Short communication

Effects of T-2 Toxin on the Expression of Connexin 32 in the Mouse Liver

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Abstract: In this study we investigated the effect of T-2 toxin (4 or 8 mg/kg/b.w.), a kind of trichothecene mycotoxins produced by the genus *Fusarium*, on the expression of connexin 32 (Cx32), a major protein of gap-junction, in mice. The area of total Cx32-positive spots on the immunostained section showed a tendency to be reduced from 6 to 36 hours after T-2 toxin inoculation (HAI), and it was significantly reduced at 36 HAI, irrespective of the dose of T-2 toxin. The value of Cx32 protein detected by Western blot analysis showed a tendency to be reduced from 3 to 48 HAI except at 6 HAI, and it was significantly reduced at 12 HAI, irrespective of the dose of T-2 toxin. Such reduction in Cx32 protein expression may be related to the depression of protein synthesis and induction of free radicals by T-2 toxin. (J Toxicol Pathol 2002; 15: 221–224)

Key words: T-2 toxin, connexin 32, mouse, liver

Gap-junctions are intercellular channels between neighboring cells that allow the passage of ions and small molecules such as calcium and cyclic AMP, and are believed to play an important role in the maintenance of tissue homeostasis¹. One of the major components of gap-junctions in the liver is a 26–28kDa protein called connexin32 (Cx32), and the expression of Cx32 has been reported to be down-regulated in response to inflammatory or injurious stimuli^{2–4}.

T-2 toxin is a type of trichothecene mycotoxin produced by various species of the genus *Fusarium*⁵. In addition to the induction of apoptosis in the tissues which contain a large number of actively dividing cells such as hematopoietic and lymphoid tissues⁶, T-2 toxin is also known to induce hepatotoxicity⁷. The present study was carried out to examine the effect of T-2 toxin on the Cx32 expression in the liver of mice.

Five-week-old 90 ICR: CD-1 male mice (Charles River Japan Inc., Kanagawa) were used. They were kept under controlled conditions (temperature, 23 ± 2°C; relative humidity, 55 ± 10%, 14–16 air changes per hour and 12 : 12 hr light: dark cycle) using an isolator caging system (Niki Shoji Co., Tokyo) and fed commercial pelleted chow that

was free from mycotoxin contamination (CE-2, Clea Japan Inc., Tokyo) and water *ad libitum*. Thirty mice were orally inoculated with 4 mg/kg b.w. of T-2 toxin (Sigma Chemical Co., St. Luis, MO) dissolved in 20% ethanol in 0.01 M phosphate-buffered saline (PBS) (T-2 (4) group), and 30 mice were given 8 mg/kg b.w. of T-2 toxin in the same way (T-2 (8) group). In addition, 30 mice were administered with 20% ethanol in 0.01 M PBS and used as controls (control group). Five animals of each group were sacrificed by heart puncture under ether anesthesia at 3, 6, 12, 24, 36 and 48 hours after inoculation (HAI), respectively. Pieces of the left lateral lobe of the liver obtained from each animal were embedded in Tissue-tek OCT compound (Sakura Fine technical Co., Ltd., Tokyo), rapidly frozen in dry-ice acetone, and stored at –80°C until used.

Frozen sections (6 µm) were subjected to immunohistochemical staining for the detection of proliferating cell nuclear antigen (PCNA) by the avidin-biotin-peroxidase complex method using the ABC kit (Vector Labs., Burlingame, CA) and mouse anti-rat PCNA antibody (Novocastra, Newcastle, UK) as the primary antibody.

Frozen sections were also subjected to immunohistochemical staining for Cx32 by fluorescent antibody method using monoclonal mouse anti-Cx32 antibody being cross reactive to mouse antigen (Zymed Laboratories, San Francisco, U.S.A.) as the primary antibody and FITC-conjugated goat anti-mouse IgG antibody (Sigma, Saint Louis, U.S.A.) as the secondary

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antibody. The fluorescence positive cells were observed by using the LSM510 (Carl Zeiss Co., Ltd.) and the percentage of area of the positive spots to the whole area were calculated using a software, NIH image (National Institute of Health, U.S.A).

The remaining liver stored at -80°C was used for Western blot analysis of Cx32 protein according to the method of Neveu *et al.*⁸ using polyclonal rabbit anti-rat Cx32 antibody being cross-reactive to mouse antigens (Zymed Laboratories) as the primary antibody and anti-rabbit IgG peroxidase-linked species specific whole antibody (Amersham Pharmacia Biotech, UK) as the secondary antibody. The obtained bands of Cx32 were quantified with a software, Quantity One (PDI Inc., NY, U.S.A), and the value of Cx32 protein was expressed as the ratio to the control.

Data of PCNA index and Cx32 protein expressions were expressed as mean $\pm$ standard error (SE), and statistical analysis was done using the statistical software package Statcel (Excel 2000 for Windows; Seiunsys, Tokyo).

PCNA index showed a tendency to increase at 12, 36 and 48 HAI, irrespective of the dose of T-2 toxin. Especially, it was significantly elevated in T-2 (4) group at

Fig. 1. Changes in PCNA index. $\circ$: control group, $\blacksquare$: T-2 (4) group, $\blacktriangle$: T-2 (8) group. * $p < 0.05$: Significantly different from control group.

Fig. 2. Distribution of Cx32-positive hepatocytes in control group at 12 HAI (a), T-2 (4) group at 12 HAI (b), control group at 36 HAI (c), and T-2 (8) group at 36 HAI (d). Immunostaining. Bar=50 μm .

36 and 48 HAI (Fig. 1).

The area of total Cx32-positive spots detected by immunohistochemistry showed a tendency to decrease from 6 to 36 HAI, irrespective of the dose of T-2 toxin, and it decreased significantly at 36 HAI (Figs. 2 and 3). In addition, the value of Cx32 protein detected by Western blot analysis showed a tendency to decrease from 3 to 48 except at 6 HAI, and it decreased significantly at 12 HAI, irrespective of the dose of T-2 toxin (Fig. 4). The time showing the minimum level of Cx32 expression was at 12 HAI by Western blot analysis and at 36 HAI by immunohistochemistry, respectively.

Gap-junction intercellular communication (GJIC) is well known to be closed by free radicals, and the closure of GJIC by free radicals may be due to oxidation of the channel-forming protein or of regulatory molecules, and it may be a rapid cellular response to injury¹. Furthermore, GJIC is known to be closed at S phase in the process of regeneration of the liver such as after partial hepatectomy^{1,9}, and carcinogenesis^{1,10,11}. In the case of hepatectomy, the expression of Cx32 significantly decreases at S phase, and a drastic reduction in Cx32 expression was also reported under proliferative conditions in the case of hepatocarcinoma. Furthermore, the expression of Cx32 has been observed to be down-regulated in response to inflammatory states²⁻⁴. On the other hand, T-2 toxin is well known to produce free radicals after metabolized by cytochrome p-450⁵ and to suppress protein synthesis. It is also known that T-2 toxin has a suppressive effect on glutathione synthesis in the liver¹².

The reduction of Cx32 protein detected by immunohistochemistry and Western blot analysis following T-2 toxin treatment in the present study seems to be due to the inhibition of GJIC by free radicals produced by T-2 toxin. However, the relationship between the Cx32 protein expression and the changes in the PCNA index is obscure.

References

1. Spay DC, Saez JC, Hertzberg EL, and Dermietzel R. Gap junction of the Liver. In: *The Liver: Biology and Pathobiology*, 3rd ed., Arias IM, Boyer N, Fausto N, Jakoby WB, Schachter DA, and Shafritz DA (eds), New York: Raven Press Ltd., 951-966, 1944.
2. Gingalewski C, Wang K, Clemens MG, and de Maio A. Posttranscriptional regulation of connexin 32 expression in liver during acute inflammation. *J Cell Physiol* 1996; **166**: 461-467.
3. Temme A, Traub O, and Willecke K. Downregulation of connexin 32 protein and gap-junctional intercellular communication by cytokine-mediated acute-phase response in immortalized mouse hepatocytes. *Cell Tissue Res* 1998; **294**: 345-350.
4. Temme A, Ott T, Haberberger T, Traub O, and Willecke K. Acute-phase response and circadian expression of connexin 26 are not altered in connexin 32-deficient mouse liver. *Cell Tissue Res* 2000; **300**: 111-117.
5. Ueno Y. Mode of action of trichothecenes. *Ann Nutr Alim*

Fig. 3. Changes in percentage of Cx32-positive areas in the liver. See the foot note of Fig. 1.

Fig. 4. Results of Western blot analysis of Cx32 in the liver after T-2 toxin inoculation. Lane 1 and 11: Standard; Lane 2, 5, 8, 12, 15 and 18: control; Lane 3, 6, 9, 13 and 19: T-2 (4) group; Lane 4, 7, 10, 14, 17 and 20: T-2 (8) group. Lane 2-4: at 3 HAI; Lane 5-7: at 6 HAI; Lane 8-10: at 12 HAI; Lane 12-14: at 24 HAI; Lane 15-17: at 36 HAI; Lane 18-20: 48 HAI.

Fig. 5. Changes in Cx32 protein expression in the liver after T-2 toxin inoculation (ratio to standard). See the foot note of Fig. 1.

- 1977; **31**: 885–900.
6. Shinozuka J, Suzuki M, Noguchi N, Sugimoto T, Uetsuka K, Nakayama H, and Doi K. T-2 toxin-induced apoptosis in hematopoietic tissues of mice. *Toxicol Pathol* 1998; **26**: 647–681.
7. Quiroga MA, Doi C, Han JS, Akai H, Tamura K, and Doi K. Acute hepatotoxicity of T-2 toxin in mice. *J Toxicol Pathol* 1991; **4**: 35–44.
8. Neveu MJ, Hully JR, Babcock KL, Herzberg EL, Nicholson BJ, Paul DL, and Pitot HC. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. *J Cell Sci* 1994; **107**: 83–95.
9. Kojima T, Yamamoto M, Mochizuki C, Mitaka T, Sawada N, and Mochizuki Y. Different changes in expression and function of connexin 26 and connexin 32 during DNA synthesis and redifferentiation in primary rat hepatocytes using a DMSO culture system. *Hepatology* 1997; **26**: 585–597.
10. Krutovskikh VA, Mesnil M, Mazzoleni G, and Yamasaki H. Inhibition of rat liver gap junction intercellular communication by tumor-promoting agents *in vivo*. *Lab Invest* 1995; **72**: 571–577.
11. Krutovskikh V and Yamasaki H. The role of gap junctional intercellular communication (GJIC) disorders in experimental and human carcinogenesis. *Histol Histopathol* 1997; **12**: 761–768.
12. Rizzo AF, Atroshi F, Ahotupa M, Sankari S, and Elavaara E. Protective effect of antioxidants against free radical-mediated lipid peroxidation induced by DON or T-2 toxin. *Zentralbl Veterinarmed A* 1994; **41**: 81–90.