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Free Radical Involvement in the DNA Damaging Activity of Fumonisin B1

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ABSTRACT *: Fumonisin B1, a mycotoxin, is thought to induce esophageal cancer in humans and apoptosis in animal cells by inhibiting ceramide synthase. Fumonisin B1 may also generate reactive oxygen species directly or indirectly, leading to DNA damage and lipid peroxidation. In this study, a DNA fragmentation assay, dichlorofluorescein (DCF) analysis, and single cell gel electrophoresis (SCGE) were used to investigate the involvement of cellular free radicals, specifically hydrogen peroxide, in the DNA damaging activity of fumonisin B1. From an in vitro DNA fragmentation assay, E. coli DNA damage by fumonisin B1 was increased by the addition of superoxide dismutase (SOD) and decreased by catalase. SCGE and DCF analysis in vivo showed that the nuclear DNA damage and intracellular free radicals in cultured rat hepatocytes treated with fumonisin B1 were increased with the concentration of fumonisin B1. DNA damage and free radical generation were inhibited by the addition of catalase. Fumonisin B1, in the presence of SOD, produces hydrogen peroxide causing oxidative DNA damage and protein malfunction, leading to genotoxicity and cytotoxicity of the toxin.*

Key Words *: Fumonisin B1, DNA damage, Free radicals, Hydrogen peroxide*

I. INTRODUCTION

Fumonisin B1 is a mycotoxin that is found occasionally in cereals and corns contaminated with the mold, *Fusarium moniliforme* (Gelderblom *et al.*, 1988). It has been reported to induce esophageal cancer in South Africa (Sydenham *et al.*, 1991) and China (Chu and Li, 1994), through the ingestion of Fusarium-contaminated corn in the diets of those regions. It was shown that fumonisin B1 can also induce porcine pulmonary edema (Fazekas *et al.*, 1998) and equine leukoencephalomalacia (Kellerman *et al.*, 1990). In experiments with monkey kidney cells, CDK2 (cyclin-dependent kinase 2) activity was inhibited by fumonisin B1 (Ciacci-Zanella *et al.*, 1998), but the *p21* promoter, a CDK inhibitor, was activated by the mycotoxin (Zhang *et al.*, 1999). TNF (tumor necrosis factor) alpha secretion was also stimulated by fumonisin B1 Dugyala *et al.*, 1998), but myelin formation in an aggregating brain cell culture was inhibited by fumonisin B1 (Monnet-Tschudi *et al.*, 1999). The effect of the mycotoxin on the cellular macromolecule synthesis in the hamster kidney cell was also reported (Abeywiekrama *et al.*, 1998). It has been suggested that fumonisin B1, as a ceramide synthase inhibitor, induces apoptosis by the accumulation of a cellular free spingoid base (Tolleson *et al.*, 1999). Another report on the neurosystem indicated a decrease in the dendrite growth rate of hippocampal neurons (Schwarz and Futerman, 1998). Cancer-promoting activity in rat liver (Gelderblom *et al.*, 1996), lipid peroxidation, DNA damage and protein synthesis inhibition by fumonisin B1 have also been reported (Abado-Becognee *et al.*, 1998; Sahu *et al.*, 1998). Although the cytotoxicity and genotoxicity of fumonisin B1 were suggested, the specific mechanisms involved in fumonisin B1 toxicity remain unclear. Due to the lack of knowledge of the molecular mechanism of the toxin, a molecular biological study on both the DNA damaging activity and the genotoxicity of the toxin was carried out in a microbial system and in animal cells. An *in vitro* DNA fragmentation experiment of *E. coli* was performed to determine if reactive oxygen species are involved in the DNA damaging activity of fumonisin B1. In animal cell experiments with rat hepatocytes, dichlorofluorescein (DCF) analysis and single cell gel

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electrophoresis were used to support the correlation of results obtained from the *in vitro* experiment.

II. MATERIALS AND METHODS

1. Strains, experimental animals and chemicals

E. coli chromosomal DNA was isolated from *E. coli* W3110 cultured in LB medium. For the isolation of rat hepatocytes, 180gr to 200gr male Sprague-Dawley rats were used. All other chemicals such as fumonisin B1, superoxide dismutase (Fe^{2+}-SOD, from *E. coli*), catalase (from *A. niger*), 2',7'-dichlorofluorescein diacetate, collagenase (type II) and Hepes were from Sigma-Aldrich Chemical Co. Williams medium E powder, insulin and fetal bovine serum were the products of Gibco company.

2. DNA fragmentation assay

A 10 μl of reaction mixture for the assay consisted of 0.16 μg *E. coli* chromosomal DNA, 0.5 μg fumonisin B1, 5 units superoxide dismutase and 0.2 unit catalase in TE buffer (10 mM Tris-Cl, pH 7.4, 1mM EDTA, pH 8.0). After 3 hrs of incubation at 37°C, 2 μl of loading buffer was added to the reaction mix. The total reaction mix was introduced to 1.0% agarose gel electrophoresis at 100 V/60 mA for 3 hrs. The gel was stained with ethidium bromide (1.0 μg/ml TBE buffer) for 30 min and destained in water for 1 hr.

3. Primary rat hepatocytes culture

Rat hepatocytes were cultured using slight modifications of published methods (Shen *et al.*, 1996). Isolated hepatocytes from the Sprague-Dawley rat were washed with Hepes buffer, and the hepatocyte (5×10^5 cells/ml) suspension was cultured in complete Williams' medium E (10% fetal bovine serum, 36 mM sodium bicarbonate, 0.02 U/ml of insulin, 100 U/ml of penicillin, 100 μg/ml of streptomycin, pH 7.4) for 2 hrs. Further incubation was carried out for 6 hrs in serum-free Williams medium E.

4. Single cell gel electrophoresis (SCGE)

Single cell gel electrophoresis was modified from the method of Ding *et al.* (1999). Rat hepatocytes (1×10^4 cells/ml) suspended in phosphate buffered saline (PBS, 0.8% NaCl, 0.02% KCl, 0.014% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) with 5 μM fumonisin B1, 500 units/ml of SOD and 500 units/ml of catalase were incubated at 37°C for 2 hrs. The incubated cell suspension was mixed with 0.7% low melting agarose gel and embedded on a glass slide. Cells on the glass slide were lysed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Triton X-100, 1% DMSO, pH 10) at 4°C for 1 hr, followed by storing in electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH 7.5) for 20 min. After electrophoresis of the glass slide at 20 V/300 mA for 20 min, the solution was neutralized with 0.4 M Tris buffer, pH 7.5 for 10 min and stained with propidium iodide (5 μg/ml) for 1 hr. The slides were observed under green light (510 to 560 nm) using a fluorescence microscope (Olympus, Model BX50, Japan).

5. Dichlorofluorescein (DCF) analysis

Rat hepatocytes, cultured in serum-free complete Williams medium E, were washed twice in PBS. 10 mM fumonisin B1 and 500 units/ml of catalase were added to the hepatocytes (3×10^5 cells/ml) suspended in PBS. The reaction mix was incubated for 3 hrs at 37°C, followed by further incubation with 1 μM DCF-DA (2',7'-dichlorofluorescein diacetate) for 4 hrs without light. The fluorescence intensity was measured at wavelength of 485 nm and 530 nm using a fluorometer (Kontron, Model SFM25, Switzerland).

III. RESULTS

Fumonisin B1 (0.5 μg/assay) did not affect *E. coli* chromosomal DNA (0.16 μg) (Fig. 1, lane 2). However, after the addition of superoxide dismutase (SOD), *E. coli* DNA was cleaved in a concentration dependent manner (1.0 unit to 20 units) (Fig. 1, lane 3 to lane 7). At a concentration of 20 units of SOD/assay, *E. coli* DNA was completely fragmented as shown in lane 7 of Fig. 1. To determine whether fumonisin B1 plays a role in the production of hydrogen peroxide from superoxide radicals by superoxide dismutase, the catalytic enzyme of hydrogen peroxide, catalase, was used (Fig. 2). Lane 5 of Fig. 2 shows the inhibition of DNA damage by the addition of 0.2 units of catalase

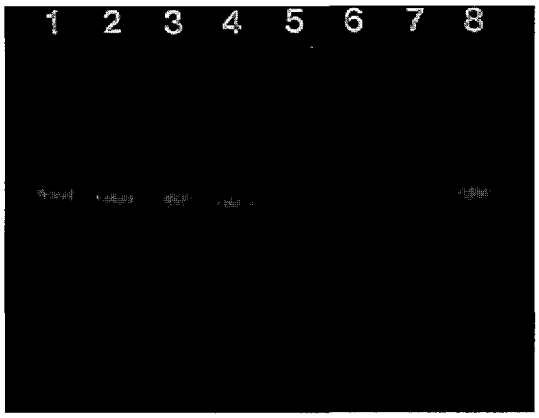


Fig. 1. Effects of superoxide dismutase (SOD) on DNA damaging activity caused by fumonisin B1 (FB1). Lane 1: control, lane 2: FB1 (0.5 μg), lane 3: FB1 (0.5 μg) + SOD (1 unit), lane 4: FB1 (0.5 μg) + SOD (2 units), lane 5: FB1 (0.5 μg) + SOD (5 units), lane 6: FB1 (0.5 μg) + SOD (10 units), lane 7: FB1 (0.5 μg) + SOD 20 (units), and lane 8: SOD (20 units).

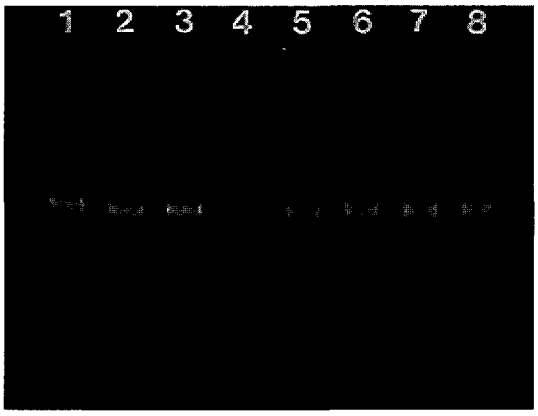


Fig. 2. Inhibitory effects of catalase on DNA damage caused by fumonisin B1 (FB1) and superoxide dismutase (SOD). Lane 1: control, lane 2: FB1 (0.5 μg), lane 3: SOD (5 units), lane 4: FB1 (0.5 μg) + SOD (5 units), lane 5: FB1 (0.5 μg) + SOD (5 units) + catalase (0.2 unit), lane 6: FB1 (0.5 μg) + catalase (0.2 unit), lane 7: SOD (5 units) + catalase (0.2 unit), and lane 8: catalase (0.2 unit).

per assay, compared with lane 4, where *E. coli* DNA was treated with 0.5 μg of fumonisin B1 and 5 units of superoxide dismutase per assay.

To confirm the possible involvement of fumonisin B1 in the generation of reactive oxygen species (ROS) in rat hepatocytes, single cell gel electrophoresis (Fig. 3) and dichlorofluorescein (DCF) analysis (Fig. 4) were performed. From the results of single cell gel electrophoresis with rat hepatocytes in 5 μM and 10 μM fumonisin B1, nuclear DNA damage of rat hepatocytes was dependent on the concentration of fumonisin B1 (B and C of Fig. 3). The DNA damage of rat

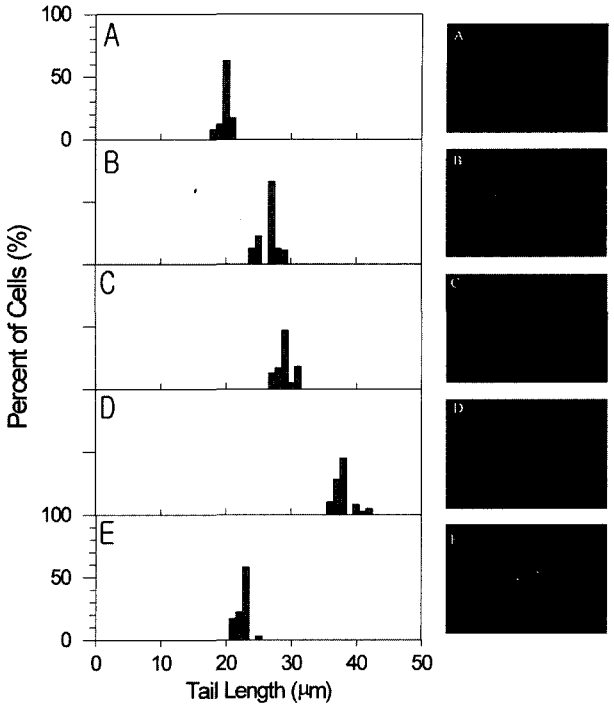


Fig. 3. Effects of superoxide dismutase (SOD) and catalase on rat hepatocyte DNA damage caused by fumonisin B1. Cell number: 50~60 cells/plate, Magnification: ×400. (A) control, (B) fumonisin B1 (5 μM), (C) fumonisin B1 (10 μM), (D) fumonisin B1 (5 μM) + SOD (500 units/ml), (E) fumonisin B1 (5 μM) + SOD (500 units/ml) + catalase (500 units/ml).

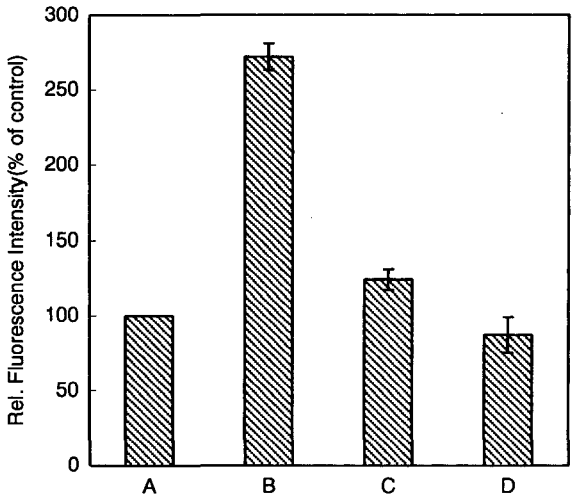


Fig. 4. Effects of catalase on free radical generation by fumonisin B1 in rat hepatocytes. The results are the mean± the SD of triplicate experiments. (A) control, (B) FB1 (10 μM), (C) FB1 (10 μM) + catalase (500 units/ml), (D) catalase (500 units/ml).

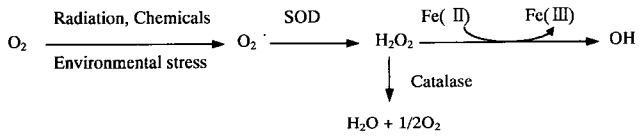
hepatocytes by 5 μM fumonisin B1 and 500 units/ml of superoxide dismutase (SOD) was seen more clearly in D than in B and C of Fig. 3. The increase in DNA

damage by fumonisin B1 in association with SOD was inhibited by 500 units/ml of catalase in E of Fig. 3.

As shown in Fig. 4 from the result of DCF analysis, the cellular relative fluorescence intensity of rat hepatocytes was increased by fumonisin B1, indicating the production of the reactive oxygen species by the toxin. The increased ROS level by 10 μM fumonisin B1 was lowered by 500 units/ml of catalase, a hydrogen peroxide catalytic enzyme.

IV. DISCUSSION

From the *in vitro* reaction of fumonisin B1 and superoxide dismutase, DNA was fragmented by fumonisin B1 and DNA fragmentation was inhibited by catalase, as shown in Fig. 1 and 2. This result indicates that hydrogen peroxide is generated by fumonisin B1 and superoxide dismutase. Furthermore, in Fig. 3, fumonisin B1 induced nuclear DNA damage in rat hepatocytes and DNA damage was increased by SOD. DNA damage by fumonisin B1 and SOD was also inhibited by catalase, suggesting the involvement of hydrogen peroxide in the DNA damaging activity of fumonisin B1. As shown in Fig. 4, fumonisin B1 increased the level of the cellular reactive oxygen species. Fumonisin B1 not only caused DNA fragmentation, but the reactive oxygen species level elevated by fumonisin B1 was decreased by catalase in rat hepatocytes in Fig. 4. This result suggests that the intracellular superoxide dismutase and fumonisin B1 in rat hepatocytes generate hydrogen peroxide, leading to nuclear DNA damage. Hydrogen peroxide is known to form 2'-deoxyadenosine N-1-oxide by the oxidation of 2'-deoxyadenosine and to increase mitochondrial DNA damage in human retinal pigment epithelial cells (Ballinger *et al.*, 1999). DNA damage by the oxygen free radical is known to be one mechanism of carcinogenesis (Sahu, 1990). In the case of the carcinogenicity of aflatoxin B1, the reactive oxygen species appears to be one of the factors causing the carcinogenic effect (Shen *et al.*, 1996). Reactive oxygen species produce hydroxyl radicals by the following pathway (Koppenol, 1994; Pierre 1995).



In the radical chain reaction from superoxide radicals through hydrogen peroxide to hydroxyl radicals, fumonisin B1 and superoxide dismutase may be responsible for the formation of hydrogen peroxide. Consequently, the DNA damaging activity of fumonisin B1 results from the action of hydrogen peroxide which is produced by the enzyme superoxide dismutase and fumonisin B1.

It was also reported that fumonisin B1 caused DNA damage in rat liver nuclei by oxidative stress (Sahu *et al.*, 1998) and induced oxidative damage in primary rat hepatocytes and in the rat liver (Abel and Gelderblom, 1998). In addition, fumonisin B1 acts as a ceramide synthase inhibitor, by which apoptosis (Tolleson *et al.*, 1999) as well as a cancer promoting activity (Gelderblom *et al.*, 1996) can be induced. DNA damage by fumonisin B1 could be induced mainly by hydrogen peroxide, although it was known that hydroxyl radical is also a direct DNA damaging agent. It is likely that fumonisin B1 could induce DNA damage by reactive oxygen species, hydrogen peroxide, leading to genotoxicity and cytotoxicity of the toxin.

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