Studies on the induction of apoptosis by T-2 toxin and its metabolites in HL-60 human promyelotic leukemia cells

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Summary

The objective of this study is to investigate the structural basis of apoptosis-inducing activity of T-2 toxin in HL-60 human promyelotic leukemia cells. DNA fragmentation pattern in gel electrophoresis and quantitative DNA assay were used to examine the apoptosis-inducing ability of T-2 and its metabolites. Of six kinds of mycotoxins tested, T-2 was found to be the most potent apoptotic agent. The rank order of the potency was T-2 > HT-2 > 3'-OH-T-2 = NEOS = 3'-OH-HT-2 = TOL = vehicle control. Since either hydroxylation of T-2 at carbon 3' (C-3') of isovaleryl group (3'-OH-T-2) or hydrolysis of T-2 at C-8 (NEOS) abrogated the apoptosis-inducing ability, it was suggested that intact isovaleryl group of T-2 molecule played an important role in induction of apoptosis in HL-60 cells in vitro.

Key words : apoptosis, T-2 toxin, T-2 tetraol, HL-60, DNA fragmentation, DNA fragment, trichothecene mycotoxin, Trichothecene mycotoxin

Introduction

T-2 toxin, one of the toxic Fusarium trichothecenes, exhibits profound immunosuppressive effects through the killing of circulating numbers of both B and T lymphocytes and significantly reduces circulating IgG and IgM antibody levels1-3). At present, however, the mechanism of T-2-induced immunosuppression remains poorly understood. Recently, we reported that acute exposure of T-2 caused severe thymic atrophy in vivo in mice through a mechanism of apoptotic cell death4). Additionally, it was shown that neither endogenous glucocorticoid nor tumor necrosis factor-α appeared to be involved in the apoptotic process but it was dependent on the de novo protein synthesis4). Ueno's group also has reported that T-2 is a potent inducer of apoptosis in in vitro and in vivo6-8). Doi's group also reported the in vivo apoptotic phenomena in the
Fig. 1 Metabolic pathways of T-2 in mammals. The pathways shown by the solid-line arrows have been verified experimentally, and the dotted-line arrow indicates a hypothetic pathway.

lymphoid organs of mice induced by T-2. The reported evidence described above has suggested that T-2 might injure a variety of animal cells through apoptotic mechanism.

As for the structure-function relationship of toxin structures, we have found that there is no correlation between the toxicity in terms of LD50 values of trichothecene mycotoxins and the potential to induce thymic apoptosis in vivo. This finding prompted us to investigate the structure-activity relationship of trichothecenes to induce apoptosis in animal cells. Regarding in vivo experiment, we have already reported that 3'-OH-T-2 is able to induce thymic apoptosis in mice as potent as T-2.

In the present study, HL-60 human promyelotic leukemia cells were used as a simple system and we evaluated the apoptosis inducing-ability of T-2 and five of its metabolites (3'-OH-T-2, HT-2, 3'-OH-HT-2, NEOS and TOL; Fig. 1) on this cell line. We show here that T-2 and HT-2 were capable of inducing apoptosis in HL-60 cells significantly.

Materials and Methods

Mycotoxins and reagents Trichothecene mycotoxins including T-2, 3'-OH-T-2, HT-2, 3'-OH-HT-2, NEOS and TOL were prepared and purified in the laboratory of Dr. Yoshizawa, Kagawa
University. The purity of these mycotoxins was over 98% determined by gas-liquid chromatography.

**Cell cultures** HL-60 cells (Riken Cell Bank, Tsukuba) were grown in suspension in a RPMI 1640 cell culture medium (Nissui Pharmaceutical Co., Tokyo) supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol (0.05 mM). The cells were maintained at 37°C under a humidified atmosphere of 5% CO₂ in air.

**DNA fragmentation analysis** Four ml of the cell suspension (7.5 x 10⁶ cells/ml) was inoculated onto 6-well culture plate (Corning Co.) and mycotoxins: T-2, 3'-OH-T-2, HT-2, 3'-OH-HT-2, NEOS and TOL (0.01 μg/ml), diluted in dimethyl sulfoxide (0.1%), was mixed to the cell suspension. After 6 hr of incubation, DNA from HL-60 cells was extracted by a method as described⁴-¹¹. In brief, cells (1.5 x 10⁶) were lysed in 0.1 ml lysing buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). The lysate was treated separately by RNase A and proteinase K (0.4 μg/μl for 1 hr at 37°C), followed by isopropanol precipitation. Pellets were suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and each sample was electrophoresed on 2% agarose gel in 1 x TBE buffer (90 mM Tris-borate, 2 mM EDTA).

**DNA quantitation** DNA fragmentation in HL-60 cells was quantified by colorimetric reaction as described⁴,¹². Briefly, cells (1.5 x 10⁶) were lysed in 0.2 ml of lysing buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0) at 4°C for 15 min and the lysates were centrifuged at 13,000 x g for 30 min to separate intact from fragmented chromatin. Intact and fragmented DNA were treated with 5% perchloric acid, and this was followed by the reaction with diphenylamine reagent to develop a dark blue color. Optical density was measured at 600 nm and percent DNA fragmentation was calculated using the formula:

% Fragmented DNA = OD supernatant/(OD supernatant + OD pellet) x 100.

**Statistical analysis** Results were expressed as means ± SEM. Treatment groups were compared by two-tailed Student’s t-test. Data are representative of two separate experiments.

**Results and Discussion**

Although it has recently been observed that apoptosis can occur even in the absence of internucleosomal fragmentation¹³,¹⁴, oligonucleosomal cleavage accompanies apoptosis in most systems. In this study, agarose gel electrophoresis was used to assess the degradation pattern of nuclear DNA in T-2 metabolites-induced cell death. HL-60 cells (7.5 x 10⁶ cells/ml) were cultured with various concentrations of T-2 (0.001, 0.005, 0.01, or 0.1 μg/ml) for 6 hr and DNA fragmentation pattern was analyzed by agarose gel electrophoresis as shown in Fig. 2. A substantial amount of DNA was cleaved into a ladder of discrete fragments of nucleosomal DNA corresponding to about 200 base pairs. The extent of DNA fragmentation increased with the concentration of T-2 (Fig. 2). DNA fragmentation was detected 3 hr after exposure to 0.01 μg/ml of T-2 and this fragmentation was quite marked after 6 hr (Fig. 3). No DNA fragmentation was detected in the control cells during culture. Based on these results, 0.01 μg/ml of any of the six kinds of mycotoxins (T-2, 3'-OH-T-2, HT-2, 3'-OH-HT-2, NEOS, or TOL) and 6 hr time point have been chosen in order to elucidate their structure-function relationships.

The relative apoptosis-inducing ability as assessed by either agarose gel electrophoresis or...
Fig. 2  DNA fragmentation by the T-2 on HL-60. The cells were exposed to various concentrations of T-2: lane 1, vehicle control; lane 2, 0.001; lane 3, 0.005; lane 4, 0.01; lane 5, 0.10 µg/ml for 6 hr. DNA was electrophoresed (5×10⁵ cells per lane) by the procedure described under Materials and Methods. Molecular size markers are shown on the left (M). Data are representative of two separate experiments.

DNA quantitation is summarized in Fig. 4. The result indicates that among the mycotoxins tested, T-2 was the most potent agent. Hydrolysis of acetyl ester at C-4 position of T-2 to produce HT-2 could induce apoptosis significantly but it was less potent than T-2. Intact
isovaleryl group at position C-8 of T-2 presumed to be important, since the apoptosis-inducing ability of T-2 was lost when the isovaleryl group was hydroxylated at C-3' position (3'-OH-T-2). The activity to induce apoptosis was lost when isovaleryl group at C-8 on T-2 molecule was removed (NEOS) or when isovaleryl group of HT-2 was hydroxylated at C-3' position (3'-OH-HT-2). Complete hydrolysis of the side groups of T-2 (TOL) resulted into no effects like vehicle
control.

It must be noted that the sequence of apoptosis-inducing ability of T-2 and its metabolites presented here did not completely parallel the in vivo sequence of our previous study. Previously we have reported that T-2 and 3'-OH-T-2 have similar effects in inducing thymic apoptosis in mice in vivo. Hydroxylation at C-3' of isovaleryl group of T-2 (3'-OH-T-2) did not reduce the extent of DNA fragmentation at all in the mouse thymus\(^9\). But in the present in vitro HL-60 system, the hydroxylated T-2 derivative, 3'-OH-T-2, did not induce apoptosis significantly (Fig. 4). Our in vitro data are consistent with a growth inhibition study of *Tetrahymena pyriformis* GL, where 3'-OH-T-2 was 76 times less toxic than that of T-2\(^{10}\). It is reported that the toxicity of T-2 and its metabolites varied species to species and route of inoculations of the toxins\(^{15}\). T-2 and HT-2 are metabolized to 3'-OH-T-2 and 3'-OH-HT-2, respectively, in various animal species and the metabolic conversion is cytochrome P-450-dependent\(^{16-20}\). However, we do not know the metabolic system of HL-60 cells but there is a report that benzene metabolites did not induce P-450 (CYP) 1A1 mRNA in HL-60 cells instead it was induced in other two human cells\(^{21}\). In order to elucidate the functional dissimilarity of 3'-OH-T-2 in in vivo mouse model\(^9\) and in vitro HL-60 system presented herein, additional experiments are required to look into the metabolic system and the hypothetical trichothecene mycotoxin-receptors in both systems.

**References**