TOXIC EFFECTS OF T-2 TOXIN ON THE PRIMARY HEPATOCYTE CULTURE FROM MICE

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Abstract: The effects of T-2 toxin on hepatocytes were investigated *in vitro*. Cultured mouse hepatocytes were treated with several doses of T-2 toxin (1, 10, and 100 $\mu\text{g/ml}$) for 6 and 24 hr. There was no significant change in the release of GPT and LDH from cultured hepatocytes into medium. However, LPO contents in hepatocytes increased dose-dependently at 6 and 24 hr after T-2 toxin administration. A decrease in cell density in hepatocyte cultures due to atrophy and necrosis was conspicuous at 24 hr-exposure to 100 $\mu\text{g/ml}$ of T-2 toxin. Electron microscopically, an increase of autosegrisomes was observed in hepatocytes at 24 hr-exposure to 1 $\mu\text{g/ml}$ of T-2 toxin. Apart from their severity, the nature of ultrastructural changes was common to hepatocytes exposed to 10 and 100 $\mu\text{g/ml}$ of T-2 toxin. Namely, degeneration of rER, deformation of mitochondria, and formation of autosegrisomes developed at 6 hr and became more prominent at 24 hr. (J Toxicol Pathol 7: 43~50, 1994)

Key words: Mouse, T-2 toxin, Primary hepatocyte culture

Introduction

T-2 toxin [3α -hydroxy- 4β , 15-diacetoxy-8- α -(3-methylbutyryloxy)-12, 13-epoxytrichothec-9-ene] is a member of the trichothecene mycotoxin produced by *Fusarium* species¹. This toxin causes pathological and biochemical disorders mainly in gastrointestinal mucosa and lymphoid and hematopoietic tissues due to its radiomimetic and immunosuppressive effects². In addition, it was reported that T-2 toxin could also produce hepatic changes such as enlargement, fatty degeneration, and/or necrosis^{3,4}. On the other hands, *in vivo* studies on the toxic effects of T-2 toxin are scarce. Segal et al.⁵ described that T-2 toxin induced hemolysis of erythrocytes obtained from many animal species by generating free radicals, and Trusal and O'Brien⁶ reported that rat hepatocytes exposed to T-2 toxin *in vitro* showed ultrastructural changes in rough endoplasmic reticula and mitochondria. The purpose of this

study is to clarify the biochemical and morphological effects of T-2 toxin on cultured mouse hepatocytes.

Materials and Methods

Hepatocyte cultures

Hepatocytes were prepared by collagenase perfusion of the liver isolated from 7-to 8-week-old male ICR: CD-1 mice (30-35 g) according to the methods of Seglen⁷. Cells were suspended in Williams medium E containing 5% fetal calf serum (FCS), 50 $\mu\text{g/ml}$ kanamycin, 1 μM dexamethasone, and 100 U/ml insulin at a concentration of 4×10^5 cells/ml, and 1.5 ml of the suspension was introduced into 35 mm dishes with collagen-coated glass coverslips.

After incubated at 37°C for 2 hr, medium together with nonadherent cells was aspirated and replaced with fresh medium without FCS.

Biochemistry

Activities of glutamic pyruvic transaminase (GPT) and lactose dehydrogenase (LDH) and contents of lipid peroxides (LPOs) in culture

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medium and cells lysed with Triton X-100 were measured. Each data point represents triplicated wells. Activities of GPT and LDH were analyzed by an autoanalyzer, Monarch (Instrumentation Laboratory, USA) and contents of LPOs were estimated as malondialdehyde (MDA) concentration by TBA method according to Uchiyama and Mihara⁸. Statistical analysis was performed using student's *t*-test and values were expressed as mean $\pm$ SD of 3 replicate determination.

Toxin

T-2 toxin (Sigma) was maintained at a stock concentration of 5 mg/ml in 20% ethanol. Just prior to use, the toxin was diluted to the desired concentrations (i.e., 1, 10 or 100 μ g/ml) in the serum-free culture medium and applied to 24 h culture of hepatocytes. Control hepatocytes received 0.1% ethanol which was equivalent to the concentration found in a 100 μ g/ml T-2 toxin group.

Morphology

After exposed to T-2 toxin for 6 hr or 24 hr,

hepatocytes on collagen-coated coverslips were fixed in 99% methanol and stained with Giemsa for light microscopic and morphometric examinations. Morphometric analysis was performed on the number of hepatocyte nuclei and the total area of hepatocytes in randomly chosen 5 areas (10,000 μ m² each) on Giemsa-stained section using an image analyzer (SP500: Olympus Co. Ltd., Tokyo).

For electron microscopy, cultures were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in phosphate buffer (pH 7.4), post fixed in 1.0% osmium tetroxide in the same buffer and embedded in epoxy resin, Quetol 812 (Nisshin EM Co. Ltd. Tokyo). Ultrathin sections were double-stained with uranyl acetate and lead citrate.

Results

GPT and LDH activities

There was no significant change in the release of GPT and LDH from cultured hepatocytes into medium between control and T-2 toxin-exposed cultures except for the GPT activity in cultures at

Fig. 1. GPT leakage from cultured mouse hepatocytes treated with T-2 toxin. Each bar represents a ratio of enzyme activity (mean $\pm$ SD); [(Medium/Cell + Medium) $\times$ 100(%)]. Analysis of significance by Student's *t*-test (*; $p < 0.05$). ■; not-treated, ■; 1 μ g/ml, ■; 10 μ g/ml, ▨; 100 μ g/ml of T-2 toxin.

6 hr-exposure to 10 $\mu\text{g/ml}$ (Figs. 1 and 2).

LPO contents

Except for the content of LPOs in cultured hepatocytes at 6 hr exposure to 1.0 $\mu\text{g/ml}$ of T-2 toxin, the LPO level was significantly higher in cultured hepatocytes exposed to T-2 toxin compared with that in controls (Fig. 3a). On the other hand, the LPO level in culture medium was always significantly higher compared with that in controls (Fig. 3b).

Light microscopic findings

Control cultures showed a nearly confluent monolayer of polygonal hepatocytes which had one or two nuclei and fine or small-sized vacuoles at 6 hr exposure (Fig. 4a), and they exhibited a very slight decrease in cell density at 24 hr-exposure (Fig. 4b). Cultures exposed to 1 $\mu\text{g/ml}$ of T-2 toxin showed a similar picture to that in control ones.

At 6 hr-exposure to 10 $\mu\text{g/ml}$ of T-2 toxin, medium and large-sized cytoplasmic vacuoles increased in number in addition to fine or small sized

ones (Fig. 4c). Cell density decreased mainly due to slight to moderate atrophy of hepatocytes at 24 hr-exposure to 10 $\mu\text{g/ml}$ of T-2 toxin (Fig. 4d).

Except for an increase in number of necrotic cells, the picture of cultures exposed to 100 $\mu\text{g/ml}$ of T-2 toxin was similar to that exposed to 10 $\mu\text{g/ml}$ of T-2 toxin at 6 hr-exposure (Figs. 4c and 4e). At 24 hr-exposure to 100 $\mu\text{g/ml}$ of T-2 toxin, cell density decreased markedly, and the remaining hepatocytes generally showed apparent atrophy. In addition, atrophic hepatocytes with elongated cytoplasm were also observed (Fig. 4f).

Morphometric findings

As shown in Table 1, the number of hepatocyte nuclei did not prominently differ by exposure-time and dose of T-2 toxin. In coincidence with the above-mentioned light microscopic findings, a decrease in the total area of hepatocytes was conspicuous at 24 hr-exposure to 100 $\mu\text{g/ml}$ of T-2 toxin (Table 1).

Electron microscopic findings

Control hepatocytes had well-developed

Fig. 2. LDH leakage from mouse hepatocytes. Each bar represents a ratio of LDH activities (mean $\pm$ SD); [(Medium/Cell + Medium) $\times$ 100(%)]. Analysis of significance by Student's *t*-test (*; $p < 0.05$). ■; not-treated, ■; 1 $\mu\text{g/ml}$, ■; 10 $\mu\text{g/ml}$, ▨; 100 $\mu\text{g/ml}$ of T-2 toxin.

Fig. 3. a) Contents of LPO in cultured mouse hepatocytes lysed with Triton X-100.
b) Contents of LPO in cultured mouse hepatocyte medium.

■; not-treated, ▨; 1 µg/ml, ■; 10 µg/ml, ▩: 100 µg/ml of T-2 toxin.

stacked arrays of rough endoplasmic reticulum (rER) around the nucleus and contains some lipid droplets (Fig. 5). Although hepatocytes exposed to 1.0 µg/ml of T-2 toxin showed almost similar pictures to those of control hepatocytes at 6 hr-exposure (Fig. 6), an increase of autosegrisomes was observed at 24 hr-exposure (Fig. 7).

Apart from their severity, the nature of ultra-structural changes of hepatocytes was common to cultures exposed to 10 and 100 µg/ml of T-2 toxin for 6 hr and 24 hr. Namely, the most prominent alteration was marked degeneration of rER result-

ing in a disappearance of well-developed stacked arrays of rER (Fig. 7). Intracytoplasmic vacuolization and formation of autosegrisomes and atrophy and deformation of mitochondria were simultaneously observed with varying severity. Moreover, dilatation of bile canaliculi was sometimes noticed.

Discussion

Toxic effects of T-2 toxin on the primary hepatocyte culture from mice were investigated

Fig. 4. a) Control mouse hepatocyte culture at 6 hr. Confluent monolayer of polygonal hepatocytes. Giemsa $\times 200$.
 b) Control mouse hepatocyte culture at 24 hr. A very slight decrease in cell density. Giemsa $\times 200$.
 c) Mouse hepatocyte culture at 6 hr-exposure to $10 \mu\text{g/ml}$ of T-2 toxin. Medium- and large-sized intracytoplasmic vacuoles. Giemsa $\times 200$.
 d) Mouse hepatocyte culture at 24 hr-exposure to $10 \mu\text{g/ml}$ of T-2 toxin. A decrease in cell density due to moderate atrophy of hepatocytes. Giemsa $\times 200$.
 e) Mouse hepatocyte culture at 6 hr-exposure to $100 \mu\text{g/ml}$ of T-2 toxin. Necrosis and atrophy of hepatocytes. Giemsa $\times 200$.
 f) Mouse hepatocyte culture at 24 hr exposure to $100 \mu\text{g/ml}$ of T-2 toxin. A marked decrease in cell density. Giemsa $\times 200$.

biochemically and morphologically for the first time. Previous reports described that LPO levels elevated in the liver of many strains of male mice *in vivo* after T-2 toxin-intoxication⁹. The present study revealed that T-2 toxin also enhanced LPO levels in primary cultured mouse hepatocytes at least after 6 hr-exposure and an increase in LPO content may have some relations to such ultrastructural changes in rER and mitochondria as mentioned below. On the other hand, interestingly, T-2 toxin had no significant effect on release of

GPT and LDH from cultured hepatocytes into medium.

Light microscopic changes in hepatocyte cultures were well correlated with morphometric ones. And the most characteristic change was a decrease in cell density mainly due to atrophy of hepatocytes (at 24 hr-exposure to $10 \mu\text{g/ml}$) and to atrophy and necrosis of hepatocytes (at 24 hr exposure to $100 \mu\text{g/ml}$). An increase in number of medium- and large-sized vacuoles was also observed in hepatocyte cultures exposed to 10 and 100

Table 1. Morphometric Findings of Primary Mouse Hepatocyte Cultures Exposed to T-2 Toxin

	Control		Toxin dose ($\mu\text{g/ml}$)					
			1		10		1,000	
	6h	24h	6h	24h	6h	24h	6h	24h
Number of hepatocyte nuclei #	130.0 ± 5.15	119.7 ± 1.04	120.6 ± 3.46	120.9 ± 4.66	124.2 ± 3.50	114.1 ± 2.21	112.4* ± 3.41	112.2* ± 3.16
Total area of hepatocytes (μm^2) #	9335.8 ± 167.48	8343.2 ± 124.5	8292.8* ± 107.7	7866.8 ± 103.44	8204.8* ± 89.71	7356.2* ± 6.55	8234.2* ± 86.02	4967.0* ± 199.62

: Mean $\pm$ SD/10,000 μm^2 of randomly chosen 5 areas in 3 samples.

* ; Significantly different from controls ($p < 0.05$).

Fig. 5. Control mouse hepatocyte at 24 hr. Well developed stacked arrays of rER and some lipid droplets. Bar = 2 μm

Fig. 6. Mouse hepatocyte at 6 hr-exposure to 1 $\mu\text{g/ml}$ of T-2 toxin. Similar picture to control hepatocyte. Bar = 2 μm .

Fig. 7. Mouse hepatocyte at 24 hr-exposure to 1 $\mu\text{g}/\text{ml}$ of T-2 toxin. An increase of autosegrisomes. Bar = 2 μm .

Fig. 8. Mouse hepatocyte at 24 hr-exposure to 100 $\mu\text{g}/\text{ml}$ of T-2 toxin. Marked degeneration of rER, deformation of mitochondria, and many autosegrisomes. Bar = 2 μm .

$\mu\text{g}/\text{ml}$ of T-2 toxin. Trusal¹⁰ reported that T-2 toxin induced mitochondrial changes characterized by condensed configurations and cristae alterations in CHO and VERO cells, and Oldham *et al.*¹¹ described that T-2 toxin brought about irregularities in the nuclear envelope and chromatin margination patterns in human fibroblasts. Furthermore, Trusal and O'Brien⁶ reported that T-2 toxin produced ultrastructural changes in rER (degranulation of attached ribosomes without dilatation of cisternae) and mitochondria (appearance of translucent foci or electron dense cores in matrix)

in primary hepatocyte cultures from rats. It is considered that degranulation of attached ribosomes from rER is consistent with inhibition of protein synthesis caused by the toxin. Although ultrastructural changes in primary hepatocyte cultures from mice were also detected in rER and mitochondria like hepatocyte cultures from rats, their intensity was apparently higher in mouse hepatocytes than in rat ones. In addition, intracytoplasmic vacuolization was also prominent in mouse hepatocytes. One possible explanation for such difference in the severity of ultrastructural

changes between mouse and rat hepatocytes was that the exposure time was longer and the dose was higher in the present study compared with those of Trusal and O'Brien⁶. The exposure time and dose of T-2 toxin we used seem to overwhelm the ability of hepatocytes to detoxify T-2 toxin. More detailed investigation on the pathogenesis of T-2 toxin-induced hepatotoxicity *in vitro* is now in progress using a combination of lower dose and shorter exposure time.

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