Original

Development of Apoptosis and Changes in Apoptosis-Related Genes Expression in the Thymus of Nivalenol-Treated Mice

Amnart Poapolathep¹, Kazuhiko Suzuki¹, Kei-ichi Katayama¹, Ryoichi Ohtsuka¹, Takayuki Nagata¹, Koji Uetsuka¹, Hiroyuki Nakayama¹, and Kunio Doi¹

¹Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Abstract: Nivalenol (NIV) is a potent toxic trichothecene mycotoxin produced by *Fusarium nivale*, and our previous study clarified first that NIV induces apoptosis in lymphoid organs of mice in a dose-dependent manner. In this study, the development of apoptosis and changes in the expression of apoptosis-related genes (*fas*, *c-fos*, *c-jun*, *p53*, *bcl-2* and *c-myc*) mRNAs were examined in the thymus of mice for up to 12 hours after inoculation (HAI) of NIV (15 mg/kg b.w.) to elucidate the relationship between the molecular genetic regulatory mechanisms and NIV-induced lymphocyte apoptosis. The number of apoptotic lymphocytes evaluated by TUNEL method clearly increased from 3 HAI and peaked at 9 HAI. The DNA ladder formation by agarose gel electrophoresis was clearly observed at 6 and 9 HAI. The expression of *c-fos* and *c-jun* mRNAs clearly elevated from 0.5 HAI and peaked at 1 HAI. The expression of *fas*, *p53*, *bcl-2* and *c-myc* mRNAs showed no significant changes. These results indicate that *c-fos* and *c-jun* may play an important role in NIV-induced lymphocyte apoptosis. (J Toxicol Pathol 2001; 14: 299–304)

Key words: Nivalenol, mouse, thymus, apoptosis, apoptosis-related genes

Introduction

Nivalenol (NIV) is a kind of type B trichothecene mycotoxins produced by many species of *Fusarium*. Contamination of agricultural products by trichothecene mycotoxins is occurring worldwide, and spontaneous outbreaks of *Fusarium* mycotoxicoses, especially due to deoxynivalenol (DON) and Nivalenol (NIV), have been regularly reported throughout the world¹. In our previous study, we clarified from the multiple viewpoints that NIV induces apoptotic cell death in lymphoid organs in a dose-dependent manner². The pathways by which apoptosis is induced vary depending on stimulators and cell types, and it is well known that some proto-oncogenes called apoptosis-related genes regulate the induction and/or inhibition of apoptosis^{3–5}.

In this study, the development of apoptosis and the sequential changes in the expression of apoptosis-related genes (*fas*, *c-fos*, *c-jun*, *p53*, *bcl-2* and *c-myc*) mRNAs were investigated in order to elucidate the relationship between the molecular genetic regulatory mechanisms and NIV-

induced lymphocyte apoptosis in the thymus of mice.

Materials and Methods

Animals

Forty-five 4-week-old male ICR:CD1 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan) and acclimatized to the environment for 1 week. They were kept in an air-conditioned animal room (temperature: 23 ± 2°C; humidity: 55 ± 5%) with a 12 h-light/ 12 h-dark cycle and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum* throughout the experimental period.

Chemicals

Nivalenol (NIV, lot no. KSE7430) was purchased from Wako Pure Chemical Industries Ltd. (Kyoto, Japan), and it was dissolved with 10% dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries Ltd.) in physiologic saline to the final concentration of 1 mg/ml for oral administration.

Treatments

After overnight fasting, 40 5-week-old male ICR:CD1 mice (body weight: 28.3 ± 1.4 g) were orally administered with NIV at the dose level of 15 mg/kg body weight. The dose of NIV was determined based on the results of the previous study². Five mice were sacrificed by heart puncture under ether anesthesia at 0.5, 1, 2, 3, 4, 6, 9 and 12 hours

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Mailing address: Amnart Poapolathep, Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
TEL: 81-3-5841-5401 FAX: 81-3-5841-8185
E-mail: fvetamp@hotmail.com

after inoculation (HAI), respectively. In addition, the remaining 5 mice which were inoculated with 10% DMSO in physiologic saline in the same way and killed at 12 HAI served as controls.

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.

Histology

At necropsy, the thymus was taken from each mouse, and fixed in 10% neutral buffered formalin. Paraffin sections (4 μ m) were stained with hematoxylin and eosin (HE). For electron microscopic examination, small pieces of the thymus were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Ohken Co., Ltd., Tokyo). Ultrathin sections were double stained with uranyl acetate and lead citrate and observed under a JEM-1200 EX electron microscope (JEOL Co., Ltd., Tokyo). Some of paraffin sections were subjected to *in situ* detection of fragmented DNA as mentioned below. In addition, a part of the fresh thymus was stored at -80°C until used for agarose gel electrophoresis and reverse transcriptase-polymerase chain reaction (RT-PCR).

In situ detection of fragmented DNA

In situ detection of fragmented DNA was carried out on the paraffin sections of thymus by the modified TUNEL method first proposed by Gavrieli *et al.*⁶ using a commercial apoptosis detection kit (Apopotag *In situ* Apoptosis Detection Kit; Interger Co.). In brief, the procedure was as follows: multiple fragmented DNA 3'-OH ends on the paraffin sections were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the sections. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. Counterstain was performed with methyl green. The TUNEL index (%) (positive cells / 1,000 lymphocytes $\times$ 100) was calculated on each section.

Agarose gel electrophoresis for the detection of DNA ladder formation

Dneasy Tissue Kit (QIAGEN GmbH, Germany) was used for DNA extraction from the frozen thymus samples.

Each sample was placed in a 1.5 ml microcentrifuge tube with lysis buffer and proteinase K and incubated at 55°C until being completely dissolved. Then, it was added with AL buffer and EtOH, and the lysate in a spin column was centrifuged at 8000 *rpm* for 1 min. After being washed with AW buffer and centrifuged, spin column was placed in a clean 1.5 ml collection microtube and then DNA was eluted in microtubes with TE buffer preheated to 70°C . The eluate was used as DNA sample for agarose gel electrophoresis.

DNA samples were electrophoretically separated on 2% agarose in Tris boric acid-EDTA solution (TBE), Tris base, boric acid and 0.5 M EDTA (pH 8.0). After electrophoresis, gels were stained with 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide (Sigma Chemical Co.) for 20 min, visualized with a UV light (312 nm) transilluminator, and photographed with VIDEO Graphic Printer UP-880.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was extracted from the thymus homogenate of each mouse using Isogen (Nippon Gene Co. Ltd., Toyama, Japan). Reverse transcriptase (RT) reaction for the first strand cDNA synthesis was carried out using oligo(dT)₁₂₋₁₈ primer 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 mRNA (Table 1). PCR was carried out in a 50 μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U ampliTaQ Gold™, 10 $\times$ PCR buffer, and dNTP mixture (Perkin Elmer, NJ., USA). This was immediately followed by pre-heating at 95°C for 7 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). Annealing temperature and cycle number are shown in Table 1. PCR products were identified by electrophoresis on 2% agarose gel (Nippon Gene Co., Ltd., Toyama, Japan) followed by ethidium bromide (Sigma Chemical Co.) staining. Fluorescence image of gel was visualized under a 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 using a Luminous Imager software (AISIN COSMOS R & D Co., Ltd., Tokyo). The average of 5 mice was calculated, and the results were shown as a relative ratio

Table 1. Primer Sequences, Annealing Temperature (Tm) and Cycle Numbers

	Sense primer	Antisense primer	Tm	Cycle No
Fas	GCTGGCTCACAGTTAAGAGTTC	TGCACTTGGTATTCTGGGTCAG	54	29
<i>c-fos</i>	AATGGTGAAGACCGTGTGTCAGG	GTTGCTGATGCTCTTGACTGG	58	31
<i>c-jun</i>	GATGGAAACGACCTTCTACGAC	ACGTTCTTGGGGCACAAGAAGT	58	31
<i>c-myc</i>	CCTATTTCATCTGCGACGAGGA	TGGTTCACCATGTCTCCTCCAA	52	30
<i>p53</i>	AGCCAGGAGACATTTTCAGGCT	AAGCCATAGTTGCCCTGGTAAG	53	28
<i>bcl-2</i>	AGAACAGGGTATGATAACCGGG	ACATGACCCACCGAACTCAA	54	29
GAPDH	TGAAGGTCGGTGTGAACGGATTGGC	CATGTAGGCCATGAGGTCCACCAC	59	22

to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Statistical analysis

The results of TUNEL index and mRNA expression levels were shown as mean $\pm$ standard deviation (SD) of 5 mice. Statistical analysis was performed using the Student's or Welch's t-test.

Results

Histological and electron microscopical findings

In the thymus of NIV-group, a small number of lymphocytes showing pyknosis or karyorrhexis were found in the cortex from 0.5 to 2 HAI. The number of such lymphocytes clearly increased at 3 HAI and peaked at 9 HAI (Fig. 1). No inflammatory reactions were detected in the thymus until 12 HAI.

In electron microscopical examination, the number of cortical lymphocytes characterized by shrinkage of cell body, condensation of nuclear chromatin and/or margination of condensed chromatin along the nuclear membrane increased at 3 HAI (Fig. 2). Later, many cell nuclei were fragmented into small pieces, some of which were ingested by adjacent epithelial cells and macrophages (Fig. 3).

Fig. 1. Thymic cortex of a control mouse (a) and a NIV-inoculated one at 9 HAI (b). Compared with (a), many pyknotic or karyorrhectic lymphocytes are seen in (b). HE $\times$ 200.

Findings of *in situ* detection of fragmented DNA

In the thymic cortex, almost all of the nuclei of lymphocytes which showed pyknosis or karyorrhexis were stained by the modified TUNEL method (Fig. 4), and the TUNEL index clearly increased at 3 HAI, and peaked at 9 HAI (Fig. 5).

Findings of agarose gel electrophoresis

The DNA ladder formation was not detected at 3 HAI, but it was clearly observed at 6 and 9 HAI (Fig. 6).

Expression of apoptosis-related genes mRNAs

The levels of *c-fos* and *c-jun* mRNAs were significantly

Fig. 2. Thymic cortex of a NIV-inoculated mouse at 3 HAI. Lymphocytes showing nuclear chromatin condensation and/or margination of condensed chromatin along the nuclear membrane are seen. $\times$ 2600.

Fig. 3. Thymic cortex of a NIV-inoculated mouse at 9 HAI. Many apoptotic bodies are ingested by epithelial cells. $\times$ 4000.

Fig. 4. Thymic cortex of control mouse (a) and a NIV-inoculated one at 9 HAI (b). Only a few TUNEL-positive lymphocytes are seen in (a) while many TUNEL-positive ones are seen in (b). TUNEL-staining. $\times 200$.

Fig. 5. Changes in TUNEL-index in the thymus after NIV-inoculation. Each value represents mean $\pm$ SD of 5 mice. (*), $p < 0.05$, (**), $p < 0.01$: Significantly different from controls.

elevated at 0.5 HAI, peaked at 1 HAI, and decreased thereafter (Figs. 7 and 8). On the other hand, there were no significant changes in the expression of *fas*, *p53*, *bcl-2* and *c-myc* mRNAs throughout the observation period (Figs. 7 and 8).

Fig. 6. Agarose gel electrophoresis of DNA extracted from the thymus of NIV-inoculated mice. Ladder formations is clearly observed at 6 HAI (lane 5) and 9 HAI (lane 6).

Discussion

As mentioned earlier, our previous study clarified that NIV is able to induce apoptosis in lymphoid organs in a dose-dependent manner². In the present study, we examined the development of lymphocyte apoptosis and the changes in the levels of apoptosis-related genes mRNAs in the thymus of mice following NIV-inoculation, because we thought that the induction of genes coding transcriptional factors may participate in NIV-induced lymphocyte apoptosis.

As a result, TUNEL-positive thymic cortical lymphocytes clearly increased from 3 HAI and peaked at 9 HAI, and distinct DNA ladder formation was observed at 6 and 9 HAI by agarose gel electrophoresis. In addition, thymic cortical lymphocytes showed ultrastructural pictures characteristic for apoptotic cells. Moreover, there were no inflammatory reactions in the thymus. These findings suggest that at least the majority of TUNEL-positive lymphocytes are apoptotic ones in the present study, although some investigators have recently demonstrated that the TUNEL-technique is not specific for apoptic cell death⁷.

Activator protein-1 (AP-1) was first identified as a transcriptional factor that binds to an essential *cis*-element of human metallothionein IIa (hMTIIa) promotor⁸ which is a protein complex containing the products of *c-jun* and *c-fos* proto-oncogenes^{9,10}. The AP-1 transcription factor complex may play a role in switching between the cell death pathway and the cell cycle¹¹. In addition, these proto-oncogenes are rapidly activated by a large series of signals in proliferation, differentiation and functional activation which are especially regulated by intracellular second messenger systems such as calcium mobilization and protein kinase-C activation¹².

Fig. 7. Sequential changes in the apoptosis-related genes mRNAs expression from controls.

Fig. 8. Sequential changes in the apoptosis-related genes mRNAs expression in the thymus of mice following NIV-inoculation. $\circ$: *c-fos*; $\square$: *c-jun*; $\triangle$: *p53*; $\times$: *Fas*; *: *bcl-2*; $\bullet$: *c-myc*. Each value represents mean $\pm$ SD of 5 mice. (*), $p < 0.05$, (**), $p < 0.01$: Significantly different from controls.

The activation of specific genes including immediate-early response genes such as *c-fos*, *c-jun* and *c-myc* is also one of the early responses to acute cell injury¹³. Some studies reported that the expression of *c-fos* precedes the initiation of apoptosis and it also concomitants with apoptosis in various systems^{14–16}. Moreover, the *c-fos* and *c-jun* could be induced in the very early phase of apoptosis in hematopoietic cells after growth factor deprivation, and blocking of these sequences by antisense oligonucleotides could prevent cell death¹⁷. These findings also suggest that the induction of *c-fos* and *c-jun* may play an important regulatory role in the process of apoptosis¹⁷.

Gene induction during apoptosis has been investigated in the lymphoid tissues, and it has been clarified that some

cell surface antigens and proto-oncogenes regulate the induction of apoptosis. Fas antigen, a cell-surface antigen, is also reported to be related to the negative selection (clonal deletion) and elimination of CD4⁺CD8⁺ T lymphocytes by apoptosis through combining with Fas ligand¹⁸. The *p53* expression is also reported in apoptosis induction in lymphocytes and other cells by radiation and carcinostatics^{5,19}. It was found that expression of *p53* could inhibit the neoplastic transformation of primary culture cells²⁰. Interestingly, it is reported that *bcl-2* is a novel oncogene which promotes cell survival rather than proliferation, and its primary role in human follicular lymphoma appears to enable the cells which have acquired a 14, 18 translocation to resist apoptosis in the lymphoid germinal center²¹. On the other hand, *myc* induces apoptosis of myeloid 22 and fibroblastic cell lines⁴. In addition, it was found that *myc* expression was induced by a heat shock promotor²³.

In the present study, the expression of *c-fos* and *c-jun* mRNAs prominently elevated prior to the clear development of cortical lymphocyte apoptosis following NIV-inoculation to mice. On the other hand, the expression of *fas*, *p53*, *bcl-2* and *c-myc* mRNAs showed no significant changes. Similar findings were also reported by our research group in T-2 toxin-induced lymphocyte apoptosis¹⁶ and epidermal basal cell apoptosis²⁴.

In conclusion, the present results indicate that the expression of *c-fos* and *c-jun* mRNAs may represent an important early event in the activation of genetic program of lymphocyte apoptosis by NIV.

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