Fumonisins and *Alternaria alternata* lycopersici toxins: Sphinganine analog mycotoxins induce apoptosis in monkey kidney cells

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Fumonisins and *Alternaria alternata lycopersici* toxins: Sphinganine analog mycotoxins induce apoptosis in monkey kidney cells

(CV-1 cells/programmed cell death/cancer/cell cycle/food safety)

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ABSTRACT  *Fusarium moniliforme* toxins (fumonisins) and *Alternaria alternata lycopersici* (AAL) toxins are members of a new class of sphinganine analog mycotoxins that occur widely in the food chain. These mycotoxins represent a serious threat to human and animal health, inducing both cell death and neoplastic events in mammals. The mechanisms by which this family of chemical congeners induce changes in cell homeostasis were investigated in African green monkey kidney cells (CV-1) by assessing the appearance of apoptosis, cell cycle regulation, and putative components of signal transduction pathways involved in apoptosis. Structurally, these mycotoxins resemble the sphingoid bases, sphingosine and sphinganine, that are reported to play critical roles in cell communication and signal transduction. The addition of fumonisin B₁ or AAL toxin, TA, to CV-1 cells induced the stereotypical hallmarks of apoptosis, including the formation of DNA ladders, compaction of nuclear DNA, and the subsequent appearance of apoptotic bodies. Neither mycotoxin induced cell death, DNA ladders, or apoptotic bodies in CV-1 cells expressing simian virus 40 large T antigen (COS-7) at toxin concentrations that readily killed CV-1 cells. Fumonisin B₁ induced cell cycle arrest in the G₂ phase in CV-1 cells but not in COS-7 cells. AAL toxin TA did not arrest cell cycle progression in either cell line. The induction of apoptosis combined with the widespread presence of these compounds in food crops and animal feed identifies a previously unrecognized health risk to humans and livestock. These molecules also represent a new class of natural toxicants that can be used as model compounds to further characterize the molecular and biochemical pathways leading to apoptosis.

Fumonisins and *Alternaria alternata lycopersici* (AAL) toxins, a recently discovered group of mycotoxins structurally related to sphinganine, are produced by *Fusarium moniliforme* (1–5) and strains of *Alternaria alternata* (6, 7), respectively (Fig. 1). These fungi are ubiquitous saprophytes and economically important pathogens of many food crops. *F. moniliforme* causes both stalk and ear rot of maize worldwide. Fumonisin contamination has been detected in substantial amounts (≥4 μM) in 74% of corn in the United States and in all areas of the world where corn is produced, with levels ranging as high as 2 mM or greater (3, 4, 8–10). The incidence of contamination of maize by the fumonisins is exacerbated by the endophytic life style of the fungus, which enables the fungus and its toxin to be present in maize plants and stored grain in the absence of visible disease symptoms (9).

Ingestion of fumonisins through contaminated feed, or as pure fumonisin B₁ (FB₁), induces a variety of responses in the challenged animals including neuro-, renal, and hepatotoxicosis and neoplasms as well as cell death (4, 11–13). Consumption of fumonisin-contaminated corn also has been linked to esophageal cancer in humans in the Transkei region of South Africa, China, and other countries (14). Because of the frequency and level of contamination in maize and the toxicity of fumonisin to animals, serious concern has developed over the role of this mycotoxin in animal and human disease. AAL toxins play an important role in the stem-canker disease of tomatoes but a role in animal disease is, at present, unresolved (15).

At present little is known concerning the mechanism by which these mycotoxins induce toxicogen or carcinogenic effects. The structural relationship to sphinganine, an intermediate in the biosynthesis of the sphingosine backbone of more complex sphingolipids such as ceramides, sphingomyelin cerbrosides, gangliosides, and sulfatides, is the basis for current models linking mode of action to alterations in the synthesis of sphingoid bases (16, 17). There is considerable evidence that ingestion of these mycotoxins leads to disruption of sphingolipid metabolism (18–20). An important site of inhibition is the reaction catalyzed by sphingosine N-acetyltransferase (ceramide synthase). Both FB₁ and AAL toxin TA (TA) inhibited ceramide synthase in rat hepatocytes (16) and in microsomal preparations from green tomato fruit (21).

Abbreviations: AAL, *Alternaria alternata lycopersici*; FB₁, fumonisin B₁; TA, AAL toxin TA; PKC, protein kinase C; SV40, simian virus 40; TUNEL, terminal deoxynucleotidyltransferase end labeling.

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Sphingolipids have been implicated as playing a role in cell contact, growth, and differentiation (22). Sphingolipids also can influence the proliferative potential of mammalian cells by induction or suppression of apoptosis, a specific form of programmed cell death (23). Recent findings suggest the regulatory coupling of proliferation and mitosis to apoptosis (24–26). Based on the fact that fumonisins and AAL toxins resemble sphingoid bases, such compounds could elicit cell death or carcinogenesis by inducing apoptosis and/or by altering the cell cycle.

We reported previously that FB1 repressed expression of two isoforms of protein kinase C (PKC) (α and δ) in cultured African green monkey kidney cells (CV-1) and inhibited transcription by activator protein 1 (AP-1) 10-fold within 3 hr after toxin treatment (27). These results identified possible molecular targets of this toxin and suggested that inappropriate regulation of signal transduction pathways associated with apoptosis may be responsible for the deleterious effects of FB1 in CV-1 cells.

In this report we provide evidence that FB1 and TA induce apoptosis in CV-1 cells and that FB1 but not TA arrests the treated cells in the G1 phase of the cell cycle. Moreover, CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (COS-7) are unaffected by the same levels of FB1 or TA. Since large T antigen has pleiotropic effects on cell cycle regulation and induction of apoptosis (reviewed in ref. 28), large T antigen may overcome the antiproliferative and apoptotic properties of FB1.

**MATERIALS AND METHODS**

Fumonisin and TA. FB1 was purchased from Sigma (>98% pure) or was a generous gift from R. D. Plattner (U.S. Department of Agriculture, Peoria, IL) (>90% pure). Both preparations gave identical results. AAL toxin TA was produced and purified as described (7).

Cells and Media. CV-1 cells and African green monkey kidney cells, were obtained from Robert Su (National Institutes of Health). COS-7 cells were obtained from American Type Culture Collection. Cells were grown in Earl’s modified Eagle’s medium supplemented with 5% fetal bovine serum. All media contained penicillin (10 units/ml) and streptomycin (100 µg/ml).

Histochemical Detection of Nuclear DNA Fragmentation and Apoptotic Bodies. The terminal deoxynucleotidyltransferase end-labeling (TUNEL) technique was used for *in situ* evaluation of CV-1 and COS-7 cells for DNA fragmentation and the appearance of apoptotic bodies (29). Slides were stained with DNA counterparts, Hoechst 33342 (Sigma), and propidium iodide. The slides were viewed under a Nikon SA fluorescence microscope and view-fields were captured by C-Imaging System (Compix, Cranberry Twp., PA).

Cell Cycle Analysis. Cells were plated at a density of 5 × 10⁵ cells per 100-mm dish and allowed to attach to the dish for 3 hr prior to treatment with FB1 or TA. Cells were prepared for flow cytometry by scraping monolayers from tissue culture dishes, washing the cells in isotonic buffer, and resuspending in Vindelov’s reagent (40 mM Tris, pH 7.6/100 mM NaCl/10 µg of RNase A per ml/7.5% propidium iodide/0.1% Nonidet P-40). Flow cytometry was conducted at the Cell Analysis Core Facility at the University of Nebraska Medical Center using a Becton Dickinson Cell Sorter.

**RESULTS**

The pivotal roles assigned currently to apoptosis for maintenance of cell stability (23) and of lipid-based signal molecules in regulation of apoptosis (30) suggested the action of FB1 and TA involve apoptosis as the functional paradigm of cell death. CV-1 and COS-7 cells were chosen as models to study the effects of FB1 and TA on apoptosis because they are well characterized cell lines. CV-1 cells have many features of normal cells because they are not tumorigenic, do not grow in soft agar, and are not contact inhibited.

**Appearance of DNA Ladders and Apoptotic Bodies.** CV-1 and COS-7 cells were treated with either FB1 or TA at various concentrations and analyzed over time for the presence of ordered DNA fragmentation and apoptotic bodies. To determine whether FB1 or TA induced cell death with features consistent with apoptosis, toxin treated CV-1 cells were labeled with digoxigenin-DUTP by terminal deoxynucleotidyltransferase using the TUNEL procedure and stained for total nuclear DNA. Free 3’-OH groups, generated during the endonuclease-catalyzed DNA fragmentation, were detected by fluorescein-conjugated anti-digoxigenin antibodies and visualized by fluorescence microscopy in cells treated with either FB1 or TA. Propidium iodide, a vital dye (31), was added to the cell cultures to permit visualization of DNA in dying cells, which could then be outlined against the fragmented DNA detected by the TUNEL reaction. Apoptotic bodies arising from apoptotic nuclei appear subsequent to the detection of DNA fragmentation. The combined use of propidium iodide and Hoechst enabled confirmation of the presence of TUNEL-positive signal based on fluorescein isothiocyanate (FITC) fluorescence. Propidium iodide suppressed the natural autofluorescence of the cell which was excited at the same wavelength as FITC and interfered with FITC unless suppressed by propidium iodide. Within 3 hr after treatment of CV-1 cells with either FB1 or TA at 1 µM, both DNA ladders (Fig. 2) and apoptotic bodies were detected (Fig. 3). Temporal analysis of DNA fragmentation by agarose gel electrophoresis indicated that smaller sized fragments (180 bp) increased in abundance up to 24 hr after toxin treatment. The lowest concentration of FB1 or TA tested that induced these effects was 10 nM where the observed TUNEL-positive cells were 10% (3 hr), 15% (6 hr), and 30% (24 hr) at the indicated time points. In separate experiments, the counting of >5000 individual CV-1 cells treated with FB1 or TA revealed 45% and 74% TUNEL-positive cells, respectively, at 2 and 5 µM, compared to 4% in the untreated control (least significant difference = 3%). There were no apparent differences in the kill rate or number of apoptotic cells between the two mycotoxins at any concentration tested.

![Fig. 2](image-url) Southern blot analysis of DNA ladders induced by FB1 and TA. CV-1 cells were treated with 5 µM FB1 or TA for various time periods. DNA was extracted and subjected to agarose gel electrophoresis and analyzed by Southern blot hybridization with total monkey DNA. Control (C) and cells treated with FB1 (lanes 1–3 were sampled at 3, 6, and 24 hr, respectively) and TA (lanes 4–6 were sampled at 3, 6, and 24 hr, respectively). Arrowheads mark 500- and 1000-bp locations based on ethidium bromide staining of DNA size markers run on the original gel.
**Fig. 3.** *In situ* detection of fragmented nuclear DNA in CV-1 cells treated with FB₁ and AAL toxin TA. (Upper) Toxin-treated CV-1 DNA that has been end labeled with digoxigenin/fluorescein by the TUNEL technique. (Lower) Same view-fields of CV-1 cells but counterstained with Hoechst 33342. Fragmented DNA and apoptotic bodies (viewed at 510–560 nm) appear as yellow to greenish membrane-bound vesicle-like elements. Red fluorescence represents intact DNA stained by propidium iodide. Total DNA, anti-digoxigenin/fluorescein-labeled and unlabeled DNA was revealed as bright white fluorescence of Hoechst at 346–460 nm. (A) CV-1 cells cultured for 24 hr in the absence of toxin. (B) Cells cultured for 3 hr in the presence of 1 μM TA. (C) Cells cultured for 3 hr in the presence of 1 μM FB₁. (D) Cells cultured for 24 hr in the presence of 5 μM FB₁.

**Temporal Progression of DNA Damage and Formation of Apoptotic Bodies.** In additional experiments, we observed that cell death induced by either toxin occurred in stages with chromatin condensation and cell shrinkage (Fig. 4A and B). These events were followed by the appearance of large bodies which reacted positively to the TUNEL procedure (Fig. 4C), indicating that they contained fragmented DNA. The initial compaction of DNA and positive end labeling were observed generally within the first 3–6 hr after toxin treatment. Fluorescence intensity varies with the extent of fragmentation and increasing fragmentation is reflected by changes in the color of the fluorescence from yellow to green. The presence of distinct, well separated apoptotic bodies (23) indicative of later stages of programmed cell death, were observed (Fig. 4 D and E) within 18 hr in each case. CV-1 cells treated with equivalent concentrations of TA displayed morphological characteristics of apoptosis identical to cells treated with FB₁.

**Effect of FB₁ and TA on Cell Cycle Progression.** Two recent studies using mouse embryo cells (Swiss 3T3), which examined the mitogenic potential of fumonisin, concluded in one case

**Fig. 4.** Progression of nuclear DNA fragmentation in CV-1 cells treated with FB₁. CV-1 cells were treated with 5 μM FB₁ for 24 hr and viewed over this time period. (Upper) Toxin-treated CV-1 DNA that has been end labeled by the digoxigenin/fluorescein TUNEL technique. Fragmented DNA and membrane-bound vesicle-like elements were viewed at 510–560 nm to reveal yellow to greenish fluorescence of anti-digoxigenin/fluorescein. Unlabeled DNA stained by propidium iodide appears as red fluorescence material within the cells. (Lower) Same view fields using Hoechst 33342 DNA counterstain. Total DNA, anti-digoxigenin/fluorescein-labeled, and unlabeled DNA were viewed at 346–460 nm as bright white fluorescence of Hoechst. (A) Control cells. (B and C) Early stages of apoptosis where nuclear DNA fragments and nucleus budding are revealed as yellow fluorescence. (D and E) Late stages of apoptosis ultimately revealing DNA fragments contained within membrane-bounded apoptotic bodies with bright green fluorescence.
Table 1. Cell cycle analysis in CV-1 or COS-7 cells after fumonisin treatment

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<td>56 25 19</td>
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CV-1 or COS-7 cells were plated at a density of $5 \times 10^5$ cells per 100-mm dish and allowed to attach to the dish for 3 hr. Control, cells treated with phosphate-buffered saline; 12 hr, cells treated for 12 hr with 5 $\mu$M FB$_1$; 24 hr, cells treated for 24 hr with 5 $\mu$M FB$_1$; 48 hr, cells treated for 48 hr with 5 $\mu$M FB$_1$. Nuclei were prepared from the respective cultures and flow cytometry was performed after nuclei were incubated with a propidium iodide solution. Values are expressed as % total cells and are from a representative experiment. At least three independent determinations were performed.

that FB$_1$ was mitogenic (17) while the other concluded that FB$_1$ was not mitogenic (32). To investigate this interaction, we examined the effect of FB$_1$ and TA treatment on cell cycle progression. Flow cytometry was performed after CV-1 cells were treated with 5 $\mu$M FB$_1$ or TA for 12, 24, or 48 hr. Our results indicated that only FB$_1$ treatment induced arrest of cell cycle in G$_1$ (Table 1). By 48 hr after treatment with FB$_1$, only 1% of CV-1 cells were in S phase of the cell cycle. In contrast, control cultures of CV-1 cells contained ~15% of the cells in S phase at the same time point. Similar concentrations of FB$_1$ (or TA) did not increase the proportion of COS-7 cells in the G$_1$ phase of the cell cycle following toxin treatment (Table 1).

As described above, COS-7 cells are monkey kidney cells transformed by SV40 (33). When COS-7 cells were treated with very high concentrations of FB$_1$ (25 $\mu$M), 16% of the cells were in S phase compared to 34% in control cultures, suggesting higher levels of FB$_1$ will also arrest cell cycle in COS-7 cells. In contrast to FB$_1$, similar concentrations of TA did not dramatically alter the ratio of CV-1 or COS-7 cells in S phase 48 hr after treatment (Table 2). Even when treated with up to 25 $\mu$M TA, they were not arrested in G$_1$ (data not shown). In summary, FB$_1$, but not TA, inhibited cell cycle progression of CV-1 cells while COS-7 cells were more resistant to the cell cycle inhibitory effects of FB$_1$.

**DISCUSSION**

These studies demonstrate that fumonisin B$_1$ and TA induced the stereotypical hallmarks of apoptosis in CV-1 cells, while FB$_1$, but not TA, induced cell cycle arrest at G$_1$ in CV-1 cells.

The observation that FB$_1$ can induce apoptosis raises the question of how proliferating cells manage to avoid triggering the apoptotic pathway that fumonisin can initiate in normal cells. This paradox may be reconciled by the fact that certain genes involved in the induction of cell death also are responsible for neoplastic transformation (34). For example, c-Myc, a ubiquitous and essential mediator of cell proliferation also induces programmed cell death (34). Tumor necrosis factor α and mitosis-inducing agents such as caffeine and okadaic acid, long recognized as tumor suppressors, can promote apoptosis in HeLa cells (35, 36). The commitment of a cell to undergo apoptosis is, in part, related to availability of growth factors, age of the cell, cell type, stage of the cell cycle, and state of differentiation (25, 34). It is unclear what specific genetic or environmental factors determine whether FB$_1$, or TA-treated cells undergo proliferation or death.

Based on our previous results which indicated that FB$_1$ inhibits AP-1-dependent transcription (27) and that the onset of this inhibition temporarily precedes the onset of cell death, it is possible that the transcription factor AP-1 plays an important role in determining whether CV-1 cells undergo apoptosis or progress through the cell cycle. AP-1 binding factors can promote G$_1$ progression in some cell types but induce differentiation in others (36). In addition, two isoforms of PKC, α and δ, are repressed by fumonisin (27). Since PKC activation results in rapid dephosphorylation of c-jun, a trans-activator of AP-1 sites, it is possible that repression of AP-1-dependent transcription by FB$_1$ is important for apoptosis. Consistent with this possibility is the fact that the same concentration of toxin (5 $\mu$M) that inhibited PKC activity and AP-1 transcription (27) also induced apoptosis. In addition, a study by Walker et al. (37) demonstrated a correlation between the presence of AP-1 DNA binding activity and repression of apoptosis. Moreover, the ability of glucocorticoids to induce apoptosis requires a glucocorticoid receptor that can repress AP-1-dependent transcription (38). Also, the activation or inhibition of PKC has been associated with the induction of apoptosis (e.g., see ref. 39). Thus, it is possible that FB$_1$ induces growth arrest and cell death, at least in part, by repressing AP-1 activity via inhibition of PKC.

An intriguing aspect of these data concerns the differing results between CV-1 and COS-7 cells following FB$_1$ treatment. The SV40 large T antigen, which is expressed in COS-7 cells, binds to two tumor suppressor proteins known to regulate cell cycle progression and apoptosis (p53 and Rb, the retinoblastoma susceptibility gene product). It is possible that the association of large T antigen with one or more of these proteins is responsible for the relative insensitivity of COS-7 cells to FB$_1$, although we cannot rule out differential uptake of the toxin.

TA is a structural analog of fumonisin B$_1$ (Fig. 1), and traditionally these toxins have been considered functionally interchangeable with respect to specificity and biological activity in both plant and animal cells. For example, both toxins inhibit ceramide synthase and both toxins produce similar symptoms on leaves and fruit of isogenic lines of tomato (21). In this report, we have shown that both toxins cause apoptosis in animal cells. Previously, we have reported that both toxins also can initiate a cell death program in plants (7, 21). Although both toxins induce apoptosis, only FB$_1$ affects the cell cycle.

Fumonisins are a serious health threat since corn and corn-based food products are a common staple in the diet of livestock animals and humans. Fumonisins are found frequently in healthy as well as diseased corn plants. Preliminary data indicate that both fumonisins and AAL toxins can be found in concentrations sufficient to induce cell death in CV-1 cells and field samples of processing tomatoes and corn (refs. 4 and 40; B. Ward and D.G.G., unpublished data). The effect of chronic exposure to these molecules on the health of animals or humans is unresolved.

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