TOXIC EFFECTS OF CARBON TETRACHLORIDE ON THE PRIMARY HEPATOCYTE CULTURE FROM MONGOLIAN GERBIL (*Meriones unguiculatus*)

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Abstract: Carbon tetrachloride (CCl_4) hepatotoxicity to the primary hepatocyte culture of Mongolian gerbils was studied. Transaminase leakage from cultured cells appeared at 3 h-exposure to 0.5 or 1.0 mM CCl_4 , remarkably increasing at 24 h. With 0.5 mM CCl_4 , there were marked increase of lipid droplets, aggregation of swollen smooth-surfaced endoplasmic reticuli (sER), dilatation of ER lumen, metamorphosis of mitochondria, and reduction of periodic acid-Schiff (PAS) reactivity. Cholesterol clefts were produced in ER areas at 3h-exposure, increasing in size and number with time course. These morphological changes due to CCl_4 were enhanced by previous treating of the culture with phenobarbital. (J Toxicol Pathol 4: 137~144, 1991)

Key words: Carbon tetrachloride, Hepatocyte culture, Hepatotoxicity, Mongolian gerbil

Introduction

CCl_4 is known to produce necrotizing lesions in the liver of many but not all animal species¹⁻⁶. Interspecies difference in its hepatotoxicity has been ascribed to difference in ability to biotransform the chemical to reactive metabolites either covalent binding with cellular constituents or promoting lipid peroxidation process at the cellular membrane³. Although isolated or cultured hepatocytes are being widely used for studying the mechanism of chemically induced cytotoxicity, some discrepancy has been noticed between experimental results *in vitro* and those *in vivo*⁷.

The Mongolian gerbil, *Meriones unguiculatus*, was reported to metabolize CCl_4 more intensively than the rat and to show higher sensitivity to the chemical^{8,9}. The present paper is to describe the morphological sequelae observed in cultured ger-

bil hepatocytes exposed to CCl_4 to have further information to the CCl_4 hepatotoxicity in this species of animals showing characteristic metabolism of lipids or cholesterol^{8,10-12}.

Materials and Methods

1. Animals

Male Mongolian gerbils were bred in a conventional environment of this laboratory and fed commercial pellets (MF; Oriental Yeast, Tokyo) and given tap water *ad libitum*. Animals aged 9 to 12 weeks weighing 60 to 70 g were used.

2. Hepatocyte culture

According to a two step perfusion method proposed by Berry and Friend¹³, the liver of pentobarbital sodium-anesthetized gerbils was perfused *in situ* for 7 min with a modified Krebs-Ringer bicarbonate buffer (119.8 mM NaCl, 23.8 mM NaHCO_3 , 0.6 mM MgSO_4 , 0.6 mM KH_2PO_4 and 5 mM HEPES, pH 7.4) containing 0.1 mM EGTA (KR-buffer) previously warmed at 37°C. The perfusate was introduced from the hepatic portal vein and drained via the inferior vena cava. The organ was then perfused for 7 min with the

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Accepted for publication: June 23, 1991

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same buffer supplemented with collagenase (50 mU/ml) and CaCl₂ (1.0 mM) (KR-C buffer). After perfusion, the liver was minced and tissues were dispersed in KR-C buffer. After gentle stirring, the suspension was filtered through a stainless mesh, and the filtrate was centrifuged at $40 \times g$ for 90 sec. The sediment was resuspended in William's medium E (WME) and washed three times with WME by centrifuge. The final sediment was suspended in WME supplemented with swine serum (10%), insulin (5 μ g/ml), dexamethasone (4 ng/ml), and kanamycin (50 μ g/ml). The cell preparation showed a viability of 85% or higher in trypan blue exclusion test. It was introduced at a density of 7.5×10^5 cells/plate into 35 mm-plastic culture dishes, which contained a collagen coated cover slip. After incubation at 37°C for 3 h in a humidified environment at 5% CO₂-95% air, the cultures were rinsed with warmed complete WME to remove free cells and debris. Then 2 ml of the same medium were added to the cultures, which were incubated at 37°C.

Fig. 1. Enzyme leakage from cultured gerbil hepatocytes treated with CCl₄. Indicated amounts of CCl₄ in DMSO were added to one day-cultures with 2 ml medium in 35 mm dishes. Each bar represents a ratio of enzyme activity; $[(\text{Cell} : \text{Cell} + \text{Medium}) \times 100 (\%)]$. Mean $\pm$ SD from 3 animals. * $P < 0.05$, ** $P < 0.01$ as compared with non-treated control (Student's *t*-test).

3. Exposure to CCl₄

CCl₄ (Wako Pure Chemical Industries, Osaka) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 M. The solution was diluted serially with serum-free complete WME, and 2 ml of each dilution was added to 24 h-culture of hepatocytes which were previously rinsed with warmed serum-free medium. In another experiment, cell cultures were pretreated with 2.5 mM phenobarbital sodium (PB) (Wako Pure Chemical Industries, Osaka) for 6 h, rinsed with the medium, and then exposed to 0.5 mM CCl₄ for 3 h.

4. Assays for glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT)

At 3, 6, and 24 h-exposure to CCl₄, the incubation medium was removed, and the cells were scraped into 2 ml phosphate buffer (100 mM, pH 7.2) containing 0.1% Triton X-100 using a teflon policeman and homogenized. The suspension was centrifuged at $10,000 \times g$ for 20 min. The supernatant and incubation medium were stocked at -20°C , and they were assayed for GOT and GPT activity according to Reitmann-Frankel method¹⁴.

5. Cytopathology

After exposing to CCl₄, hepatocyte cultures on collagen coated cover slips were observed by phase-contrast microscopy (PCM). Then cultures were fixed in formalin and stained with periodic acid-Schiff (PAS), Sudan black B(SBB), and oil red O (ORO) for light microscopy. For electron microscopy some cultures were washed with WME and fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2 and postfixed in 1% osmium tetroxide in the same buffer. They were dehydrated by a graded ethanol series and embedded in Epon 812 resin. Ultrathin sections were made in parallel to the culture surface and stained with uranyl acetate and lead citrate. Observation was made using a JEM-1200EX electron microscope (EM).

Results

At 3 h-exposure to 0.5 mM or 1.0 mM CCl₄, the

Fig. 2. Untreated gerbil hepatocyte culture at 24 h of incubation, stained with a) PCM, b) SBB, and c) PAS.

GOT and GPT levels of the culture medium were significantly higher as compared with those of controls (Fig. 1). The enzyme leakage was much more remarkable at 24 h-exposure. Even with 0.1 mM CCl_4 , exposure some leakage was seen.

Untreated cultures showed a nearly confluent monolayer of polygonal hepatocytes and three to six neighbouring cells contacted each other by PCM (Fig. 2a). Each cell had one or two nuclei and clear cytoplasm with a PAS-positive marginal zone and some SBB- or ORO-positive lipid droplets (Figs. 2b and 2c). At 24 h-culture with or without 0.1% DMSO, lipid droplets were either decreased in number or disappeared, while PAS positivity still remained.

At 3 h-exposure to 0.5 mM CCl_4 , cytoplasmic lipid droplets were increased in number, and the cytoplasm of some cells was diffusely PAS-positive at various degrees. EM revealed lucid cholesterol clefts appearing in the lumen of aggregated smooth-surfaced endoplasmic reticulum (sER) (Fig. 3).

At 6 h-exposure, lipid droplets were numerous and diffusely distributed in the irregular-shaped cytoplasm (Figs. 4a and 4b). A number of cells were PAS negative (Fig. 4c). EM revealed an increased number of enlarged cholesterol clefts and various sized lipid droplets in aggregated sER and Golgi complex (Fig. 5). A few cells showed de-

Fig. 3. Gerbil hepatocyte culture at 3 h-exposure to 0.5 mM of CCl_4 , showing some cholesterol clefts (arrows) in sER area. TEM. Bar = 2 μm .

granulation of rough-surfaced endoplasmic reticulum (rER) with dilated lumens, where myelin body structures were present (Fig. 6a). Aggregated cholesterol clefts were surrounded by peroxisomes (Fig. 6b). There were swollen and

Fig. 4. Gerbil hepatocyte culture at 6 h-exposure to 0.5 mM of CCl₄, showing lipid droplets (a, b) and reduced PAS positivity (c) in the cytoplasm. a) PCM, b) SBB, c) PAS.

Fig. 5. Gerbil hepatocyte culture at 6 h-exposure to 0.5 mM of CCl₄, showing an increased number of cholesterol clefts and lipid droplets. TEM. Bar = 2 μ m.

metamorphosed mitochondria with poor cristae (Fig. 6c). Glycogen granules were reduced in number as compared with non-treated control cultures.

At 24 h-exposure to 0.5 mM CCl₄, certain cells were necrotized being detached from the plate. PCM revealed that most cells had a great number of larger lipid droplets (Figs. 7a and 7b) showing considerably metamorphosed organelle. Most

cells were PAS-negative and a few cells showed a dark cytoplasm. By EM a number of lipid droplets were shown to be deformed, and degranulated rER were disorganized with dilated lumen. A few cells were shown to have aggregated sER or rER, irregular-shaped mitochondria, and remarkably larger cholesterol clefts (Fig. 8).

After exposure to 0.1 mM CCl₄ for 6 h, lipid droplets were slightly increased in number, and

Fig. 6. Gerbil hepatocyte culture at 6 h-exposure to 0.5 mM of CCl_4 , showing dilated ER lumen (a), aggregated cholesterol clefts surrounded by peroxisomes (b), and swollen mitochondria (c). TEM. Bar = 2 μm .

Fig. 7. Gerbil hepatocyte culture at 24 h-exposure to 0.5 mM (a, b) or 0.1 mM (c, d) of CCl_4 . SBB (a, c) or ORO (b, d) stain.

small cholesterol clefts appeared within the lumen of sER. At 24 h-exposure a few cells had enlarged lipid droplets being diffusely positive for SBB and ORO (Figs. 7c and 7d). The number of PAS-positive cells were smaller as compared with untreated control cultures.

After treating with 2.5 mM PB for 6 h, cultured hepatocytes seemed to be metabolically activated showing abundant ER areas (Fig. 9a). The subsequent exposure to 0.5 mM CCl_4 for 3 h, the number of cytoplasmic droplets and vacuoles were increased more remarkably than in case of non-PB

Fig. 8. Gerbil hepatocyte culture at 24 h-exposure to 0.5 mM of CCl₄, showing numerous large cholesterol clefts. TEM. Bar=2μm.

Fig. 9. Gerbil hepatocyte culture at 3 h-exposure to 0.5 mM of CCl₄ at 6 h-treating with 2.5 mM PB (a, b), and at 3 h-exposure to 0.5 mM of CCl₄ after 6 h-pretreating with 2.5 mM PB (c). PCM.

treated control cultures (Figs. 9b and 9c). The lumens of ER, Golgi complex, and nuclear membrane were considerably distended and a number of larger cholesterol clefts were produced within aggregated sER in PB-treated and CCl₄-exposed cultures (Fig. 10).

Discussion

The primary culture of rat or mouse hepatocytes has been reported to be a useful model for CCl₄ hepatotoxicity¹⁵⁻¹⁸. Nakamura *et al*¹⁵, reported LDH and GOT leakage and morphologic changes in the primary rat hepatocyte cultures after expo-

Fig. 10. Gerbil hepatocyte culture at 3 h-exposure to 0.5 mM of CCl_4 after 6 h-pretreating with 2.5 mM PB, showing dilated EM lumen, aggregated sER and remarkable production of cholesterol clefts. TEM. Bar = 2 μm .

sure to more than 5 mM of CCl_4 . In this study, cultured hepatocytes from gerbils, which were known to be susceptible to CCl_4 , were shown to be very sensitive to the chemical. The leakage of GOT and GPT as well as cytopathic changes were observed already after 3 h-exposure to 0.5 mM of CCl_4 . Exposure to 0.1 mM of CCl_4 also induced a mild enzyme leakage and some lipid accumulation at 24 h. These findings agreed with liver cell injury induced *in vivo* in the gerbil treated with CCl_4 .

The fat accumulation in the liver occurring shortly after CCl_4 treating, might result from blocking of lipoprotein secretion in consequence of covalent bindings of CCl_4 metabolites with the cell structure^{16,19,20}. However, it was reported that no severe fatty liver was produced in the gerbil after CCl_4 administration⁷. In the present cytopathic study, the cultured gerbil hepatocytes were shown to have remarkable lipid deposits being ORO and SBB-positive after CCl_4 exposure. In gerbils naturally living in dry environment, lipid catabolism might be of importance for energy production, and this species of animals normally has a higher lipid content in the liver than the rat⁸. The lipid droplets in gerbil hepatocytes were reduced in culture without reduction of glycogen.

The lipid accumulation in cultured gerbil hepatocytes exposed to CCl_4 , seemed to result from blocking of lipid metabolism, lipoprotein secretion, or lipid catabolism.

In the present study, characteristic cholesterol clefts appeared in ER areas of cultured gerbil hepatocytes at a very early stage of exposure to 0.1 or 0.5 mM CCl_4 . The clefts increased in size and number with time course at higher concentration of CCl_4 , suggesting that CCl_4 inhibited cholesterol metabolism. The hepatocyte secretion of very low density lipoprotein (VLDL)^{13,14} integrating cholesterol might be inhibited by CCl_4 , while no cholesterol clefts have been described in the rat hepatocytes exposed to CCl_4 . The gerbil is known to respond promptly to dietary cholesterol showing a marked rise of its serum level and considerable accumulation of cholesterol ester in the liver¹⁰⁻¹². These findings might be due to a low grade turnover of cholesterol including its catabolism in the gerbil liver. The appearance of intracellular clefts of cholesterol ester in CCl_4 -treated gerbil hepatocyte culture might result from the inhibition of cholesterol catabolism.

Cai and Mehendale⁹ reported that neither chlordecone, phenobarbital nor mirex affected hepatotoxic and lethal effects of CCl_4 in the gerbil

while rather enhancing CCl₄ hepatotoxicity in the rat. In this study, acute and severe morphologic changes were induced in gerbil hepatocytes, when the culture was previously treated with PB before CCl₄. As the cytochrome P-450 content of cultured hepatocytes is known to be reduced with incubation time^{21,22}, the more remarkable morphologic changes induced by PB plus CCl₄ were assumed to be due to accelerated production of bioactive CCl₄ metabolites resulting from increased level of monooxygenase.

On the other hand, gerbil hepatocytes showed a very low degree of necrotic changes after 24 h-exposure to CCl₄ in spite of remarkable enzyme leakage. The gerbil liver was reported to be comparatively resistant to necrogenic effect of CCl₄ as compared with the rat liver⁸, suggesting that the homeostasis of hepatic calcium is more stable in the gerbil.

The cultured gerbil hepatocytes might provide a good tool for studying the mechanisms of CCl₄ hepatotoxicity.

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