FINE STRUCTURAL CHANGES AND APOPTOTIC CELL DEATH BY T-2 MYCOTOXIN

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It is now established that the form of cell death is divided into necrosis and apoptosis, and the latter apoptosis is closely related to various biological phenomena such as aging, development, cancer and immuno-modulation. Presently, apoptosis is characterized by as follows; the morphological change - condensation and fragmentation of chromatin, formation of apoptotic bodies etc., and the biochemical change - DNA fragmentation at nucleosomal length. Apoptotic cell death, however, is hard to observe with light microscope, since the cellular damage is completed in a short period and apoptotic bodies are phagocytosed by another cells, i.e. macrophages.

T-2 toxin, a trichothecene mycotoxin, is major toxic metabolite of Fusarium species. It is reported that the high cytotoxicity of trichothecenes would be related to an induction of cancer and other diseases. The trichothecene mycotoxins induce inflammatory reaction and impairment of immunoresponse. Morphologically, cytotoxicity of T-2 toxin had been previously characterized by necrotic lesion and karyorrhexis of actively growing cells in bone marrow, thymus and spleen. (1)

Recently, based on the concept of apoptosis, this lesion has been observed as apoptotic cellular damage, therefore we reexamined cytotoxicity of T-2 toxin in thymus, spleen and liver of mice. In this study, we observed the ultrastructural characteristics and the organ specificity of apoptosis and removal of apoptotic bodies induced by T-2 toxin. In addition, we analyzed DNA fragmentation by TUNEL method and expression of Fas and Fas-ligand by immunostaining.

MATERIALS AND METHODS

Toxin and Animals
T-2 toxin was isolated from the culture filtrate of F. sporotrichioides (2). Female Balb/C mice were purchased from Sankyo Labo Service Ltd. (Shizuoka, Japan), and used at 5-week old. The toxin dissolved in olive oil was given at a dose of 5.0 mg/kg intraperitoneally, and the mice were sequentially sacrificed at intervals of several hours under ether anesthesia. Control mice received olive oil alone at a dose of 0.1 ml/10 g body weight

Histopathology and Electron Microscopic Analysis
Thymus, spleen and liver were fixed in 10% neutral formalin, embedded in paraffin, sliced into 3-5 mm section, and stained with haematoxylin-eosin, for histopathology.

For electron microscopy, the tissues were fixed in 1% glutaraldehyde-4% formalin over 6 hr at 4°C, rinsed in 0.1 M cacodylate buffer overnight, postfixed in 1% osmium tetroxide for 1 hr at 4°C, and then dehydrated in a sequential ethanol series. The fixed tissues were embedded in Epon 812, and sectioned using an Ultratome Nova (LKB, Bromma, Sweden). The sections were double stained with uranyl acetate and lead citrate, and examined under a 1200 EX electron microscope (JEOL, Tokyo).

DNA Fragmentation
DNA fragmentation was analyzed by the TUNEL method using an in situ nick translation kit (Behlinger GmbH, Frankfurt, Germany) (3). This cytochemical technique is based on nick end labeling (TdT-mediated dUTP-biotin nick end labeling), and was carried out according to the manual's instructions.

FAS and FAS-ligand
Fas and Fas-ligand were analyzed by immunostains using rabbit polyclonal IgG for mouse Fas (SANTA CRUZ,USA) and rabbit polyclonal IgG for mouse, rat and human Fas-ligand (SANTA CRUZ,USA) as primary antibodies.
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Fig. 1. Electron micrograph of thymus at 12 hr after 5 mg/kg body weight of T-2 toxin. Mag. × 5,000. Some apoptotic bodies (*) are present.

Fig. 2. Electron micrograph of thymus at 12 hr after 5 mg/kg body weight of T-2 toxin. Mag. × 5,000. A macrophage (M) phagocytes apoptotic bodies (*).
Fig. 3. Electron micrographs of thymocytes in various steps of formation into apoptotic bodies (a-d), and diagram representing the process of formation.
Fig. 4. DNA fragmentation analysis by TUNEL method in liver of mice given 5 mg/kg body weight T-2 toxin.

a. Liver at 1 hr after administration. Mag. ×400. Positive hepatocytes (arrow head) and positive Kupffer cells (arrow) are present.

b. Liver at 2 hr after administration. Mag. ×400. Only Kupffer cells are positive (arrow).
Fig. 5. Electron micrographs of liver 2 hr after 5 mg/kg of T-2 toxin.
a. An apoptotic hepatocyte (*) among normal hepatocytes (H) is present. Mag. ×5,000.
b. A Kupffer cell (K) phagocytes an apoptotic body (*). Mag. × 5,500.
RESULTS AND CONCLUSIONS

Our present findings are summarized as follows:

1. Apoptotic cell death was induced by T-2 toxin in thymus, spleen and liver. Especially in liver, it was induced earlier compared with other tissues we observed.

2. DNA fragmentation analysis by TUNEL method were positive in thymus, spleen and liver. Especially in liver, only hepatocytes showed positive reaction at 15 min. after T-2 toxin. Positive hepatocytes and positive Kupffer cells were observed at 30 min. and 1 hr after the administration. The liver after 2 hr of the administration showed only few positive hepatocytes.

3. Apoptotic bodies were phagocytosed by macrophages in thymus and spleen, and by Kupffer cells in liver.

4. Light and electron microscopic observation showed the activation and mobilization of Kupffer cells.

5. Immunoperoxidase stainings for Fas and Fas-ligand were negative.

6. In the process of formation of apoptotic bodies, the "crescent-shaped spaces" were found around nuclei with irregular condensation of chromatin. These perinuclear spaces were observed in an early stage of the process ahead of nuclear interdigitation.

Our data suggests that karyorrhexis is reported as the characteristic finding of cytotoxicity induced by the trichothecenes (1), that it is based on the concept of Apoptosis. Concerning liver in particular, it is affected by the toxin much earlier than the other organs are, and apoptotic bodies are phagocytosed by Kupffer cells rapidly. These findings suggest that the evaluation for toxicity of chemical compounds and medicines to liver needs to be reconsidered. Also apoptosis is originally the form of cell death defined by morphological observation under electron microscope, and it has been recently reported that the other cells as well as apoptotic cells showed positive reactions in some case. In this analysis by TUNEL method, Kupffer cells showed positive reactions in liver of mice given T-2 toxin, however apoptosis of Kupffer cells were not observed ultrastructurally. There is a possibility that DNA fragmentation of apoptotic hepatocytes phagocytosed by Kupffer cells showed positive reaction, and it is suggested that the induction of apoptosis should be confirmed by TUNEL method together with electron microscopical observation.

As to the mechanism of apoptotic cell death, the expression of apoptosis-related genes and proteins is proposed, i.e. Bcl-2, while Fas and Fas-ligand were negative in this study. However, the detailed mechanism of apoptosis induced by the trichothecene is not defined yet. It is reported that apoptosis induced by T-2 toxin or nivalenol is related with rapid elevation of intracellular calcium ion (4, 5), therefore it is possible the alteration in intracellular ionic balance is the trigger for induction of apoptosis. Besides, the crescent-shaped spaces on the process of formation into apoptotic bodies appear because the construction in tissues limits cytoplasmic alteration. This characteristic is observed frequently in early stage of the apoptotic process, and this finding will be useful for the further morphological observation of apoptosis.

Foods and feed stuff are contaminated worldwide by various mycotoxins, thus we are exposed to a low level for a long term. It has been clarified that trichothecenes in particular, cause the decrease in immunoresponse and apoptosis is concerned as the reason. And this is considered as the base of various diseases and it is very important to observe the cytotoxic mechanism of mycotoxins.

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