

Figure 2. Apoptosis shown with hematoxylin and eosin staining in cells cultured HSM. Each cell line was cultured in HSM for two days and subjected to hematoxylin and eosin staining. Arrows indicate pyknotic nuclei. (A) HLF, (B) PLC/PRF/5, (C) MIA-Paca2, (D) PANC-1, (E) MKN45 and (F) MKN74 cells. Original magnification, x400; scale bar, 50 μm. HSM, hepatocyte selection medium.

in a three-dimensional environment (31). The current study used HSM, which does not contain glucose or arginine, and the results revealed that that deprivation of glucose and arginine may synergistically suppress cell proliferation and induce apoptosis. Therefore, targeting the metabolism of glucose and arginine appears to be a promising approach for cancer treatment.

In addition, the current study observed that the motility of cancer cells was suppressed in HSM. The effects of glucose or arginine deprivation on the motility of cancer cells remain to be elucidated; however, glucose deprivation is able to suppress

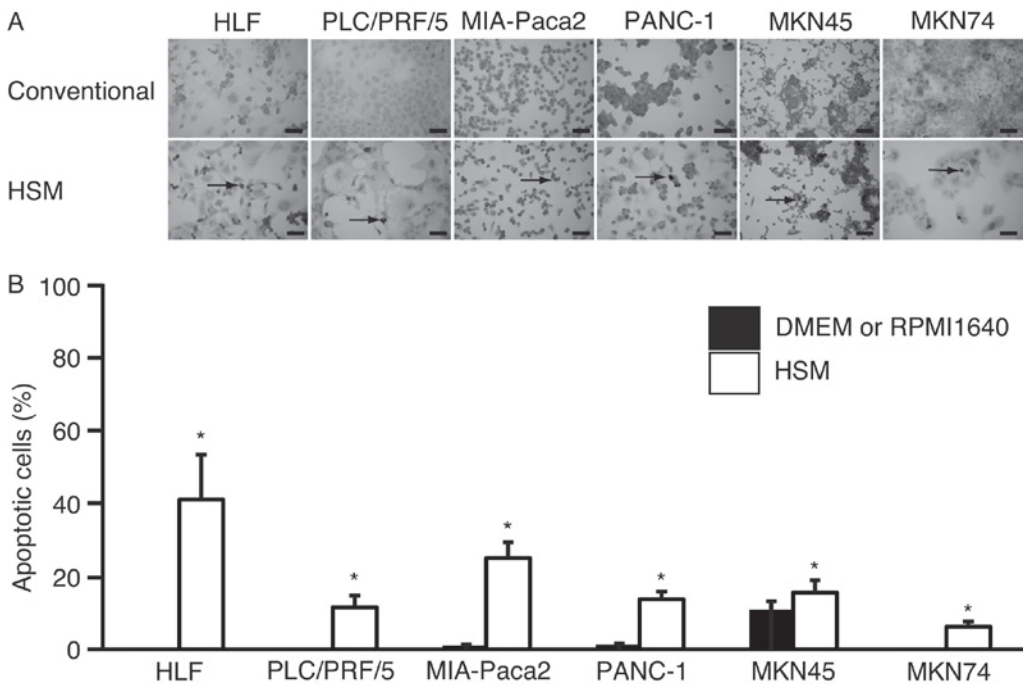

Figure 3. Ratios of apoptotic cells increased with hepatocyte selection medium. Results of the TUNEL assay. Each cell line was cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum, or in HSM for three days. (A) Images were captured (original magnification, x100; scale bar, 200 μm) for each cell line following the TUNEL assay. Arrows indicate TUNEL-positive cells. (B) Cells were counted and the proportion of apoptotic cells was calculated. Data are presented as the mean ± standard deviation. Error bar, standard deviation. *P<0.05; n=3. DMEM, Dulbecco's modified Eagle's medium; conventional, culture in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; HSM, hepatocyte selection medium.

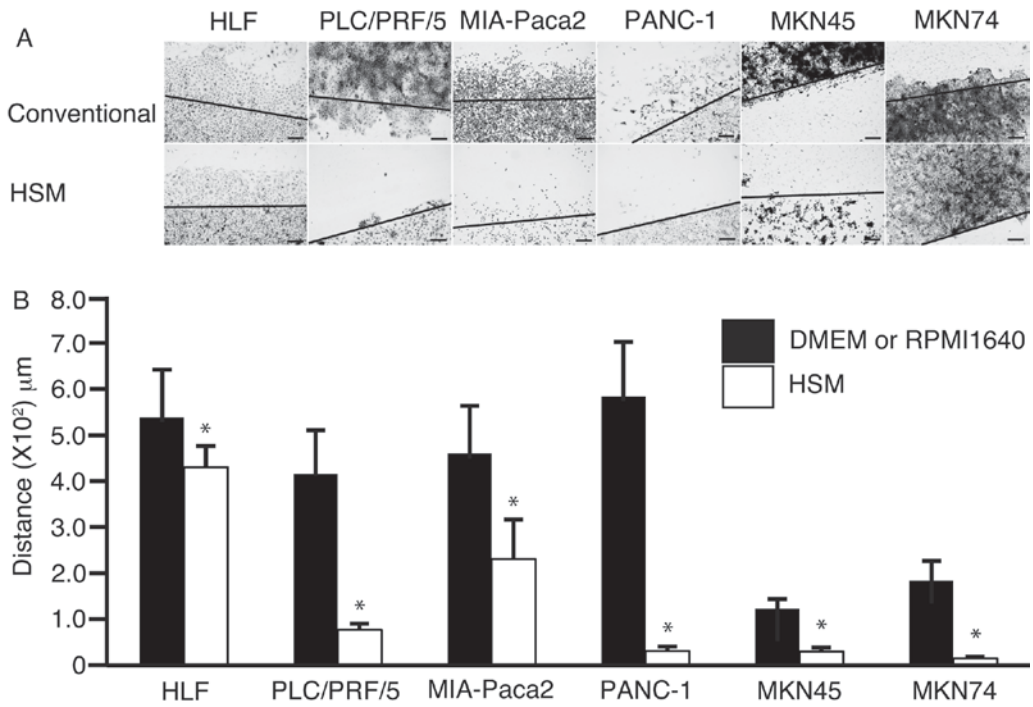

Figure 4. Cell motility suppressed in cells grown in HSM. (A) Each cell line was cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum or hepatocyte selection medium following scratching of the cell monolayer. Original magnification, x100; scale bar, 200 μm. (B) The cells were subjected to hematoxylin and eosin staining after two days of culture, and cultured in DMEM or RPMI-1640 (closed bar), or hepatocyte selection medium (open bar) for two days. DMEM, Dulbecco's modified Eagle's medium; HSM, hepatocyte selection medium. *P<0.05 in cells cultured in HSM compared with those in DMEM or RPMI1640.

the motility of microglia in the mouse brain (32). Whether the results obtained for microglia have the same biological significance in cancer cells requires further study. However, the results of the current study suggested that the deprivation of glucose and arginine in combination inhibited the motility of cancer cells.

An advantage of HSM culture is that it allows hepatocyte survival due to the presence of galactose and ornithine (21). Therefore, it was hypothesized in the present study that primary or metastatic liver cancer may be treated without associated hepatotoxicity by using HSM. Transcatheter arterial chemoembolization (TACE) is an established technique, whereas balloon occluding of the intrahepatic artery is a relatively novel technique that obstructs the artery with a micro-balloon and immerses the cancer cells in chemotherapeutic agents during TACE (33). Therefore, the current study hypothesizes that cancer cells may undergo apoptosis when cancerous tissues are immersed in HSM using balloon-occluded TACE.

In conclusion, the proliferation and motility of hepatocellular carcinoma cells, pancreatic cancer cells and gastric cancer cells were suppressed in a medium without glucose and arginine, but supplemented with galactose and ornithine (HSM). HSM may have potential as a new treatment of hepatocellular carcinoma.

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