The ultrastructural effects and immunolocalisation of fumonisin B₁ on cultured oesophageal cancer cells (SNO)

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Introduction

Cancer of the oesophagus (OC) follows the increasing incidence of cancer worldwide. There is a high incidence in the black population in certain parts of Transkei, South Africa and in parts of China; both have increased in recent times.¹ ² Maize is the staple food of the population of Transkei.³ Fumonisins are mycotoxins produced by Fusarium verticillioides and other Fusarium fungi, found worldwide on maize and maize-based foods.⁴ ⁵ Maize from an area of high OC incidence in the Transkei contained higher levels of fumonisin B₁ (FB₁) (44 ppm) than did commercial maize meal (<10 ppm).⁶ Fumonisin B₁, a strongly polar compound,⁷ is the most prevalent of the fumonisin mycotoxins.⁸ ¹⁰ The polarity of the toxin determines its level of carcinogenicity¹¹ i.e. the more polar the molecule, the greater the cytotoxic response. In addition to polarity, other determinants, such as the presence of a free amino group, carboxyl groups and the location of the hydroxyl group, could also affect the biological activity of these compounds. Thus, both the amino group and the intact molecule play an important role in the toxic and cancer-promoting activity of fumonisins.¹² ¹³ This would be compatible with the association of FB₁ with both soluble and insoluble portions of the cell.¹⁴

Numerous investigations have shown that fumonisin B₁ (FB₁) is the causal agent in a range of animal toxicities, including leucoencephalomalacia, pulmonary oedema and renal and hepatic cancer in rats and mice. Fumonisin B₁ has also been implicated in the aetiology of oesophageal cancer in South Africa. Human data are lacking, however, and the International Agency for Research on Cancer has accordingly classified this mycotoxin as a Type 2B carcinogen. This study investigated the ultrastructural effects of FB₁ on cultured oesophageal carcinoma cell line (SNO). The pathological changes induced by FB₁ were determined using transmission and scanning electron microscopy. Immunocytochemistry was used to immunolocalise FB₁ (monoclonal anti-FB₁) within the cells. The results showed marked pathological changes that included enlargement or microsegregation of the nucleus, microsegregation of the nucleolus, and swelling and elongation of mitochondria, as well as signs of membrane damage. These cytotoxic effects were associated with the action of FB₁, since the toxin was internalised in nuclei, mitochondria and the cytoplasm of affected cells. This study shows that FB₁ may exert its biological effects in SNO cells through binding to cellular macromolecules or membrane components within the affected organelles.

Key words: oesophageal cancer, fumonisin B₁, cell culture, immunocytochemistry

Materials and methods

Reagents

Fumonisin B₁ (98%) was purchased from Sigma (Johannesburg, South Africa). A monoclonal FB₁ antibody was purchased from Neogen (Michigan, U.S.A.). All other immunochemicals were purchased from Adcock Scientific (Durban, South Africa). All HPLC and other chemicals were purchased from Merck (Johannesburg, South Africa).

Maintenance of the SNO cell line

The SNO epithelial cells²⁶ (a well-differentiated squamous carcinoma line) were grown and maintained in Eagle’s minimum essential medium (EMEM) containing 0.25 mM Hapes
buffer supplemented with 5–10% foetal calf serum (FCS), 1% L-glutamine and 1% penstrep fungizone (complete culture media). The cells were maintained in a 37°C incubator and grown to confluency (25 cm² flasks) before use.

Transmission electron microscopy

Confluent 25 cm² flasks of SNO cells were treated with FB₁ at concentrations of 1 µM, 2 µM, 4 µM, 8 µM, 16 µM or 32 µM for 24 h; these concentrations were in keeping with a previous in vitro study.²⁵ Flasks containing untreated cells served as controls. After a 24-h incubation period, the treatment medium was removed and stored for subsequent HPLC analysis. The cells were washed twice with Hanks’ balanced salt solution (HBSS) (3 ml), and the cells were then fixed with 1% glutaraldehyde in HBSS for transmission electron microscopy (TEM) and immunocytochemistry (ICC). After a 30-min fixation period, the epithelial cells in flasks were washed twice with HBSS (5 ml), and then processed for microscopy. Briefly, cells were post-fixed in 1% osmium tetroxide in distilled water (4°C) (omitted for TEM), counterstained with Reynold’s lead citrate, and viewed and photographed using a JEOL-JEM 100S transmission electron microscope. Sections (0.5 µm) were also picked up on 200 mesh nickel grids and probed immunochemically for FB₁.

Immunocytochemistry

Non-osmicated sections on 200 mesh nickel grids were etched with 50 mM Tris containing 1% BSA (pH 7.2). The secondary antibody used was goat anti-mouse IgG conjugated to a 10 nm gold probe (at the same concentrations as those used for TEM), fixed and dehydrated as described for TEM. Coverslips were then critical point-dried, coated to 10 nm thickness with gold particles, and viewed using a Hitachi S520 scanning electron microscope.

Results and discussion

SNO epithelial cells in culture have to be firmly attached to the substrate on which they are grown. Such anchorage-dependence was evident in untreated control cells where the cellular processes were well defined (Fig. 1a). In FB₁-treated cells (Figs 1b–d), there was a retraction of cellular processes that was more pronounced with increasing concentrations of the toxin; this may have been associated with the progression towards cell death. Treatment of SNO cells with 4 µM and 8 µM FB₁ resulted in blebbing or vesication of the plasma membrane (Figs 1b and 1c). The treated cells broke up into smaller bodies with no swelling (Figs 1b and 1c). Blebbing of the plasma membrane into smaller membrane-bound like apoptotic bodies is a common structural feature of apoptotic cells.²⁶,²⁷ Thus, at the lower concentrations of FB₁, apoptosis was the likely mechanism of cell death, since the integrity of the plasma membrane was maintained. At higher concentrations of FB₁ (16 µM), the SNO cells exhibited gross pathology such as swelling (Fig. 1d); the plasma membrane is a target of FB₁, and may therefore have been the major site of damage.²⁸ Loss of membrane integrity prevents cells from regulating osmotic pressure, causing them to swell and rupture (Fig. 1d). Since necrosis is the death of cells through external damage, usually mediated via the destruction of the plasma membrane or the biochemical supports of its integrity,²⁹ it is likely that necrosis was the mechanism of death at higher concentrations of FB₁. Loss of cells due to cell death mechanisms (apoptosis or necrosis) may initiate a compensatory mitosis that could contribute to the onset of cancer.²⁵,³⁰

Transmission electron microscopy revealed that untreated SNO cells contained several Golgi bodies and mitochondria (Fig. 2a); a single large prominent nucleolus was present in the nucleus (Fig. 2b). Nuclear and nucleolar alterations were some of the toxic effects in FB₁-treated SNO cells. Nuclear alterations of FB₁-treated cells included enlargement, irregular profiles, and invagination of the nuclear membrane that often led to microsegregated nuclei (Fig. 2c). Nuclear effects included an increase in the size and number of nucleoli (Fig. 2c). Mitochondria were elongated (Fig. 2d) and/or had swollen cristae (Fig. 2e). Endoplasmic reticuli (ER) were more abundant and some cisternae of ER were vesiculated (Fig. 2e) or swollen (Fig. 2f). Other cellular alterations of FB₁-treated cells included the presence of numerous and occasionally swollen cellular processes (Fig. 3d).

Using ICC, FB₁ was identified by the presence of 10 nm gold probes. Both the untreated SNO cells and method controls showed no positivity for FB₁, whilst gold-labelled FB₁ was immunolocalised in the treated cells (Figs 3a–d). Fumonisins B₁ were present in the cytoplasm (Fig. 3a). Within nuclei of FB₁-treated cells (Fig. 3b), label was associated with the nuclear membrane, nucleoplasm and nucleolus. There was an abundance of FB₁ in mitochondria that showed pathological changes (Fig. 3c), but FB₁ label was also localised to a limited extent in mitochondria that appeared normal. Label also was present within swollen processes or membrane blebs of FB₁-treated cells (Fig. 3d).

The presence of the gold probes within the cytoplasm, mitochondria and nucleus of treated cells (Figs 3a–c) shows that FB₁ gained entrance into the cell. Studies with ¹⁴C-labelled FB₁,
conducted by Cawood et al.\textsuperscript{13} showed that FB\textsubscript{1} bound tightly to microsomes and plasma membranes of rat liver after 1 h incubation, and FB\textsubscript{1} remained in the membranes even after extensive washing of these fractions. Fumonisin B\textsubscript{1} is a highly polar molecule consisting of four carboxyl groups, one amine group and several hydroxyl groups. Even allowing for complete chelation of the carboxyls with elements such as calcium, it does not seem possible that FB\textsubscript{1} could freely permeate membranes. Two possibilities exist for the passage of FB\textsubscript{1} through the cell membrane, namely, that a trans-membrane transport system existed or that it was metabolically modified to allow permeation. Because of the unusual structure of FB\textsubscript{1}, it is tempting to suggest that it entered the cell via some sort of endocytic process, possibly mimicking sphingolipid-type membrane-binding agents. Continuity between the outer membrane of the nuclear envelope and the sacs of the ER were frequent, presumably providing FB\textsubscript{1} access to the nucleus and nucleolus. Alternatively, FB\textsubscript{1} in the soluble portion of the cell may have diffused into the nucleus via the nucleopore, bearing in mind the strong resemblance of the toxin to membrane lipids.

The higher levels of FB\textsubscript{1} found in nuclei and nucleoli in this study correlated well with the ultrastructural observation that FB\textsubscript{1} targeted the nucleolus, resulting in enlargement and microsegregation of the nucleolus. In addition, some of the ultrastructural alterations observed, particularly swelling of organelles, were manifestations of membrane damage, which may have been caused by the action of FB\textsubscript{1} on these cells. Cellular membranes are postulated to be one of the principal targets for the fumonisins \textit{in vivo}.\textsuperscript{30} Fumonisin B\textsubscript{1} has been shown to exert its effects through a disruption in sphingolipid metabolism.\textsuperscript{34,35} Fumonisin B\textsubscript{1} is a competitive inhibitor of the enzyme, ceramide synthase, which catalyses the acylation of sphinganine (Sa) in the \textit{de novo} biosynthesis of sphingolipids and the

\textbf{Fig. 1.} The effects of FB\textsubscript{1} on SNO cells as shown by SEM. \textbf{a}, Control cells were anchored to the coverslip by well-defined cellular processes (C). \textbf{b, c}, Treatment of cells with increasing concentrations of FB\textsubscript{1} (\textit{B} = 8 \mu M, \textit{C} = 16 \mu M) resulted in retraction of cellular processes and blebbing of the plasma membrane (B). \textbf{d}, Cells treated with 32 \mu M FB\textsubscript{1}, were rounded and the cellular processes were almost totally retracted.
re-utilisation of sphingosine (So) derived from sphingolipid metabolism. As a result, FB1 causes an increase in the amount of free Sa and a decrease in the formation of complex sphingolipids such as So and ceramide. Complex sphingolipids have been implicated in cell–cell interactions. Therefore, a blockage of de novo sphingolipid synthesis might weaken intercellular interactions and make membranes leaky. This would allow increased penetration of plasma components into underlying tissues.

Fumonisin B1 has also been implicated in the disruption of a variety of cellular responses including mitogenesis and cytotoxicity. Other in vitro investigations have demonstrated that FB1 inhibited cell proliferation and induced either cell necrosis or apoptosis in SNO cells, LLC-PK1 cells, cultured turkey lymphocytes, chicken macrophages and CV-1 African green monkey kidney cells. A 4-h exposure of FB1 caused significant cytoplasmic blebbing and varying degrees of nuclear disintegration in vitro.

Long-term studies on FB1 exposure in rodents and nonhuman primates showed no oesophageal lesions in either animal model. In our indigenous population, however, maize is the staple diet and often heavily contaminated maize (maize not suitable to be milled into flour) is brewed into a traditional beer. This coupled to chronic FB1 exposure, alcohol consumption, smoking, nitrosamines and scalding hot food or drinks may all be aetiological agents in OC in South Africa.

**Conclusion**

The cellular pathology observed suggests that FB1 specifically targeted mitochondria, the nucleus and nucleolus in SNO cells. The high levels of label (gold probes) found in these organelles further suggests that ultrastructural alterations occurred as a result of FB1 toxicity. The known mechanism of FB1-induced toxicity is through disruption of sphingolipid metabolism. It may, however, also be possible that FB1 exerts its biological effects through binding to macromolecules in these organelles.
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Fig. 3. a, Gold-labelled FB₁ was present in the cytoplasm of 16 µM-treated cells. b, Large quantities of gold label (circled) were present in the nucleus and nucleoplasm of FB₁-treated cells. c, Positively-stained cells containing FB₁, in an elongated mitochondrion. d, Membrane blebs that detached from the cell contained labelled FB₁.


