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Cytotoxicity of millimolar concentrations of ethanol on HepG2 human tumor cell line compared to normal rat hepatocytes in vitro

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Abstract Purpose: The antiproliferative effect of high concentrations of ethanol (80–100 mmol) on liver carcinoma is well known. However, the high concentrations of ethanol affect both tumor cells and normal hepatocytes. The present study was designed to determine the effect of low ethanol concentrations (0–10 mmol) on cell proliferation and cell death (apoptosis and necrosis) in a human tumor cell line HepG2 and in normal rat hepatocytes. Methods: Primary cultures of normal rat hepatocytes and HepG2 cells cultures were used. Cells were incubated with increasing ethanol concentrations or without ethanol (control group) for 24 h and analyzed immediately (group I) or after an additional incubation time of 48 h without additional ethanol application (group II). Cell proliferation was determined by assessing 5-bromo-2′-deoxyuridine (BrdU) incorporation. Apoptosis was assessed by means of DNA fragmentation and cysteine aspartate-specific protease (caspase-3) activity. Necrosis was analyzed by quantification of lactate dehydrogenase (LDH) release into culture medium. Results: Twenty-four h exposure to 1 mmol ethanol inhibited cell proliferation in HepG2 cells by 75% (P < 0.05), while it remained unaltered in rat hepatocytes. The effect of ethanol persisted for another 48 h where cell proliferation was 5% of control in HepG2 cells and 70% of control in rat hepatocytes (P < 0.005). After 24 h incubation with 1 mmol ethanol 28% of HepG2 cells and 12% of rat hepatocytes showed DNA fragmentation as sign of apoptosis (P < 0.001). In group II 39% of HepG2 cells and 26% of rat hepatocytes were apoptotic (P < 0.001). Caspase-3 activation progressively increased after ethanol treatment in HepG2 cells and rat hepatocytes. The first significant difference was observed after 4 h (activity in HepG2 was 68% higher than in rat hepatocytes) and was maximum after 10 to 12 h where the activity in HepG2 was 180% of the activity in rat hepatocytes. Lactate dehydrogenase release into culture medium as an indicator of necrosis in HepG2 cells, increased from 0.5% in group I to 12% in group II, and from 0.1% to 8% in rat hepatocytes (P < 0.005). Increasing ethanol concentration to 10 mmol increased necrosis to 75% in HepG2 cells, and to 45% in rat hepatocytes (P < 0.05) whereas the effects on cell proliferation and apoptosis were not significantly different. Conclusions: Small ethanol concentrations (equivalent to 1 mmol) inhibit cell proliferation and increase apoptosis more strongly in HepG2 cells than in normal rat hepatocytes. These findings suggest the use of 1 mmol ethanol as a treatment for hepatocellular carcinoma because this mainly affects tumor cells but not surrounding normal tissue.

Key words Ethanol · Hepatocellular carcinoma · Cell proliferation · Apoptosis · Necrosis

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. The coexisting cirrhotic changes in the liver parenchyma, even by small tumors (diameter of 3 cm or less), make surgical treatment not an option. The use of cytotoxic drugs may be an alternative treatment. HCC is, however, an intrinsically chemoresistant tumor (Adjei et al. 1996). Therefore, such treatments have not significantly improved the survival rate of patients. Thus, the identification of more effective substances as an alternative treatment would be very helpful.

In the past decade percutaneous ethanol injection has been used in the treatment of solitary or small (less than 4 cm in diameter) HCC (Vilana et al. 1992; Shiina et al. 1993; Redvanly et al. 1993). The effect of ethanol results in inhibition of cell proliferation (Shiina et al.
1990). However, the principal problem with using ethanol is its toxic effects on non-tumor cells adjacent to the tumor area (Shima et al. 1990), especially when using higher concentrations of ethanol for treatment of tumors with more than 4 cm in diameter (Vilana et al. 1992). Ethanol is predominantly metabolized in the liver by alcohol dehydrogenase (ADH), and acetaldelyde dehydrogenase (ALDH). The cytochrome P450 2E1 or microsomal alcohol oxidizing system (MEOS) pathway (Lieber 1994a; Wu and Cederbaum 1996; Bailey and Cunningham 1998), in general, is not important where low doses of alcohol are used (Lieber 1994b). However, the MEOS system is induced through ethanol at high concentrations (Roberts et al. 1995; Ingelmann-Sundberg et al. 1994). Ethanol metabolism alters the cytosolic and mitochondrial redox status and disrupts the functions of various metabolic pathways. Generation of reactive oxygen species are thought to be responsible for most of the hepatotoxicity of high concentrations of ethanol (Lieber 1993; Kurose et al. 1997).

HepG2 cells and primary rat hepatocytes have been extensively used for the study of hepatic metabolism, regeneration, and carcinogenesis. Several studies address the effect of ethanol on HepG2 cells (Wu and Cederbaum 1996; Neuman et al. 1993; Yang and Cederbaum 1997; Cameron et al. 1998). There are many in vitro experimental models of ethanol-induced toxicity (Lieber 1994a; Neuman et al. 1993; Gutierrez-Ruiz et al. 1995). However, most of them have used high concentrations of ethanol (Kurose et al. 1997; Neuman et al. 1993; Cameron et al. 1998; Neuman et al. 1995).

The present study was designed to determine the effect of low ethanol concentrations (0.2–10 mmol) on cell proliferation and cell death (apoptosis and necrosis) in a human tumor cell line HepG2 and in normal rat hepatocytes. The results show a significant difference in the sensitivity of HepG2 cells and not hepatocytes to ethanol that might be important for the therapeutic value of a treatment of hepatocellular carcinoma with low concentrations of ethanol. In a previous study we have demonstrated that 1 mmol ethanol is superior to doxorubicin and mitomycin C in inhibition growth of HepG2 cells (Castañeda and Kinne 1999).

Materials and methods

Experiments on HepG2 cells

HepG2 cells, derived from human hepatocellular carcinoma (obtained from Deutsches Krebsforschungszentrum, Heidelberg, Germany) were seeded in 250 ml tissue culture flasks (Falcon-Becton Dickinson, Heidelberg, Germany) at 1 × 10⁴/ml concentration in 10 ml RPMI-1640 medium supplemented with 10% fetal bovine serum (Boehringer Mannheim, Mannheim, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (ICN Flow, Meckenheim, Germany) at 37 °C, in a humidified atmosphere of 5% air and 7.5% CO₂. After 7 days of cell culture, the cells were harvested with 0.05% trypsin/0.02% EDTA (Gibco BRL, Eggersheim, Germany). Cells were seeded in 96-well microtiter plates (Falcon-Becton Dickinson, Heidelberg, Germany) at concentrations of 5 × 10⁴ in 200 μl culture medium. After 24 h, ethanol (Merck, Darmstadt, Germany) was added and cells were incubated for an additional 24 h in the presence of increasing ethanol concentrations (0.2–10 mmol). Then cells and cell supernatants were analyzed either immediately after ethanol exposure (group I) or after another incubation for 48 h in ethanol-free medium (group II). Control cells were exposed for the same period of time only to RPMI culture medium.

Primary cultures of isolated rat hepatocytes

Liver cells were obtained from male Wistar rats weighing 220–280 g after exsanguination by in situ perfusion of the liver with HCO₃⁻-containing Ca²⁺-free ion Krebs-Henseleit solution at 37 °C. The Regierungspräsident Arnsberg and the Animal Care Committee approved all procedures. Hepatocytes were harvested using a perfusion technique (Petzinger et al. 1988) previously described by Wehner (Wehner and Guth 1991). Briefly, under intraperitoneal urethane anesthesia (1 g per kg body weight) the liver was perfused with a 0.05% collagenase (type A, Sigma, Deisenhofen, Germany) solution for 20 min. After dissociation, the cell suspension was used. Primary cell cultures were performed in 96-well microtiter plates at 2 × 10⁴ in 200 μl RPMI-1640 culture medium supplemented with fetal bovine serum, penicillin, and streptomycin and maintained under the same conditions as the HepG2 cells. The effect of ethanol was analyzed using the same procedure as described above.

Cell proliferation assessment

At the end of the treatment time, cell proliferation rate was measured by means of a colorimetric 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA test (Boehringer Mannheim, Mannheim, Germany), based on the measurement of BrdU incorporation during DNA synthesis. Briefly, BrdU labeling solution (20 μl/well) was added and incubated for 16 h at 37 °C. Thereafter, FixDenat solution (200 μl/well) was added and incubated for 30 min at room temperature. Finally, anti-BrdU-POD solution (100 μl/well) was added also for 30 min at room temperature. At the end of the assay, 100 μl peroxidase substrate (5-bromo-2-deoxyuridine labeling) was filled into each well. After 15 min incubation at room temperature, absorbance of the samples was assessed. Results are expressed as mean absorbance of the samples in an ELISA plate reader (Dynatech Laboratories MR5000, Denkendorf, Germany) at 450 nm with a reference wavelength of 690 nm. Measurements were made after 24 or 72 h of ethanol exposure. Results were plotted as dose-response curve using BrdU incorporation percentage of control, and ethanol concentration (0–10 mmol).

To evaluate the sensitivity of ethanol (0–2 mmol) after 24 and 72 h, we determined the concentration required to inhibit cell proliferation by 50% (IC₅₀) and 90% (IC₉₀) of control values in HepG2 cells and in isolated rat hepatocytes using the BrdU incorporation test.

Cell death assessment

Ethanol-induced necrosis was expressed as the percentage of LDH released after subtracting LDH content in culture medium (or supernatant) using LDH content in cytosolic fraction. The LDH level was determined using the method described by Decker et al. (Decker and Lohmann-Matthes 1988). Briefly, 10 μl of pyruvate (Sigma, Deisenhofen, Germany) were added to each well containing the supernatant or the lysed cytosolic fraction. The enzymatic reaction was started by the addition of 20 μl freshly prepared NADH solution (Sigma, Deisenhofen, Germany). Twenty microliters of LDH inhibitor oxamate (Sigma, Deisenhofen, Germany) was added after 7 min at room temperature. Absorbance in each well was determined using a wavelength of 340 nm in a microplate...