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Abstract

Spheroid cultures of human hepatoblastoma cells (HuH-6 line) were established by rotating 3 x 10(6) cells/3 ml culture medium in 25-ml Erlenmeyer flasks on a gyratory shaker. The size of the spheroids rapidly increased until 4 days of culture, and thereafter their size gradually increased until 8 days of culture. A considerable amount of lactate dehydrogenase (LDH) was detected in the culture medium at 24h after seeding because of cell damage by subculturing, but thereafter the amount released was small, indicating that the spheroids were in healthy condition. Albumin production, one of the differentiated functions of hepatocytes, was higher in spheroid cultures than in monolayer cultures. Using this spheroid culture model, the cytotoxic effects of alcohols on HuH-6 cells were studied by measuring the activity of LDH released in the medium from damaged cells. The results indicate that the increasing order of toxicity of the alcohols was as follows: methanol $<$ ethanol $<$ propanol.

KEYWORDS: human hepatoblastoma, spheroid cultures, albumin, alcohols, LDH

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Spheroid cultures of human hepatoblastoma cells (HuH-6 line) were established by rotating 3×10^6 cells/3ml culture medium in 25-ml Erlenmeyer flasks on a gyratory shaker. The size of the spheroids rapidly increased until 4 days of culture, and thereafter their size gradually increased until 8 days of culture. A considerable amount of lactate dehydrogenase (LDH) was detected in the culture medium at 24h after seeding because of cell damage by subculturing, but thereafter the amount released was small, indicating that the spheroids were in healthy condition. Albumin production, one of the differentiated functions of hepatocytes, was higher in spheroid cultures than in monolayer cultures. Using this spheroid culture model, the cytotoxic effects of alcohols on HuH-6 cells were studied by measuring the activity of LDH released in the medium from damaged cells. The results indicate that the increasing order of toxicity of the alcohols was as follows: methanol < ethanol < propanol.

Key words: human hepatoblastoma, spheroid cultures, albumin, alcohols, LDH

Recently, the development of alternative methods to the use of animals for studying drug toxicity has been rapidly progressing. One such method is the use of cultured cells. Among the many kinds of cultured cells reported, hepatocytes, especially of human origin, are useful for the cytotoxicity assay because many drugs are metabolized in the liver where they exert their cytotoxicity. Thus, morphological and biochemical changes in the liver are frequently used as a measure of drug toxicity.

Cultured hepatocytes which retain hepatocyte-specific functions are instrumental to drug toxicity assay. However, it is well known that hepatocytes in monolayer cultures rapidly lose their differentiated functions. To maintain as many hepatocyte-specific functions as possible, several methods such as modification of attachment substrates (1-4), new tissue culture media formulations (5, 6), supplementation of differentiation-stabilizing or -promoting substances such as phenobarbital, sodium butyrate and DMSO (7-10), co-culture with other cells (10, 11), hollow fiber membrane chambers (12), spheroid cultures (13), and heterospheroid cultures of hepatocytes and fibroblasts (14) have been developed. In most of these experiments, normal hepatocytes in primary culture were used, but they rapidly lose hepatocyte-specific functions after they are transferred to monolayer cultures. In addition, it is presently impossible to maintain normal hepatocytes as replicative cultures without the loss of their specific functions.

Thus, instead of using normal hepatocytes, the use of human hepatoma or hepatoblastoma cells which have various features specific to hepatocytes may be applicable to toxicological studies because of their ease of use and good reproducibility. This idea is based on the findings that rat hepatocyte lines derived from minimum deviation hepatoma have more hepatocyte-specific functions than those from the normal liver (15, 16). Thus, we attempted to establish spheroid cultures using a human hepatoblastoma cell line which possesses various characteristics specific to hepatocytes, and investigated the cytotoxic effects of alcohols on spheroids.

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Materials and Methods

Cell culture. The human hepatoblastoma cell line HuH-6 (17), which was established in our laboratory, was used. These cells secrete albumin, α-fetoprotein (AFP), α_1-antitrypsin, ceruloplasmin, fibrinogen, transferrin, and many other serum proteins (18). In addition, the cells synthesize bile acid and secrete it into culture medium. The cells were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % fetal bovine serum (FBS), 20 mM Hepes and 100 μg/ml kanamycin at 37 °C in a 5 % CO_2 incubator.

Preparation of multicellular spheroids by rotation culture. Multicellular spheroids were prepared as described by Moscona (19). Cells were seeded in silicon-coated 25-ml Erlenmeyer flasks containing 3 ml of culture medium with 10 % FBS at a density of 1.0×10^6 cells/ml, and the cultures were rotated at 70 rpm on a gyratory shaker to induce the formation of multicellular spheroids. Medium was replaced with fresh medium 24 h after cell inoculation, and thereafter every 24 h or 48 h by centrifugation ($30 \times g$ for 3 min). The diameter of the spheroids formed was measured microscopically every 24 h with a Burker-Turk hemocytometer (Embbo, Tokyo, Japan). The mean diameter was obtained by measuring 100 spheroids in each experiment.

Electron microscopy. Spheroids were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C, embedded in 3 % agarose, postfixed in 1 % osmium tetroxide for 1 h at 4 °C, dehydrated in a graded series of ethanol rinses, embedded in Epon 812 resin, and stained with 2 % uranyl acetate and 2.7 % lead citrate. Thin sections were observed with a transmission electron microscope (JEM-100CX, JEOL, Tokyo, Japan).

Assay of albumin production. The amount of albumin secreted into the medium was measured by an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (13). Briefly, Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with mouse anti-human albumin antibody (Cedarlane, Ontario, Canada) diluted 1:10000 in carbonate-bicarbonate buffer (pH 9.6), washed with 0.05 % Tween 20 in phosphate-buffered saline (PBS), and blocked with 2 % gelatin in PBS for 1 h at 37 °C. Assay samples (100 μl/well) were added and incubated for 1 h at room temperature. Human albumin (Sigma, St. Louis, MO, USA) was used as a standard. The plates were incubated with 100 μl of peroxidase-conjugated goat anti-human albumin antibody (Cappel, Durham, NC, USA) diluted 1:100000 in Tween/PBS for 1 h at room temperature, then they were developed with 100 μl/well of substrate solution [4 ml of phosphate-citrate buffer (pH 5.0), 2 μl of H_2O_2 and 1 tablet of ortho-phenylenediamine] for 10 min, and the reaction was stopped by adding 100 μl of 2.5 M sulfuric acid. The plates were read at 490 nm using a microplate reader (NP-500, Kurabou, Tokyo, Japan).

Lactate dehydrogenase assay. Assay medium was collected every 24 h and lactate dehydrogenase (LDH) activity was determined using a MTX "LDH" kit (Kyokuto, Tokyo, Japan). Briefly, 50 μl of the medium per well was reacted with 50 μl of coloring reagent. After 7 min at room temperature, the stop solution was added and the plates were read at 540 nm using the microplate reader.

Measurement of DNA. Cell suspensions were centrifuged at $2,000 \times g$ for 5 min at 4 °C and cells were collected as pellets. Then they were incubated at 37 °C sequentially in 0.2 ml of lysis buffer A [10 mM Tris (pH 8.0), 1 mM EDTA, 0.05 % NP40 and 30 μg/ml of RNase] for 30 min, and in 0.2 ml of lysis buffer B [10 mM Tris (pH 8.0), 1 mM EDTA, 0.1 % SDS, 10 μg/ml of proteinase K] for 90 min. Cell lysates were mixed with 0.6 ml of isopropanol and 40 μl of 3 M sodium acetate, and centrifuged at $15,000 \times g$ for 20 min at 37 °C. After DNA was recovered as pellets, 1.0 ml of 10 mM Tris (pH 8.0) and 1 mM EDTA was added. DNA concentrations were determined using a Spectrophotometer (DU Series 600, Beckman Instruments, Fullerton, CA, USA, USA).

Preparation of alcohols. Methanol, ethanol and 1-propanol were used for toxicological studies. Each alcohol was diluted with culture medium at concentrations of 0.5 %, 1.0 % and 2.0 %.

Results

The time course of the increase in the size of spheroids is shown in Fig. 1. Their average size was about 40 μm in diameter 24 h after the initiation of the cultures, rapidly increased up to 4 days of culture, and thereafter it gradually increased until 8 days, reaching about 210 μm in diameter. The size of the spheroids increased in parallel with the amount of cellular DNA per flask, indicating that the enlargement of spheroids was due to cell proliferation.

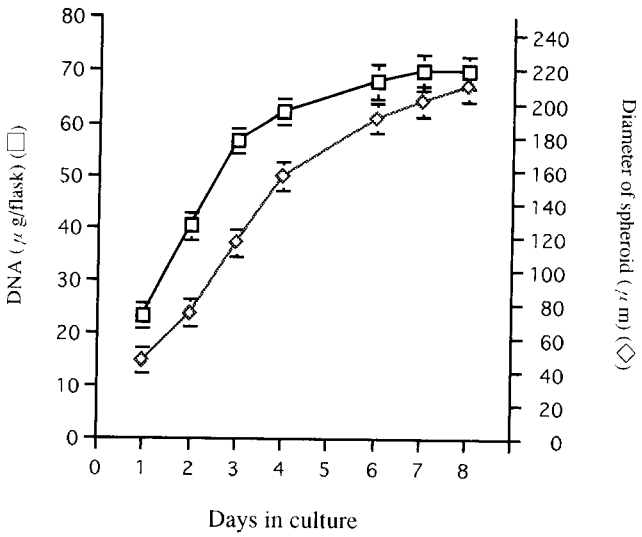

Fig. 1 Time course of increase in the size of spheroids and in DNA per flask. Each point was measured in triplicate cultures. The bars represent the standard deviation.

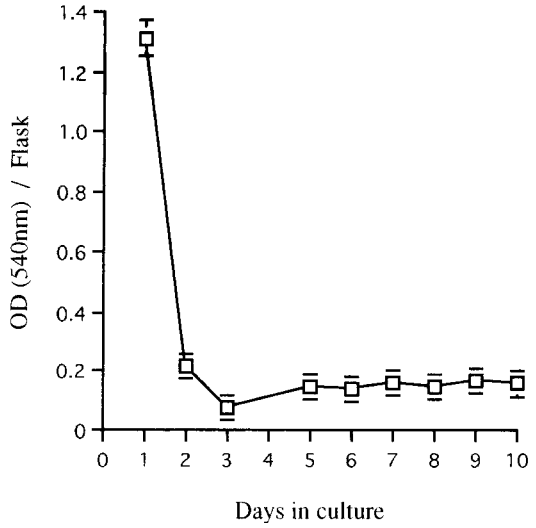

Fig. 2 Time course of lactate dehydrogenase (LDH) release in culture medium from spheroids. The bars represent the standard deviation.

There is a possibility that the cells in the center of the enlarged spheroids may not receive sufficient nutrition. Thus, LDH activity in the medium was measured until 10 days of culture. The high activity of LDH in the medium at 24 h after the start of cultures may be due to damage caused by subculturing the cells, but thereafter LDH activity was stably maintained at a low level (Fig. 2). This fact was observed in all experiments (more than ten). These findings indicate that, even if the spheroids became large, the cells which comprise the spheroids were healthy. The electron micrograph shows that the spheroids consisted of tightly packed cuboidal epithelial cells with adenomatous arrangement resembling a bile canaliculus. Numerous microvilli were observed on the surface of the cells facing the bile-like canaliculus in the center of the spheroid (Fig. 3).

Next, albumin production was assayed. As shown in Table 1, the production of the spheroid cells was two times that of the cells in monolayer cultures. These findings indicate that cells in the spheroids have more of the differentiated functions of hepatocytes than do cells in monolayers.

Using this spheroid model, the cytotoxicity of alcohols was investigated. The culture medium was replaced with fresh medium 24 h after the cells were put in rotation cultures, and then 24 h later the test samples were added to cultures because at this time there was no apparent LDH release from the cells which were presumably damaged by subculturing (Fig. 2). After exposure of the

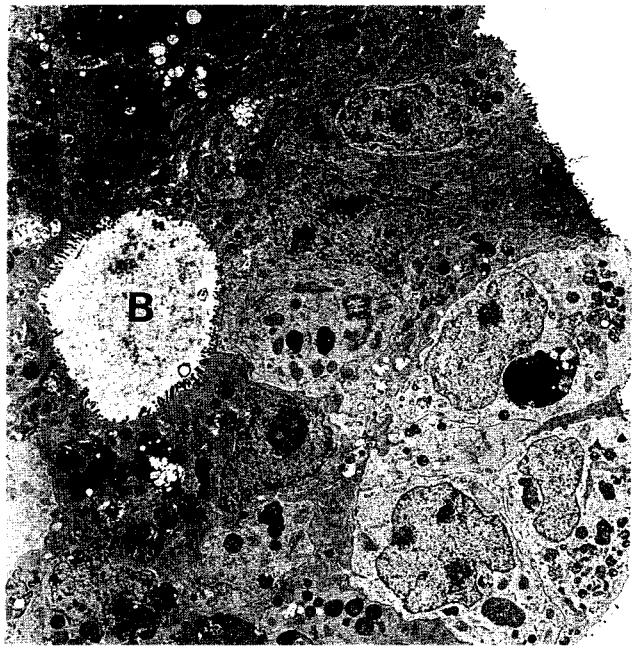

Fig. 3 Electron micrograph of a spheroid. B shows the formation of bile-like canaliculus.

Table 1 Albumin production in spheroid and monolayer cultures

Culture	Albumin produced (ng/ml)	Total albumin (ng)	DNA (μg/Flask)	Albumin/DNA (ng/μg)
Spheroid	5.36 ± 0.59	16.09 ± 1.76	59.98 ± 1.41	0.27 ± 0.04
Monolayer	3.65 ± 0.48	7.29 ± 0.96	59.38 ± 1.09	0.12 ± 0.02

Each value is the mean of triplicate cultures.

Table 2 Lactate dehydrogenase (LDH) activity in medium and cellular DNA in cultures treated with alcohols

Drugs	%(v/v)	LDH activity (OD 450nm)*	DNA (μg)/flask
Control		0.12	79.92
Ethanol	0.5	0.13	69.89
	1.0	0.15	61.56
	2.0	0.81	49.54
1-Propanol	1.0	1.12	29.37
Methanol	0.5	0.12	78.87
	1.0	0.12	78.98
	2.0	0.12	77.21

*50μl of the culture medium was assayed.

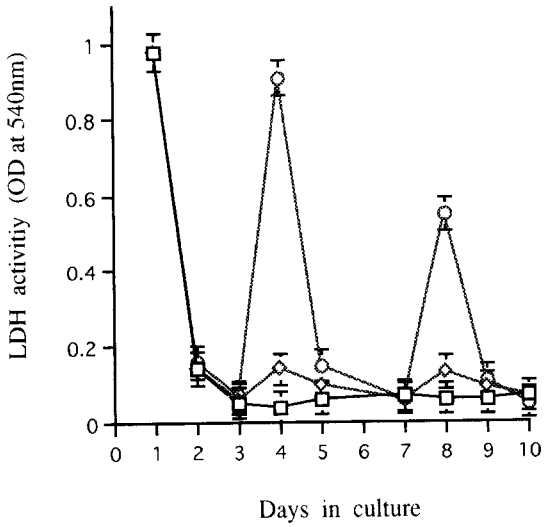

Fig. 4 LDH release in culture medium from spheroids on days 4 and 8 in the same culture. Ethanol was added to the culture on days 3 and 7 at a final concentration of 2.0% and ethanol-medium was replaced by fresh medium on days 4 and 8. The bars represent the standard deviation. LDH: See Fig. 2.
□: Control; ◇: 1% Ethanol; ○: 2% Ethanol

cells to alcohols for 24h, the amount of LDH released into the culture medium was measured. As shown in Table 2, when HuH-6 cells were treated with 2.0% ethanol or 1.0% 1-propanol, a large amount of LDH was released, whereas little release of LDH was detected in the cultures treated with 0.5% ethanol or 2.0% methanol. The degree of these cytotoxic effects was comparable to the decrease in DNA/flask of the cells treated with each alcohol. Taken together, the cytotoxicity of the alcohols examined increased as follows: methanol < ethanol < propanol.

The cytotoxic effects of ethanol were repeatedly examined in the same spheroid culture, as shown in Fig. 4. The cultures to which ethanol was added on day 3 at a final concentration of 2.0% showed a marked release of LDH on day 4. Then the ethanol-containing medium was replaced by ethanol-free medium, and 3 days later ethanol was added to the culture again. LDH release was observed 24h after the re-addition of ethanol, though the second peak of LDH activity was lower than the first peak. These findings indicate that the same spheroid cultures can be repeatedly used for toxicological studies of drugs.

Discussion

Hepatocyte cultures are an important tool for screening xenobiotics and for elucidating the mechanisms involved in toxicity. However, under conventional culture conditions, hepatocytes lose most of their differentiated functions and xenobiotic metabolisms within a few days after the initiation of cultures. Therefore, a number of attempts have been made to maintain hepatocyte-specific functions. These attempts have been made by using hepatocytes in primary culture, but the preparation of such cultures has proven difficult. In addition, the physical and biological characteristics of hepatocytes in primary culture can vary because of variations inherent in harvesting techniques and in the liver material itself. If estab-

lished hepatocyte lines which have hepatocyte-specific functions are available, they would be easy to prepare, efficient and give reproducible results (20, 21). Thus, we attempted to make spheroid cultures using HuH-6 human hepatoblastoma cells since these cells have many hepatocyte-specific functions (17, 18).

We determined the time course of formation of spheroids and their physiological conditions such as LDH release in the culture medium and albumin production, but could not detect expression of cytochrome P-450 2E1, one of the enzymes related to xenobiotic metabolism in the liver. Presumably, HuH-6 cells can not express P-450 isoforms even if they are cultured in three-dimensional conditions. Thus, our present spheroid culture model is applicable for studies on the cytotoxic effects of agents such as anti-cancer drugs and X-rays which can act on cells which lack xenobiotic metabolisms (22).

The toxicity of alcohols may be due to the length of the carbon-carbon bonds. Alcohols penetrate the cell membrane and alter the composition of the lipid bilayer, thus disorganizing the acyl chains of phospholipid groups and increasing membrane fluidity (23). These effects may alter functions associated with membrane proteins such as ion channels, receptors, and membrane-bound enzymes (24). Although we used HuH-6 cells in the present experiments, similar cytotoxic effects of alcohols were observed in human fibroblasts (25). Thus, the longer the carbon-carbon bond of alcohols, the greater the membrane damage. This speculation is supported by the present finding that propanol is the most toxic among the alcohols examined.

The average diameter of the HuH-6 spheroids was 210 μm, whereas there is a report that the mean diameter of normal hepatocyte spheroids in primary cultures was 100-140 μm (13). This may be due to differences between normal and malignant hepatocytes; normal hepatocytes usually form smaller aggregates than do malignant hepatocytes (26). Even though HuH-6 cells formed large spheroids, they could be used to test drug toxicity because the cells in the spheroid were healthy both morphologically and biochemically.

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