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Cytotoxic effects of ethanol on a human hepatoma cell line, PLC/PRF/5

KINO, K.[1], MORIYA, F.[2], KAWASHIMA, K.[1], OHNO, T.[3], and NAMBA, M.[4]*

[1]*Department of Surgery,*

[2]*Department of Legal Medicine,*

[4]*Department of Cell Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, 2–5–1 Shikata, Okayama 700, Japan,*

[3]*RIKEN Cell Bank, Koyadai, Tsukuba Science City, Japan.*

Summary

The cytotoxic effects of ethanol on the colony forming efficiency of PLC/PRF/5 human hepatoma cells were investigated. When cells were inoculated into plastic dishes and incubated in a CO_2 incubator (open system), no cytotoxicity was observed with concentrations of up to 1% of ethanol in the culture medium. However, when flasks were used and their caps were tightly closed (closed system), some cytotoxicity was demonstrated, the lethal dose of ethanol for 50% cell death being 0.64 + 0.04%. The contrasting results in the open and closed systems were due to the evaportion of ethanol, since ethanol was very rapidly lost from the culture medium under the open system, whereas there was no substantial loss of ethanol under the closed system. The present results indicate that a closed system should be used to evaluate cytotoxic effects of volatile substances such as ethanol.

Introduction

The use of cultured cells provides valuable information on the potency of the toxic effects of various substances (1). Ethanol, the principal ingredient omit in alcoholic beverages, is harmful to various types of tissues, such as those of the liver, the esophagus, and the central nervous system, and excessive intake may result in serious diseases such as liver cirrhosis, esophageal cancer, and alcoholism. In this study, we intended to assess the cytotoxicity of ethanol in human liver cells in culture. However, it is very difficult to use normal human liver cells for this purpose, since, at present, we are unable to maintain these cells as replicative cultures, even when various growth factors, such as hepatocyte growth factor (2), or EGF, are used. Therefore, instead of normal human liver cells, we used an established human hepatoma cell line PLC/PRF/5 (3), which has various features that are specific to liver cells, to examine the cytotoxicity of ethanol. Although cells of established cell lines generally show more or less abnormal characteristics in comparison with their normal *in vivo* counterparts, but their use makes experiments easier, more reproducible and less expensive. In fact, in another study, no significant differences in cytotoxicity assays were observed between established cell lines and primary or early passage cell strains (1). We used a colony formation assay as our *in vitro* cytotoxicity assays, since this has been shown to be a highly sensitive method (4).

*Correspondence should be addressed to Dr. Masayoshi Namba, Department of Cell Biology, Institute of Molecular and Cellular Biology, Okayama University Medical School, 2–5–1 Shikata, Okayama 700, Japan
Tel: 0862–23–7151 #2510
Fax: 0862–22–2846

Material and Methods

Cells and Cultures

We used a PLC/PRF/5 human hepatoma cell line which has some characteristic func-

tions of liver cells, e. g., the production of serum proteins such as α-fetoprotein, albumin, antitrypsin, and ceruloplasmin (5). The cells were cultured in a 5% CO_2 incubator at 37°C and maintained by subculturing with 0.2% trypsin (1: 250, Difco, Detroit, MI) in phosphate buffered saline (pH 7.4) when the cultures reached 100% cunfluence. The culture medium used was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml kanamycin.

Cytotoxicity assay

Cytotoxicity was determined by measuring the colony formation of the cells. We made a single cell suspension by trypsinizing the RPL/RFC/5 monolayer culture at the logarithmically growing stage, following which we seeded 500 cells with 5 ml of culture medium containing various concentrations of ethanol, into Falcon #3002 55-mm dishes, for the open system, or into Falcon #3012 flasks for the closed system. Ethanol (Nacalai Tesque. Inc., Kyoto) was directly dissolved in the culture medium on a volume/volume basis. The open and closed systems are shown in Figure 1. The cap of the flask culture was left loose for 1 hr to enable the adjustment of pH (to around 7.4) in the CO_2 incubator; the cap was then tightly closed. Cells were exposed to ethanol for the whole culture period, i.e., from the day of cell seeding to the end of the culture. On days 10 to 14, the cultures were fixed with 100% methanol for 5 min and stained with 2% Giemsa solution for 30 min, and colonies (>20 cells) were counted under a microscope. The percentage 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. Each experiment was carried out at least twice and triplicate dishes or flasks were used at each data point.

Assay of ethanol

Ethanol was determined by headspace gas chromatography, as described by Nanikawa and Kotoku (6). Three dishes or flasks were used at each data point.

Results

As shown in Figure 2, in the open system, no significant ethanol cytotoxicity of ethanol was observed up to an ethanol concentration of 1%. On the other hand, in the closed system, there was some dose-dependent cytotoxicity. From the data of four separate closed system experiments, the ethanol concentration in the culture medium that caused 50% cell death (TCLD50) was calculated to be 0.64 + 0.04%.

Since we observed no cytotoxicity in the open system, we were prompted to measure the time-course change of ethanol concentrations in the culture media. As shown in Figure 3, in the open system, little alcohol remained in the medium 24 hr after incubation, while, in the closed system, no significant reduction in alcohol concentration was observed for up to 48 hr.

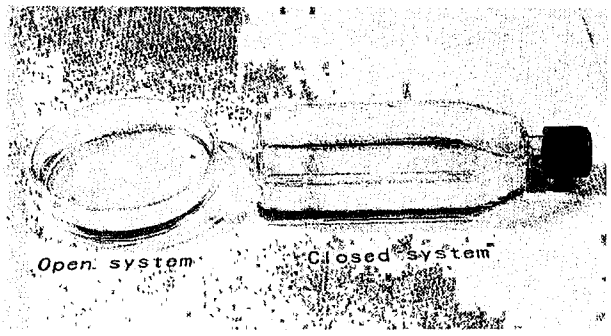

Fig. 1. Open and closed systems with 5 ml culture medium.

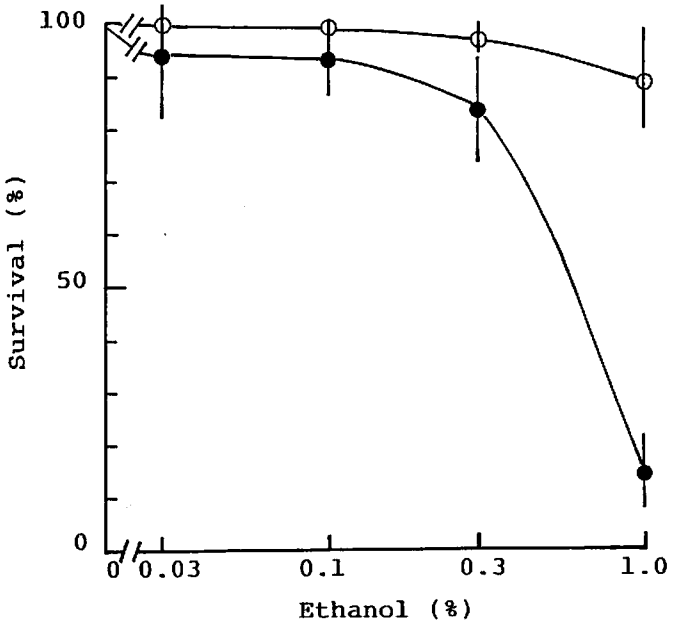

Fig. 2. Effects of ethanol on colony-forming efficiency of PLC/PRF/5 human hepatoma cells in open and closed systems.
○; open system, ●; closed system. Bars indicate standard error.

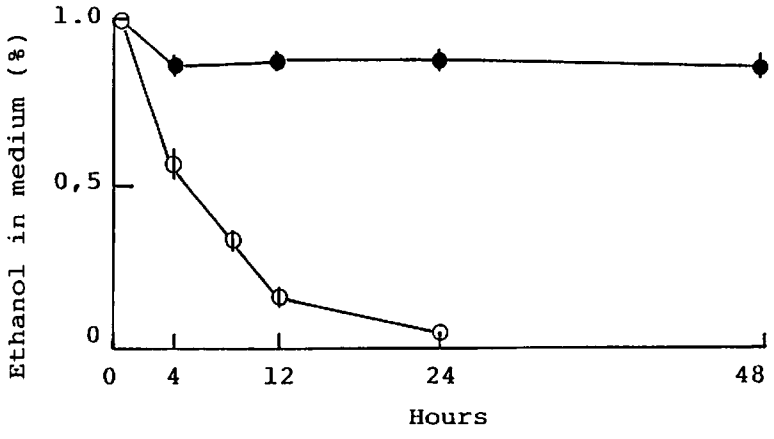

Fig. 3. Time course of ethanol concentrations in 5 ml culture medium in open and closed systems.
○; open system, ●; closed system. Bars indicate standard errors.

Discussion

As stated above, the use of cultured cells provides valuable information on the potency of the toxic effects of various substances (1). That there is a relationship between the cytotoxicity and tissue specificity of drugs is well known. This prompted us to examine the cytotoxic effects of ethanol in human liver cells. However, because of the difficulties inherent in culturing normal human liver cells, we used human hepatoma cells, which exhibit several liver-specific features in the present experiment. We measured the cytotoxicity of ethanol by determining colony forming efficiency, which reflects cell growth potential. However, in the future, whether this assay method is suitable for detecting the toxicity of

ethanol in liver cells should be clarified, since the majority of cells in the liver tissue do not grow under normal conditions. Other assay methods, such as the measurement of LDH release by damaged cells into the culture medium may also be worth evaluating (7).

The lethal concentration of ethanol in the human is around 0.3%, whereas the present *in vitro* data show that the TCLD50 was 0.64 + 0.04%. This concentration is almost twice as high as the *in vivo* one, but at present it is impossible to determine whether there is any relationship between human mortality and cell death. However, the finding that the TCLD50 ethanol concentration is fairly close to the lethal dose *in vivo* indicates that the colony formation assay may be suitable for determining the cytotoxicity of ethanol.

When the open system was used, no cytotoxicity was observed with ethanol concentrations of up to 1%, whereas some cytotoxicity was detected with the closed system. This may be due to the rapid evaporation of ethanol in the open system, since the concentration of ethanol was fairly constant in the closed flasks. These findings indicate that a closed system is more suitable than an open one for determining the cytotoxicity of volatile substances such as ethanol.

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