Original

T-2 Toxin-Induced Apoptosis and *c-fos* mRNA Expression in Con A-Stimulated Mouse Thymocyte Primary Cultures

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Abstract: The development of apoptosis and the expression of *c-fos* mRNA were investigated up to 24 hours after treatment (HAT) with 0.2 g/ml of T-2 toxin in Con A-stimulated primary thymocyte cultures prepared from BALB/c mice. Cell viability began to decrease from 1 to 3 HAT, and it was approximately 50% at 6 HAT. The level of cytoplasmic nucleosomes peaked at 3 HAT (about 2 times of control) and decreased thereafter. At 6 HAT, thymocytes showed irregular-shaped or fragmented nuclei. By RT-PCR, the level of *c-fos* mRNA began to increase within 1 HAT, and maintained a high level through the observation period. Preincubation with BAPTA/AM, a intracellular calcium ion chelator, markedly suppressed the expression of *c-fos* mRNA and the level of DNA fragmentation induced by T-2 toxin. Although less effective, preincubation with H-7, a protein kinase C (PKC) inhibitor, also depressed the above-mentioned two parameters. These findings indicate that, in mouse thymocyte primary cultures treated with T-2 toxin, *c-fos* may play an important role in the induction of apoptosis and the increase in intracellular calcium ion may be closely related with the expression of *c-fos* mRNA. In addition, these findings also suggest that PKC-dependent pathway may be involved in T-2 toxin-induced thymocyte apoptosis. (J Toxicol Pathol 2001; 14: 247–251)

Key words: T-2 toxin, apoptosis, primary thymocyte culture, *c-fos*, Calcium iron, PKC

Introduction

T-2 toxin is a kind of trichothecene mycotoxin produced by species of the genus *Fusarium*. It infects corns, wheat, barley and rice crops in the field or during storage¹ and affects human and farm animals. Oral, parenteral and cutaneous exposures of trichothecene mycotoxin produce lesions in hematopoietic, lymphoid and gastrointestinal tissues as well as functional suppression in reproductive organs^{2–5}. Our research group first clarified that T-2 toxin-induced death of lymphocytes in the thymus^{6,7}, splenic white pulp^{6,7} and Peyer's patches⁸ is apoptosis. After that, our research group also confirmed that hematopoietic cells in the bone marrow and splenic red pulp⁹, and intestinal crypt epithelial cells¹⁰ die through apoptosis following T-2 toxin-inoculation.

In the study on the sequential changes in mRNA expression of apoptosis-related genes (*fas*, *p53*, *bcl-2*, *c-myc* and *c-fos*) in the thymus of mice treated with T-2 toxin,

Shinozuka *et al.*¹¹ reported that *c-fos* mRNA expression significantly increased prior to the development of apoptotic cell death. Recent studies also reported that the expression of *c-fos* mRNA precedes the initiation of apoptosis or it is concomitant with apoptosis in many systems^{12–16}. In addition, the regulatory mechanisms underlying transcription of the *c-fos* have been extensively studied, and it is well established that both mobilization of intracellular ionized calcium ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC) lead to an accumulation of this transcript^{17–19}.

The purpose of this study was to clarify the relationship between the development of apoptosis and the expression of *c-fos* mRNA and to examine the effects of calcium ion chelators and a protein kinase C (PKC) inhibitor on the expression of *c-fos* mRNA and the DNA fragmentation in T-2 toxin-treated mouse thymocytes.

The present study was approved by The Laboratory Animal Use and Care Committee of Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Materials and Methods

Animals

Four-week-old male BALB/c mice were purchased from SLC (Shizuoka, Japan) and acclimatized for 1 week. They were kept under controlled conditions (temperature, 23

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$\pm 2^{\circ}\text{C}$; relative humidity, $55 \pm 5\%$, 14–16 air changes per hour and a 14-hr light and 10-hr dark cycle) in an animal room and fed commercial pelleted chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*.

Cell cultures

The thymus was obtained from 5-week-old male BALB/c mice. Cells were suspended in Hank's buffered salt solution (HBSS) (Nissui Pharmaceutical Co., Tokyo, Japan) with 2% fetal bovine serum (FBS) (Lot. No. 6A4002, JRH Biosciences, Australia) and cultivated in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol (ME) (WAKO Pure Chemical Co., Osaka, Japan), penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in 5% CO_2 in air. For mitogen-induced blastogenesis, 5 $\mu\text{g}/\text{ml}$ of Con A (WAKO Pure Chemical, Co., Osaka, Japan) was added to cultures and incubated at 37°C in 5% CO_2 in air for 48 hours.

Treatment with T-2 toxin

The stock solution of T-2 toxin (Lot. No. 117F4078, Sigma Chemical Co., St. Louis, USA) was dissolved in 99.5% ethanol, sterilized by filtration, and diluted to the final concentration of 0.2% in RPMI-1640 medium. Cells were incubated in the above-mentioned medium with T-2 toxin or solvent alone.

Experimental designs

Two experiments were carried out. In Experiment 1, the development of apoptosis and the expression of *c-fos* mRNA following T-2 toxin-treatment were investigated in Con A-stimulated mouse thymocyte primary cultures. In Experiment 2, the effects of chelation of calcium ion and inhibition of PKC activity on the development of T-2 toxin-induced apoptosis were investigated in the same culture system.

Experiment 1: Cells (4×10^6 cells/ml) were cultured in RPMI-1640 (supplemented as described above) in 36 mm dishes (SUMILON, Sumitomo bakelite Co., Tokyo, Japan). Then, 0.2 $\mu\text{g}/\text{ml}$ of T-2 toxin or solvent alone were added to cultures and incubated at 37°C in 5% CO_2 . At 1, 3, 6, 12 and 24 hours after treatment (HAT), cell viability was estimated by trypan blue exclusion method, and cell samples were obtained for reverse transcription-polymerase chain reaction (RT-PCR) and DNA fragmentation assays.

Experiment 2: 0.1 μg of ethylene glycol-bis (b-aminoethyl-ether)-N, N, N', N'-tetraacetic acid (EGTA) (DOJINDO Chemical Lab., Kumamoto, Japan) was used as an extracellular ionized calcium ($[\text{Ca}^{2+}]_e$) chelator, and 25 μM of bis-(o-aminophenoxy)-ethane-N, N, N', N'-tetraacetic acid tetrakis (BAPTA/AM) (DOJINDO Chemical Lab., Kumamoto, Japan) as an intracellular ionized calcium ($[\text{Ca}^{2+}]_i$) chelator. As a protein kinase C (PKC) inhibitor, 20 μM of 1-(5-isoquinoliny)sulfonyl-2-methylpiperazine dihydrochloride (H-7) (Seikagaku Co., Tokyo, Japan) was used. Calcium chelators and a PKC inhibitor were dissolved

in water or dimethyl sulfoxide (DMSO) (WAKO Pure Chemical, Co., Osaka, Japan) and diluted to the appropriate final concentration in RPMI 1640 medium.

Con A-stimulated mouse thymocytes which were preincubated with calcium chelators or a PKC inhibitor at the above-mentioned concentrations for 1 hour were treated with 0.2 $\mu\text{g}/\text{ml}$ of T-2 toxin at 37°C in 5% CO_2 in air for 3 hours. At 1 and 3 HAT, cell numbers were counted, and cell samples were obtained for RT-PCR and DNA fragmentation assays.

RNA extraction and RT-PCR method

In Experiment 1 and 2, total RNA extraction and RT reaction were carried out as described in the previous paper¹¹, using Isogen (Nippongene Co., Ltd., Toyama, Japan) and SUPERScript preamplification system (GibcoBRL, Life technology Co., USA). Polymerase chain reaction (PCR) was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of mouse *c-fos* mRNA (sense: GAATGGTGAAGAC CGTGTCAAGG; antisense: CGTTGCTGATGCTC TTGACTGG) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: TGAAGGTC GGTGTGAACGGATTTGGC; antisense: CATGTAGGCCATGAGGTCCAC CAC). PCR was carried out in a 50 μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U AmpliTaq GoldTM, $10 \times$ PCR buffer and dNTP mixture (Perkin Elmer, NJ., USA). This was immediately followed by pre-heating at 95°C for 9 min, denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 1 min using Takara PCR Thermal Cycler MP (Takara, Ohtsu, Japan). PCR products were identified by electrophoresis on 1% agarose gels (Nippon Gene Co, Ltd., Toyama, Japan) followed by ethidium bromide staining. Fluorescence image of gel was visualized under UV-CCD video system (Epi-Light UV_{FA1100}; AISIN COSMOS R&D Co., LTD., Tokyo) and the data were fed into a computer. The image was analyzed by using a Luminous Imager software (AISIN COSMOS R&D Co., LTD., Tokyo). The *c-fos* mRNA level was shown as a relative ratio to GAPDH expression.

Detection of DNA fragmentation by ELISA method

DNA fragmentation in thymocytes was examined by ELISA method using a commercial kit (Cell Death Detection ELISA, Boehringer Mannheim GmbH, Germany). The nucleosomes of samples were incubated on anti-histone-coated microtiter plate module. After removal of unbound nucleosomes by washing step, anti-DNA-peroxidase (POD) reacted with the DNA-part of the nucleosome. After removal of unbound peroxidase conjugate by washing step, the amount of peroxidase retained in the immunocomplex was determined photometrically with ABTS (2, 2'-azino-di-[3-ethylbenzthiazoline sulfonate]). From absorbance values at 405 nm using a microplate reader (Model 550, BIO-RAD, Tokyo, Japan), the specific enrichment of mono- and oligo-nucleosomes released into cytoplasm was calculated.

Statistical analysis

The results of cell viability, and levels of *c-fos* mRNA expression and DNA fragmentation were expressed as mean $\pm$ standard deviation (SD) of 3 or 4 dishes. Statistical analysis was done using the Student's and Welch's *t*-test.

Results

Cell viability and DNA fragmentation assays

The cell viability significantly decreased in ConA-stimulated mouse thymocytes incubated with 0.2 μ g/ml of T-2 toxin. No effects on the cell viability and cytoplasmic nucleosomes were observed until 1 HAT. Thereafter, the cell viability began to decrease from 1 to 3 HAT, and it was approximately 50% at 3 HAT (Fig. 1). The level of cytoplasmic nucleosomes peaked at 3 HAT (about 2 times of control), and decreased thereafter (Fig. 1). At 6 HAT, thymocytes showed irregular-shaped or fragmented nuclei.

Changes in *c-fos* mRNA expression after T-2 toxin-treatment

Sequential changes of *c-fos* mRNA expression after T-2 toxin-treatment are shown in Figs. 2 and 3. A rapid increase in *c-fos* mRNA expression was observed in Con A-stimulated mouse thymocyte primary cultures after T-2 toxin-treatment. The level of *c-fos* mRNA began to increase within 1 HAT, and remained a high level through the observation period. In control cultures, the level of *c-fos* mRNA showed no significant changes through the observation period.

Effects of calcium chelators and a protein kinase inhibitor

The effects of calcium chelators and a PKC inhibitor on *c-fos* mRNA induction by T-2 toxin are shown in Figs. 4 and

5. Chelation of $[Ca^{2+}]_i$ by BAPTA/AM resulted in a marked decrease of *c-fos* mRNA expression (one-fifth of T-2 toxin alone). On the other hand, chelation of $[Ca^{2+}]_e$ by EGTA had no effect on *c-fos* mRNA expression. A PKC inhibitor, H-7, slightly depressed the expression of *c-fos* mRNA (two-third of T-2 toxin alone).

Fig. 6 shows the effects of calcium chelators and a PKC inhibitor on T-2 toxin-induced DNA fragmentation in mouse thymocyte primary cultures. BAPTA/AM and H-7 depressed the level of fragmented DNA to approximately 50% (BAPTA/AM) and 80% (H-7) of T-2 toxin alone, respectively. However, EGTA showed no effect on the level of fragmented DNA.

Fig. 2. Sequential changes of *c-fos* mRNA expression in Con A-stimulated mouse thymocytes primary cultures which were incubated with 0.2 μ g/ml of T-2 toxin or solvent alone (control) for up to 24 hours.

Fig. 1. Effect of T-2 toxin on cell viability (■) and DNA fragmentation (○). Con A-stimulated mouse thymocyte primary cultures were incubated with 0.2 μ g/ml of T-2 toxin for up to 24 HAT. Each value represents the mean of 4 samples per groups. *, $p < 0.05$, **, $p < 0.01$: Significantly different from control.

Fig. 3. Sequential changes of *c-fos* mRNA expression in Con A-stimulated mouse thymocyte primary cultures were incubated with 0.2 μ g/ml T-2 toxin (■) or solvent alone (□) for up to 24 hours. Each value represents the mean of 4 samples per group. **, $p < 0.01$: Significantly different from control.

Fig. 4. Inhibition of T-2 toxin-induced *c-fos* mRNA by calcium chelators (EGTA and BAPTA/AM) and a PKC inhibitor (H-7) in Con A-stimulated mouse thymocyte primary cultures. Cells were preincubated in EGTA (0.1 mM), BAPTA/AM (25 μ M) or H-7 (20 μ M), and then incubated with 0.2 μ g/ml of T-2 toxin or solvent alone (control) for 1 hour.

Fig. 5. Inhibition of T-2 toxin-induced *c-fos* mRNA by calcium chelators and a PKC inhibitor in Con A-stimulated mouse thymocyte primary cultures. As to the conditions of cell cultures, see the footnote of Fig. 4. *c-fos* mRNA expression is expressed as the percent inhibition (percent of T-2 toxin alone). Each value represents the mean of 3 samples per group.

Fig. 6. Inhibition of T-2 toxin-induced DNA fragmentation by calcium chelators and a PKC inhibitor in Con A-stimulated mouse thymocytes. Cells were preincubated with calcium chelators or PKC inhibitors, and then incubated with 0.2 μ g/ml of T-2 toxin for 3 hours. The level of DNA fragmentation is expressed as the percent inhibition (percent of T-2 toxin alone). Each value represents the mean of 3 samples per group.

Discussion

The toxicity of T-2 toxin in Con A-stimulated mouse thymocytes was investigated. In the cells treated with 0.2 μ g/ml of T-2 toxin, cell viability began to decrease from 1 to 3 HAT when the level of fragmented DNA began to increase. These findings suggest that T-2 toxin can also induce apoptotic cell death in mouse thymocytes *in vitro* as reported in *in vivo* study⁷. In addition, a rapid increase in *c-fos* mRNA expression was also observed prior to the development of T-2 toxin-induced apoptotic cell death in mouse thymocytes primary cultures as described in *in vivo* study¹¹.

To understand the mechanisms of T-2 toxin-induced thymocyte apoptosis, it is important to clarify the signal transduction pathway by which *c-fos* proto-oncogene is activated. There are substantial evidences that the expression of *c-fos* is regulated by the major intracellular second messenger, Ca^{2+} ¹⁸. In the present study, *c-fos*

induction was suppressed by chelation of $[\text{Ca}^{2+}]_i$, suggesting that T-2 toxin-induced *c-fos* expression depends on $[\text{Ca}^{2+}]_i$. Similar involvement of $[\text{Ca}^{2+}]_i$ has been reported in the induction of *c-fos* after oxidative stress²⁰ and of hsp70 after heat shock²¹. Although the effect of T-2 toxin on $[\text{Ca}^{2+}]_i$ remains obscure, Yoshino *et al.*²² reported that the level of $[\text{Ca}^{2+}]_i$ was markedly elevated in HL-60 cells after exposure to T-2 toxin. They also reported that intracellular calcium chelators inhibited apoptosis induced by T-2 toxin in HL-60 cells²². In this study, BAPTA/AM depressed the increase in the level of fragmented DNA induced by T-2 toxin. As mentioned above, the mechanism of mobilization of $[\text{Ca}^{2+}]_i$ is still obscure. However, chelation of $[\text{Ca}^{2+}]_e$ by EGTA had no effect on the expression of *c-fos* mRNA and the induction of DNA fragmentation in mouse thymocyte primary cultures

following T-2 toxin-treatment, suggesting that the increase in $[Ca^{2+}]_i$ may be due not to the influx of extracellular Ca^{2+} but to the intracytoplasmic release of Ca^{2+} from endoplasmic reticula.

Although less effective as compared with BAPTA/AM, a chelator of $[Ca^{2+}]_i$, H-7, a PKC inhibitor, also showed depressive effects on T-2 toxin-induced *c-fos* mRNA expression and DNA fragmentation in mouse thymocyte primary cultures. This suggests that PKC-dependent pathway may be also involved in T-2 toxin-induced apoptosis.

In conclusion, in Con A-stimulated mouse thymocyte primary cultures, *c-fos* may play an important role in the induction of apoptosis, and $[Ca^{2+}]_i$ may be closely related with the expression of *c-fos* mRNA. PKC-dependent pathway may also be involved in T-2 toxin-induced thymocyte apoptosis.

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