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Comparison of Various Methods of Assaying the Cytotoxic Effects of Ethanol on Human Hepatoblastoma Cells (HUH-6 Line)

Tsunenori KOSAKA*, Ken-ichi FUKAYA, So TSUBOI, Hong PU, Tadao OHNO[a], Takao TSUJI[b] and Masayoshi NAMBA

Department of Cell Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, Shikata, Okayama 700, [a]Riken Cell Bank, Tsukuba Science City 305 and [b]First Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan

The sensitivity of five kinds of cytotoxicity assays using ethanol on human hepatoblastoma cells (HUH-6 line), which were cultured as monolayers or spheroids, was compared. Ethanol was chosen as a test because it acts on cell membranes directly without being metabolized and exerts its cytotoxicity. The assay methods used were as follows: 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), colony formation, cell growth and DNA assays. The sensitivity of the assays was: LDH < DNA < cell growth < MTT < colony formation. LDH assay had the advantage that the same culture could be used for multiple assays, but when a small number of cells were assayed, no significant increase in the release of LDH was detected in the assay cultures compared with the control cultures. Although the DNA and cell growth assays were more sensitive than the LDH assay, the extent of cell damage may be underestimated because the damaged cells and DNA present in the cultures are included in the assay samples. On the other hand, both MTT and colony formation assays showed a high sensitivity. The MTT assay was done within 24 h after ethanol was added to the cultures and was applicable to both monolayer and spheroid cultures, while the colony formation assay required 1-2 weeks and it was applicable only to monolayer cultures. Taken together, the MTT assay was the most suitable method to evaluate the cytotoxic effects of ethanol on HUH-6 cells cultured as either monolayers or spheroids.

Key words: human hepatoblastoma, monolayer culture, spheroid culture, cytotoxicity

The cytotoxicity of drugs is generally evaluated using animal experiments. However, alternative methods have recently been desired because animal experiments are expensive and labor-intensive, and also because the results occasionally take a long time to obtain. One of the alternative methods to animal toxicity testing is the use of cultured cells. Among the many kinds of cultured cells, hepatocytes, especially those of human origin, are useful for cytotoxicity assays because many drugs are metabolized in the liver and, as a result, that is where they tend to exert their cytotoxic effect.

Ethanol, because of its hydrophobic properties, penetrates the cell membrane and perturbs the membrane structure and functions (1). Ethanol disturbs the composition of the lipid bilayer, thus disorganizing the acyl chains of phospholipid groups and increasing membrane fluidity. Consequently, these effects may alter functions associated with membrane proteins such as ion channels, receptors, and membrane-bound enzymes, resulting in cell damage. In fact, ethanol alters the activity of adenylate cyclase, Na^+-K^+ ATPase, enolase, voltage-sensitive Na-channels, protein kinase C and other membrane-bound enzymes in both excitable and nonexcitable cells (2-7). Inhibitory effects of ethanol have also been reported on ligand-activated Ca^{2+} channels in human lymphocytes (8) and on shaw2 K^+ channels in *Xenopus* oocytes (9). Thus, the cytotoxic effect of ethanol on HUH-6 cells may be mainly due to ethanol itself and not due to acetaldehyde, one of the metabolites of ethanol. In fact, at the cellular level, the cytotoxicity of alcohols is due to the length of the

* To whom correspondence should be addressed.

carbon-carbon bonds (10). Furthermore, no significant cytotoxic effects of acetaldehyde were detected in rat hepatocytes in primary culture (11).

Various cytotoxic assays have been reported such as 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (12), lactate dehydrogenase (LDH) release (13), neutral red release (NRR) (14), ^{51}Cr release (15), colony formation (16), cell growth (17) and DNA assays (18). The ^{51}Cr release assay is inconvenient because some cell lines do not incorporate chromium well and because trypsinization might remove or at least reduce trypsin-sensitive surface antigens on the target cells and thus alter their sensitivity to lysis (19). Both the MTT and NRR assays are quantitative colorimetric cytotoxicity assays, but the results obtained by the former are more consistent with the *in vivo* data than the latter (20). MTT is a substrate which is cleaved by living cells. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. In the LDH assay, LDH activity released from damaged cells is measured. LDH exists in the cytoplasm and leaks into the medium when cells are damaged. In this study, we investigated which assay methods were suitable for evaluating the cytotoxic effects of ethanol on human hepatocytes.

Materials and Methods

Cell culture. The human hepatoblastoma cell line HUH-6 (21) established in our laboratory was used. These cells secrete albumin, α-fetoprotein (AFP) and many other serum proteins (22). In addition, the cells synthesize bile acids and secrete it into the culture medium. Neither hepatitis C virus nor hepatitis B virus was detected in this cell line. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % fetal bovine serum (FBS), 20 mM Hepes and 100 μg/ml kanamycin, and they were cultured at 37 °C in a 5 % CO_2 incubator. At the time of seeding, the concentration of FBS was increased to 10 %.

Preparation of multicellular spheroids by rotation culture. Multicellular spheroids were prepared as described by Moscona (23). 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).

Lactate dehydrogenase assay. Assay medium was collected every 24 h and LDH activity was determined using an MTX "LDH" kit (Kyokuto, Tokyo, Japan). Briefly, 50 μl of the medium per well was allowed to react 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 a microplate reader (NP-500, Kurabou, Tokyo, Japan). Condition medium was collected every 24 h and used immediately for the assay.

MTT assay. The toxicity of ethanol was evaluated using a Cell Counting Kit (Dojin, Tokyo, Japan). Ethanol was added to cultures and 24 h later MTT reagent was added at a concentration of 10 % (v/v). Then a 96-well plate was incubated for 2 h at 37 °C in a 5 % CO_2 incubator. The absorbance was measured at 450 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-HCl (pH 8.0), 1 mM EDTA, 0.05 % NP40 and 30 μg/ml of RNase] for 30 min, and in 0.2 ml 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 ℃. After DNA was recovered as pellets, 1.0 ml of 10 mM Tris (pH 8.0) and 1 mM EDTA were added. DNA concentrations were determined with a Spectrophotometer (DU Series 600, Beckman Instruments, Fullerton, CA, USA).

Cell growth assay. Cells were seeded in test tubes containing 1 ml DMEM medium with 10 % FBS at a density of 1.5×10^5 cells/ml and the test tubes were placed at an angle of 5°. Then, 24 h later, the medium was refed and the indicated concentrations of ethanol were added to cultures. Cells were counted 96 h after seeding with a Burker-Turk hemocytometer (Embbo, Tokyo, Japan).

Colony formation assay. Cells were seeded in 30-ml culture flasks containing 5 ml DMEM medium with 10 % FBS at a density of 1.0×10^3 cells/flask (Coster #3025, Cambridge, England). Then, 24 h later, the medium was changed and the indicated concentrations of ethanol were added to cultures. One week later, colonies were fixed with 100 % methanol, stained with 4 %

Giemsa solution, and were counted with a microscope.

Preparation of ethanol. Ethanol was diluted with culture medium at final concentrations of 0.5 %, 1.0 % and 2.0 %.

Results

First, various methods to assess ethanol toxicity of HUH-6 cells were evaluated using spheroid cultures. When the cytotoxicity was measured by the MTT, LDH and DNA assays, the culture medium was replaced by fresh medium 24 h after the cells were seeded. Then, 24 h later, ethanol was added to cultures. After exposure of the cells to ethanol for 24 h, LDH released from the cells was measured. As shown in Fig. 1, when the spheroids were treated with 2.0 % ethanol, a large amount of LDH was released, whereas little release of LDH from the cells treated with 0.5 % and 1.0 % ethanol was detected. Then the ethanol-containing medium was replaced by ethanol-free medium, and 3 days later ethanol was added to the culture again. The amount of LDH released was observed 24 h 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.

On the other hand, when the cytotoxicity was measured by the DNA or MTT assays in the spheroid cultures, the cytotoxicity of ethanol was detected even in low concentrations of ethanol such as 0.5 % and 1.0 % (Table 1). These results indicate that the MTT and DNA assays are more sensitive than the LDH assay. When ethanol toxicity was measured by the MTT assay, the concentration of ethanol which produces a 50 % decrease in mitochondrial dehydrogenase activity was about 1.0 % (Table 1).

Second, the various cytotoxicity assays were evaluated using monolayer cultures. As shown in Fig. 2, the cytotoxic effects of ethanol on the cell growth were detected in the presence of 2.0 % ethanol but not 0.5 % and 1.0 %. Similar results were obtained by the LDH assay, *i.e.*, the cytotoxicity of ethanol was observed only

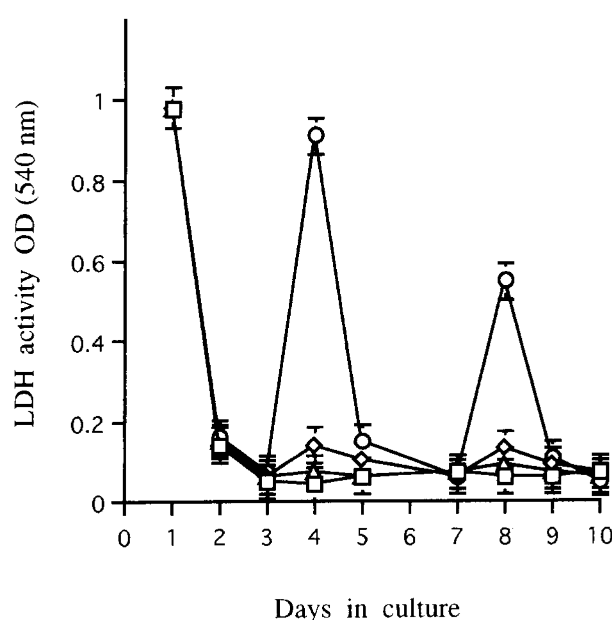

Fig. 1 Lactate dehydrogenase (LDH) released in culture medium from the same spheroid cultures on days 4 and 8. Ethanol was added to the culture on days 3 and 7 at final concentrations of 0% □, 0.5% ◇, 2.0% ○ and ethanol-medium was replaced by fresh medium on days 4 and 8. Each point is the mean of triplicate cultures (± SE). OD: Optical density.

Table 1 Comparison of sensitivity of various methods of assaying ethanol toxicity[a]

Cultures	Ethanol % (v/v)	MTT (OD)	MTT (% Control)	DNA (μg)/Flask	DNA (% Control)	LDH activity
	0	0.32+0.02	100	49.63±2.89	100	0.03±0.001
Spheroid	0.5	0.28±0.02	87.5	42.43±2.89	85.5	0.03±0.001
	1.0	0.16±0.01	50	28.20±1.09	56.8	0.04±0.001
	2.0	0.12±0.01	37.5	22.87±0.58	46.1	0.20±0.004
	0	0.78±0.02	100	15.50±0.14	100	0.19±0.010
Monolayer	0.5	0.53±0.04	68	12.28±0.85	79	0.21±0.001
	1.0	036±0.03	46	9.80±0.60	63	0.23±0.001
	2.0	0.24±0.01	31	5.78±0.49	37	0.36±0.010

[a]Each point is the mean of triplicate cultures (± SE). MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoniam bromide; OD: Optical density; and LDH: Lactate dehydrogenase.

Table 2 Effects of ethanol on plating efficiency of HuH-6 cells

	Plating efficiency (%)[a]	% Survival[b] 0.3% Ethanol	% Survival[b] 1.0% Ethanol	Ethanol (%) for $TCLD_{50}$
Exp. 1	5.7	71.0±5.0	16.5±3.9	0.57
Exp. 2	3.4	68.9±7.3	15.2±2.0	0.55

a) (Number of colonies/Number of cells seeded in the control medium) × 100

b) The percentage of survivinge of surviving cells was calculated by dividing the number of colonies formed in the medium containing ethanol by the number of colonies formed in the control medium, and multiplying by 100. Triplicate flasks were used at each date point and each experiment was carried out at least twice. Each value is the mean (± SE).

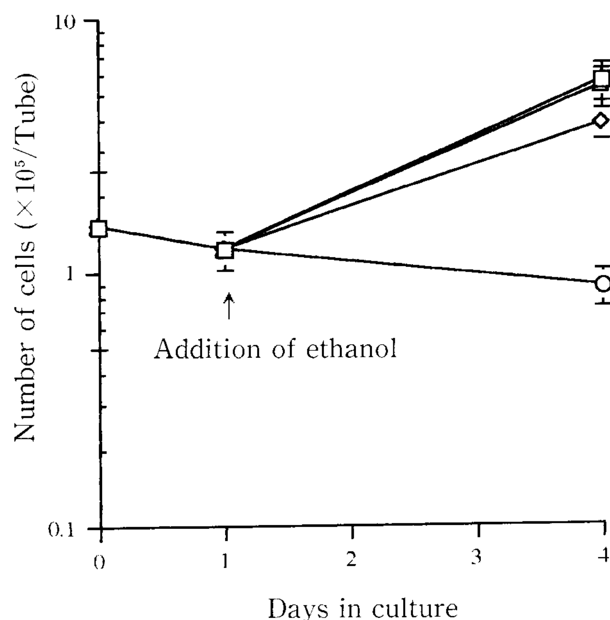

Fig. 2 Evaluation of ethanol cytotoxicity by cell growth assay. Ethanol was added on day 1 at final concentrations of 0%□, 0.5%△, 1.0%◇, 2.0%○ and cells were counted on day 4. Each point is the mean of triplicate culltures (± SE).

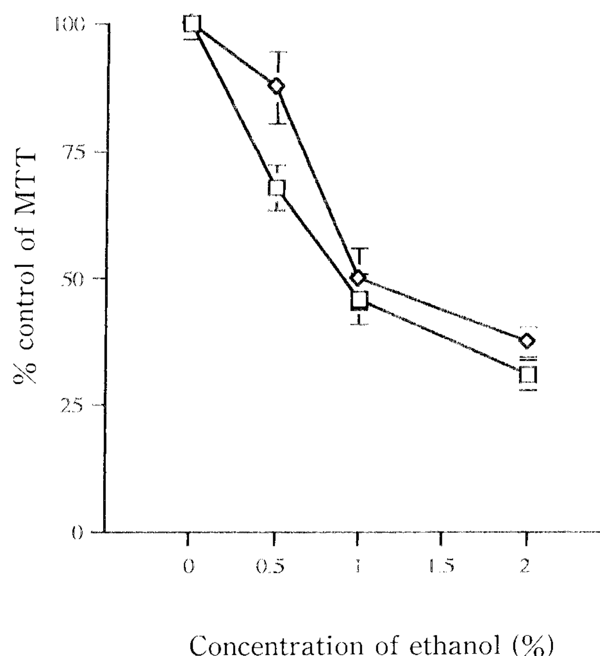

Fig. 3 Comparison of ethanol toxicity between spheroid ◇ and monolayer □ cultures by MTT assay. Each point is the mean of triplicate cultures (± SE). MTT: 3 (4, 5 dimethylthiazol-2-yl) -2, 5 -diphenyltetr 20 lium bromide.

Table 3 Ethanol concentrations (%) at $TCLD_{50}$

Assay method	Monolayer	Spheroid
Colony fomat	0.56	-
MTT	0.91	1.00
Cell growth	1.34	-
DNA	1.51	1.64
LDH	>2	>2

(−): The assay can not be done. MTT, LDH: See Table 1.

in the cells treated with 2.0 % ethanol (Table 1). In contrast, in the MTT and DNA assays, cytotoxicity was apparent even with 0.5 % ethanol and the former method was even more sensitive than the latter one. In the MTT assay, the monolayer culture was more sensitive than the spheroid culture at 0.5 % ethanol, but no significant difference in sensitivity was detected at 1.0 % and 2.0 % of ethanol (Fig. 3). Table 2 shows the percentage of surviving colonies of HUH-6 cells in the presence of 0.3 % and 1.0 % ethanol. Based on these data, the concentration of ethanol in the culture medium that caused 50 % cell death ($TCLD_{50}$: tissue culture lethal dose) was about 0.6 %. In Table 3, the sensitivity of various methods to test ethanol cytotoxicity is compared. According to the results, the MTT assay was the most suitable method to evaluate the cytotoxic effects of ethanol on HUH-6 cells cultured as either monolayers or spheroids.

Discussion

We investigated which assay method is the most suitable to evaluate the cytotoxic effects of ethanol on HUH-6 cells at the cellular level. The assay methods studied were as follows: MTT, LDH, colony formation, cell growth and DNA assays. When the cytotoxicity is measured by colony formation and cell growth assays, only monolayer cells can be used, but when it is done by DNA, MTT and LDH assays, both monolayer and spheroid cultures are available. The sensitivity of these methods to assay ethanol cytotoxicity increased in the following order: LDH < DNA < cell growth < MTT < colony formation. The LDH assay is the easiest procedure to perform, followed by MTT, cell growth, colony formation and DNA assays in this order. In terms of cost, the cell growth assay is the most inexpensive, followed in order by the colony formation, DNA, LDH and MTT assays. Although the LDH assay had the advantage that cytotoxic effects could be repeatedly examined in the same cultures, its sensitivity was influenced by the cell number. When a small number of cells was used, no significant difference in cytotoxicity was observed between the control and ethanol-treated cultures. Conversely, the DNA and cell growth assays were more sensitive than the LDH assay, but one disadvantage of these methods is that the damaged cells and DNA remaining in the surviving cell population could be measured, resulting in underestimation of the actual cell damage. Though the colony formation assay was more sensitive than the MTT assay, it was not applicable to spheroid cultures and furthermore at least 1 week was necessary until the results became available. Thus, among the various cytotoxicity assay methods examined, the MTT assay is the most useful method for evaluating the cytotoxicity of ethanol because of its high sensitivity, the short time required to know the results, and convenience.

When testing the cytotoxicity of drugs using cultured cells, the following points should be considered. a) Is the assay sensitive enough to obtain *in vivo* data? b) Do the cultured cells have characteristics and functions similar to the cells in the body? In general, cells growing in monolayers rapidly lose specific functions such as drug metabolizing and detoxifying enzyme activity that the cells originally have in the body (24). Some drugs become toxic after being metabolized. In such a case, it is meaningless to use the cells that have lost their ability to metabolize the test drugs. On the other hand, three dimensional cultures such as spheroid cultures, hollow fiber membrane chambers and multiporous microcarrier maintain the original liver tissue functions such as albumin production, glucuronidation and ureagenesis fairly well (25-28). Therefore, when the toxicity of drugs is tested using cultured cells, care must be taken in choosing the most appropriate method. Although we detected the albumin production of HUH-6 cells in spheroid cultures to a greater extent than in monolayer cultures, whether these spheroid culture can display other hepatocyte-specific functions, especially xenometabolic functions, remains to be determined. Interestingly, HUH-6 cells have low alcohol and acetaldehyde dehydrogenase activities (29). However, at the cellular level, the cytotoxic effects of ethanol on these cells may be mainly due to ethanol itself and not to its metabolites such as acetaldehyde (10, 11).

In the toxicity assay of drugs, it is important to consider the metabolic pathway of drugs and their target organs. Since the liver metabolizes many drugs, hepatocyte cultures which have hepatocyte-specific functions are important tools for screening xenobiotics and for elucidating the mechanisms involved in drug toxicity. The HUH-6 cell line, however, does not exhibit activity of cytochrome P-4502E1, one of enzymes related to ethanol metabolism. Recently, a hepatocyte line (MVh2E1) having P-4502E1 activity has been reported (30), but the enzyme activity was low. If any cell line which retains reliable xenobiotic metabolic enzymes can be established, it will be very useful for examining the toxicity of various drugs.

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