CYTOTOXICITY AND IMMUNOLocalIZATION OF FUMONISIN B₁ IN DEC99 AND BALB/C 3T3 CELL LINES

K. Todorova*, A. Georgieva, P. Dimitrov, Iv. Ivanov, R. Russev

Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT

Fumonisin is a widespread mycotoxin, produced by several species of *Fusarium* moulds that contaminate mainly crops, and food and feed based on cereals. These metabolites represent a significant health threat for humans and animals because of their toxic and carcinogenic effects. The present study aims to ascertain the cytotoxic effects of FB₁ on permanent lines and its subcellular localization. In the present study, the cytotoxic effect of fumonisin B₁ (FB₁) on DEC99 (duck embryo cells) and BALB/c 3T3 (mouse embryo fibroblasts) cell lines was assessed by Neutral Red Uptake (NRU) test and standard Pappenheim and fluorescent AO/PI double staining. Intracellular distribution of FB₁ in DEC99 cells was examined by immunofluorescence and immunoelectron microscopy methods. The results from the NRU test indicated that DEC99 cells are more sensitive to the toxic effect of FB₁ than BALB/c 3T3 cells. AO/PI double staining revealed morphological features of apoptosis of cells treated with 300 µg FB₁/ml. The immunofluorescent and immunogold labeling of FB₁ revealed the localization of the micotoxin in the cytoplasm and the nucleus of the cells. The presented results clearly indicate that the cell line DEC99 is a useful model system for studies on the cytotoxic effects of fumonisins.

Key words: fumonisins, avian cell line, toxic effect, subcellular localization

INTRODUCTION

Some of the common fungal infections of crops are caused by filamentous fungi of the genus *Fusarium*: *F. graminearum*, *F. verticillioides* (*moniliforme*), *F. poae*, *F. equiseti*, *F. culmorum*, *F. sporotrichoides*, *F. proliferatum*, *F. oxysporum*, *F. solani* and others. These moulds synthesize three main groups of mycotoxins - fumonisins, zearalenone and trichothecenes. A number of plants as wheat, maize, rye, sorghum, millet, oats, rice, potato, melon, banana, cucumber, tobacco plants etc. are subject to an infection with these molds and produced toxins fall into final products made from them, including vegetable oils, sauces, beer, cigarettes, etc.

Fumonisin has been recently isolated mycotoxin from mouldy maize in South Africa (1). Fumonisin is a low-molecular-weight chemical compound produced as secondary metabolites causing leukoencephalomalacia, pulmonary oedema, cardiovascular toxicity, hepatotoxicity,

nephrotoxicity and immunosuppression. The vital interest to these mycotoxins is also due to the fact that their distribution and high concentrations correlate with incidents of cancer of the liver and the esophagus in humans and they are considered food-borne carcinogens (2, 3). Another important point is participation in the development of infertility and embryotoxicity as a result of defects of neural tube formation (4). It has been also found that fumonisins participate in the etiology of atherosclerosis by impairing the exchange of fatty acids and phospholipids in the liver and increasing cholesterol levels in blood (5).

Fumonisin inhibits the action of the enzyme ceramide synthase (sphinganine (sphingosine) N-acyltransferase) which is involved in the synthesis of complex sphingolipids (structural components of cell membranes) from sphinganine (Sa) and sphingosine (So) (6-8). Full inhibition of ceramide synthase by fumonisins leads to a rapid increase in intracellular concentrations of sphingoid bases - sphinganine and sphingosine (6, 8, 9). While sphingosine readily crosses the cell membrane (10), sphinganine degrades more slowly and

*Correspondence to: Katerina Todorova,
katerinagencheva@yahoo.com, tel 0887986565,
02/9792375, fax+359 02 871 01 07, fax+359 02
871 90 07

accumulates in cells. This leads to alterations in cellular regulation, caused by infraction of lipid metabolic pathways that are essential for cell growth, differentiation and death (5). Several studies in vitro gave evidence of fumonisin-induced changes in key enzymes involved in cell cycle regulation, proliferation and apoptosis leading to an imbalance of these processes and hence to the development of cancer (11-15).

Fumonisin B₁ is the most prevalent member of this family of toxins, that occur mainly in maize, wheat and other cereals (16, 17). Cytotoxicity of fumonisin B₁ is established in rat hepatocytes, hepatoma cells, canine and porcine kidney cells, Rk13 cell, K562 erythroleukemia cell line, IPEC-J2 and few others (18-23). The elucidation of the mechanisms of the toxic and carcinogenic action of mycotoxins has been defined as a major priority of the mycotoxicological research. The interaction of the toxin to cell organelles and mechanisms of penetration through the membranes are not enough investigated problems. Another priority is to further exploit the species-related differences in target organs and sensitivity to fumonisins in comparative, mechanistic and genomic/epigenomic-based experiments to determine which species and toxicological outcomes provide the most relevant models for risk assessment.

The present study aims to ascertain the cytotoxic effects of FB₁ on permanent lines of avian and mammalian embryonal cells and to issue the penetration of the toxin and its subcellular localization as subsequent conditions for a breach of cellular regulation.

MATERIALS AND METHODS

Mycotoxin.

Fumonisin B₁ (Genaxxon bioscience GmbH, Germany) was dissolved in sterile double distilled water. Solution of FB₁ with concentration 1mg/ml water was further diluted with complete cell culture medium and applied at concentrations ranging from 40 to 400 µg/ml.

Cell lines and cultivation conditions.

BALB/c 3T3, a cell line of mouse embryo fibroblasts (Todaró and Green, 1963). The cells were grown in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, Germany).

DEC99, a permanent line of duck embryo cells (Patent № 63125/15.05.2001; Ivanov and Kril, 2001), was cultivated in a combination of Medium 199 and Iscove's Modified

Dulbecco's Medium (Sigma Aldrich, Germany).

The culture media were supplemented with 10% Fetal Bovine Serum (Sigma Aldrich, Germany), 100UI/ml penicillin and 100µg/ml streptomycin (AppliChem). Cells cultures were grown as monolayers in 25cm² tissue culture flasks at 37°C in a humidified atmosphere and 5% CO₂.

Light microscopy.

The morphological alterations induced by FB₁ in the cell cultures were examined by an inverted light microscope (Leica DM 5000B, Wetzlar, Germany) on native and after standard Pappenheim staining preparations.

Fluorescence microscopy.

Apoptogenic effects of FB₁ on DEC99 cells were studied after double staining with acridine orange propidium iodide (AO/PI) (Sigma Aldrich, Germany) and fluorescence microscope (Leica DM 5000 B, Wetzlar, Germany at I3 Filtercube). Cells were grown on cover slips in 30 mm Petri dishes (Corning Incorporated) and treated with 300 µg FB₁/ml for 24 hours. Untreated cells were used as controls.

Neutral Red Uptake (NRU) test.

Cells were trypsinized and counted using cell counter (Invitrogen, Thermo Fisher Scientific, USA) and plated in a 96 wells microtiter plate. The cells were treated with eight different concentrations of FB₁ ranging from 400 to 40 µg FB₁/ml (dilution factor $\sqrt[7]{10}$, 100 µl per well, six wells per concentration) for 48 h.

Cytotoxic potential of FB₁ was assessed by the Neutral Red Uptake test. Optical density was measured after 48h exposure of cells to the mycotoxin by microplate reader (TECAN, SunriseTM, Grödig/Sazburg, Austria), at wave length 540 nm. The relative cell viability, expressed as a percentage of the untreated controls, was calculated for each concentration.

Immunofluorescence.

Subcellular localization of FB₁ in the DEC99 cells, treated with 300 µg FB₁/ml for 48 hours, was examined by fluorescence microscopy (Leica DM 5000 B, Wetzlar, Germany, MicroFilter Cube I3, BP 450-490, dichromatic mirror 510, suppression filter LP515, LED wave – 470) and laser scanning confocal microscopy (Leica TCS SPE, Wetzlar, Germany, 488 nm laser scanning, immerse X 40 (1.15 aperture), x 63 (1.30 aperture)) after incubation of cells with primary anti-fumonisin

B₁ monoclonal antibodies (Ridascreen, R-Biopharm AG, Darmstadt, Germany) and secondary goat anti-mouse FITC-conjugated antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA).

Immunoelectron microscopy.

In treated with 300 µg FB₁/ml DEC99 cells for 48 hours, immune complexes of FB₁ and the anti-fumonisin B₁ monoclonal antibodies were marked using anti-mouse immunoglobulin conjugated with colloidal gold particles (10 nm in diameter) and visualized by electron microscopy (JEOL 1200 EX, Japan).

Statistical analysis.

The statistical significance of the differences in the cell viability of the control and FB₁-treated cells was assessed by One-way ANOVA, followed by Bonferroni's post hoc test (GraphPad Prism software package). Results are expressed as mean ± SD; $p < 0.05$ was accepted as the lowest level of statistical significance.

RESULTS

Light microscopic investigations on native preparations of FB₁-exposed DEC99 cells after treatment with concentrations over 150 µg/ml showed cytopathic alterations such as shrinkage, rounding up and detachment of the cells and loss of monolayer integrity. The cytopathic alterations were evident after 24 hours of treatment and progressed in time- and dose-dependent manner and were sharply expressed over concentrations of 207 µg FB₁/ml. The observed control duck cells were confluent and formed tight monolayer. In contrast, morphological changes were not observed in BALB/c 3T3 cells treated with the same concentrations of FB₁.

Pappenheim (May – Grünwald – Giemsa) staining of cells treated with 300 µg FB₁/ml revealed distinctive morphological features of cell death which correspond to apoptosis-shrunk, lysed or rounded in shape cells with pycnotic nuclei. Cells lost contact and formed altered point lace-like monolayer (**Figure 1A**).

Figure 1. Morphological alterations induced by fumonisin B₁ in duck embryo cell line DEC99 - point lace-like monolayer (A), control cells (B), May-Grünwald-Giemsa, X20.

Apoptotic, necrotic and only few viable DEC99 cells were observed under fluorescence microscope after treatment with 300 µg FB₁/ml for 24 hours and AO/PI double staining. Early apoptosis of cells was evidenced by intercalated AO within the fragmented DNA as bright-green fluorescence. Blebbing and nuclear margination and apoptotic bodies formation characteristic of late stages of cell death, and presence of red-orange color due to the binding of PI were noticed in the majority of cells after 24 hour treatment with FB₁ (**Figure 2**). In contrast, untreated cells showed intact nuclear structure.

The results from NRU assay revealed concentration-dependent cytotoxic effect of FB₁ on the cell line DEC99. The cell viability was significantly decreased at concentrations 207 µg/ml ($p < 0.05$), 288 µg/ml ($p < 0.01$) and 400 µg/ml ($p < 0.001$), as evidenced by the reduction of the dye uptake (**Figure 3A**). The experiments with BALB/c 3T3 cells showed a moderate decrease of the cell viability at the concentrations of FB₁ higher than 100 µg/ml, however statistical significance was not established (**Figure 3B**).

Figure 2. Blebbing and nuclear margination in cells after 24 hour treatment with 300µg FB₁/ml, AO/PI, X20.

Figure 3. Cytotoxic effect of fumonisin B₁ on cell lines DEC99 (A) and BALB/C 3T3 (B). Neutral red uptake test; *p<0.05, **p<0.01, ***p<0.001, compared to negative control.

The immunofluorescence microscopy studies showed that FB₁ is distributed predominantly in the cytoplasm and perinuclear region of DEC99 cells, forming clusters or confined pearl-like structures (**Figure 4B**). Fluorescent signals with lower intensity were also observed

by confocal microscopy in the nucleus of FB₁-treated cell (**Figure 4A**). Immunogold electron microscopy study indicated that FB₁ is located as in the cytoplasm as well as in the nucleus but mainly in the euchromatin (**Figure 5**).

Figure 4. Subcellular localization of fumonisin B₁ in duck embryo cell line DEC99. A and B - cell cultures exposed to 300µg FB₁/ml: A-laser scanning confocal microscopy, B-fluorescent light microscopy, C-control cells; Black bar 30µm.

Figure 5. Immunogold labeling of fumonisin B₁ in the euchromatin of cells treated with 300 µg FB₁/ml; Black bar 300nm.

DISCUSSION

Strong cytopathological effect on DEC99 cells was manifested 24 h after the application of FB₁ in concentrations higher than 150 µg /ml medium and progressed, depending on the time of exposure, leading to abundant cell death. Areas free of cells appeared in the monolayer as a consequence. The observed cytotoxicity could be also due to the accumulation of sphinganine in cells observed after fumonisin B₁ intoxication (7).

The comparative study on the toxicity of FB₁ in different cell lines showed that the duck cell line DEC99 appears to be more sensitive than the mouse fibroblast cell line BALB/c 3T3 as shown by the Neutral red uptake assay. The presented results clearly indicate that DEC99 cells are a useful model system for studies on the cytotoxic effects of fumonisins. According to data reported by Sheu et al., 1996 (24), similar to our results, relative morphological and growth stability of BALB/c 3T3 were observed even at concentrations of 500 µg FB₁/ml. These investigations support the statement that FB₁ renders a species specific action (5, 25) and its toxicity varies according to the sensitivity of the experimental object.

Fumonisin penetrates cell membranes due to their low molecular weight. Few suggestions exist how cells intake fumonisins - through active or passive ways. It is accepted that the toxin is metabolically modified by transmembrane transport system or endocytosis is also possible mimicking sphingolipid-type binding agents (25). The visualization of the toxin in different parts of the cells shows that the passage continued by some mechanism

mainly via membrane structures or some kind of intracellular transport. It is accepted that the continuity of the outer membrane of the nuclear envelope and the sacs of endoplasmic reticulum could also provide access of FB₁ to penetrate in nucleus and nucleolus (25). According to Cawood et al. (26) the toxin has the ability to bind tightly to membrane structures and remains there. In our *in vitro* experiment we demonstrated by means of immunocytochemistry that the toxin passed through the cell membrane and after 48 h was noticed in the cytoplasm, perinuclear spaces and even in the euchromatin of the nucleus of DEC 99 cells. That led to rapid cell death in DEC 99 cell line. Our results as well as the previously published data (25) confirm that FB₁ is able to penetrate in different cell types and could provoke the reported by Merrill et al., (7) initiation of competitive inhibition of ceramide synthase, thus leading to pathological effects observed after consumption of fumonisin-contaminated corn products.

CONCLUSIONS

Fumonisin B₁ induced significant dose-dependent reduction of the cell viability of DEC99 duck embryo cells.

The avian embryonal cells appeared to be more sensitive to the cytotoxic effect of FB₁ than BALB/c 3T3 mouse embryo fibroblasts.

Fumonisin B₁ is able to pass through cytoplasmic and nuclear membranes of DEC99 cells and it is localized mainly in perinuclear region of the cytoplasm and in the nucleus.

The permanent cell line DEC99 is a valuable novel model, alternative to experimental

animals, for studies on the cytotoxicity of mycotoxins.

ACKNOWLEDGMENT

This work was supported by European Social Fund and Republic of Bulgaria, Operational Programme "Human Resources Development" 2007-2013 Framework, Grant № BG051PO001-3.3.06-0048 from 04.10.2012.

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