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Sterigmatocystin induced apoptosis in human pulmonary cells *in vitro*

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ABSTRACT

Sterigmatocystin (ST) is generally recognized as a potential carcinogen, mutagen and teratogen. Studies showed that ST could induce adenocarcinoma of lung in mice *in vivo* and DNA damage, cell cycle arrest in a human immortalized bronchial epithelial cell line (BEAS–2 B cells) and a human lung cancer cell line (A549 cells) *in vitro*. Besides, ST could induce G_2 arrest (cell cycle arrest in G_2 phase) in several other cells. Cell cycle arrest may be one of the common toxic effects of ST. As cells may undergo apoptosis or death due to cell cycle arrest, we wondered whether apoptosis is another common effect of ST in different cells *in vitro*. In the present study, we studied the effects of ST on proliferation and apoptosis in A549 cells and BEAS–2 B cells with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and flow cytometric analysis (FCM). The MTT results showed that proliferation inhibition following ST treatment for 24 h was observed in both A549 and BEAS–2 B cells *in vitro*. And increased apoptosis by FCM was also found after ST treatment. Down-regulation of Bcl-2, up-regulation of Bax and the activation of caspase-3 after ST treatment were detected by western blotting analyses. The results in the present study are consistent with our previous results, which indicated that inducing apoptosis may be a common effect of ST in different cells *in vitro*.

1. Introduction

Sterigmatocystin (ST) is a biochemical precursor of aflatoxins, produced by fungi of many different *Aspergillus* species (Versilovskis and De Saeger, 2010). ST producing fungi were frequently isolated from different foodstuffs, while ST was regularly detected in human food, animal feed and even in indoor environment, such as carpet and building materials (Engelhart et al., 2002; Nielsen et al., 1999). Many studies showed that ST is carcinogenic in the animal model, and possibly carcinogenic to humans, which has been recognized as a 2 B carcinogen (possible human carcinogen) by International Agency for Research on Cancer (IARC, 1976; Versilovskis and De Saeger, 2010). Several *in vivo* and *in vitro* studies have shown that ST may induce lung adenocarcinoma in mice and induce malignant transformations in human fetal lung tissue, which suggested that ST is a potent lung carcinogen (Cao et al., 2000; Fujii et al., 1976; Huang et al., 2004; Xing et al., 2007). Recently, our study showed that ST could induce DNA damage and affected key proteins involved in cell cycle regulation to trigger cell cycle arrest in BEAS–2 B and A549 cells (Huang et al., 2014). In addition, we found that ST could also induce G_2 arrest in human gastric epithelium immortalized cells (GES-1) and human esophageal epithelium immortalized cells (Het-1A) *in vitro* (Wang et al., 2013; Zhang et al., 2013). It indicated that G_2 arrest may be the common effect of ST in different cells *in vitro*. Considering that the cells may undergo apoptosis or death due to cell cycle arrest (Cui et al., 2010), we wondered whether apoptosis is another common effect of ST in different cells *in vitro*.

The imbalance between cell proliferation and death is considered as an early and important event in carcinogenic process (Dong et al., 2009). Cell may undergo apoptosis or other death process if DNA repair systems are overburdened due to severe damages. Apoptosis is a conserved and regulated cell suicide process, the malfunction of which is closely linked with carcinogenesis. It has been generally accepted that induction of apoptosis is the most important bio-effect of many carcinogenic mycotoxins (Ayed-Boussema et al., 2008; Cui et al., 2010). In previous studies, we observed that Ochratoxin A (OTA) induced apoptosis in GES-1 and Het-1A cells *in vitro* (Liu et al., 2015). In addition, ST could induce apoptosis in GES-1 cells (Zhang et al., 2013). However, the appearance of apoptosis depends on cell type and damaging agent. So we wondered whether ST induces apoptosis in human

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pulmonary cells.

Apoptosis is controlled by a large number of genes acting as death switches. The bcl-2 and bax are two discrete members of a gene family involved in the regulation of apoptosis. The bcl-2 blocks cell death following various stimuli, demonstrating a death-sparing effect; however, overexpression of bax has a pro-apoptotic effect and bax also counters the anti-apoptotic activity of bcl-2 (Ahamed et al., 2011; Ahamed et al., 2011; Goldar et al., 2015). It has also been well documented that signaling pathway leading to apoptosis involved the sequential activation of cysteine proteases known as caspases (Ahamed et al., 2011). During apoptotic signaling pathway, activation of caspase-3 is generally considered to be an important factor, which is a key executioner of apoptotic cell death (Fu et al., 2016). However, until recently, the effect of ST on the regulation of apoptosis related factors in human pulmonary cells was not sufficiently clear.

In this study, BEAS–2 B cells (representative of human bronchial epithelial cells) and A549 cells (representative of human alveolar type II pneumocytes) were chosen because ST could induce cell cycle arrest in BEAS–2 B and A549 cells. Furthermore, using these two types of human lung cells, we evaluated the effect of ST on cell proliferation, apoptosis and apoptosis related factors using MTT assay, flow cytometric analysis and western blotting analyses.

2. Materials and methods

2.1. Chemicals and reagents

Highly purified sterigmatocystin (ST) (> 99% purity, benzene free) was purchased from ENZO Life Sciences, Inc. (Farmingdale, NY). ST was diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). The primary antibodies used for Western blot analysis were rabbit anti-human Bax, Bcl-2 and caspase-3 monoclonal antibodies (Epitomics, CA, USA).

2.2. Cell culture and treatment

BEAS–2 B cells and A549 cells were purchased from the American Type Culture Collection (ATCC, Manassas VA). BEAS–2 B and A549 cells were cultured in DMEM/F-12 and RPMI, respectively, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal bovine serum (FBS) in 5% CO_2/95% air. The cells were then treated with solvent (DMSO, final solvent concentration 0.1%) alone or with different concentrations of ST (6, 12 and 24 μM) for 24 h.

2.3. MTT assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan, which has a purple color. The reduction of MTT depends on the cellular metabolic activity due to NAD(P)H flux. Cells with a low metabolism reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction. The mechanism of reduction of MTT will also determine the amount of product. In the present study, the method of MTT was employed to evaluate the level of proliferation. Cells were seeded on 96-well culture plates at 4×10^3 cells/well and treated with ST ranging from 0.06 to 240 μM for 24 h at 37 °C. At the end of treatment (24 h), 20 μl of MTT stock solution was added to each well (final concentration: 0.5 mg/ml) for another 4 h incubation. After 4 h, the medium was replaced with 150 μl of DMSO to dissolve the converted purple dye in culture plates. The absorbance was measured on a spectrophotometer microplate reader at a wavelength of 560 nm. Cell viability was assayed as the relative formazan formation in treated wells compared to control wells [(A560 treated wells/A560 control wells) × 100%] after correction for background absorbance.

2.4. Flow cytometric (FCM) analysis

Cells from different treatment groups were collected and washed twice with cold PBS. For cell cycle analysis, the cells were fixed in 70% ethanol at 4 °C overnight. After centrifugation for 5 min at 1000 rpm at 4 °C, the pellet was treated with 2 mg/ml RNase A at 37 °C for 20 min and stained with 50 μg/ml propidium iodide (PI) containing 0.1% Triton X-100 and EDTA 0.02 mg/ml. Cells (2×10^6) were quantified to determine the distribution of different cell cycle phases using FCM analysis Multicycle AV software (FACSAria, BD Biosciences, CA, USA).

2.5. Western blotting analyses

The cells were harvested and washed with ice-cold PBS. Total protein was extracted in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA (pH = 8.0), 50 mM Tris-HCl (pH = 7.5) and protease inhibitor cocktail (Sigma-Aldrich, USA). The lysate was centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was collected. The protein concentration was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). 40–80 micrograms of protein was used for SDS-PAGE and transferred onto a PVDF membrane after electroblotting at 4 °C. Subsequently, the membrane was incubated in Tris-buffered saline containing 0.05% Tween 20 and 5% (w/v) nonfat dry milk and then incubated with the desired primary antibody at 4 °C overnight. After treatment with the appropriate secondary antibody, the immunoreactive bands were visualized using chemiluminescence and scanned using the Odyssey Fc Imaging System (Licor, USA).

2.6. Statistical analysis

All experiments were performed at least three times. All data were expressed as the mean ± standard deviation (SD). Significant differences were analyzed using one-way analysis of variance (ANOVA) and SPSS software. The dose–effect relationship was analyzed with correlation analysis. The *p*-values less than 0.05 were considered to be significant.

3. Results

3.1. ST inhibited growth of A549 and BEAS–2B cells

Cytotoxic effect of ST on A549 and BEAS–2B cells was measured by MTT assay. We found that low concentrations of ST (from 0.06 to 6 μM) had no effect on cell viability after 24 h treatment (Fig. 1A and 1B). However, higher than 6 μM ST led to a significant decrease in the viability of A549 and BEAS–2 B cells. As shown in Fig. 1, the cell viability in ST treated groups displayed a significant decrease in a concentration-dependent manner ranging from 12 to 240 μM in both A549 ($r = -0.962$ $P < 0.05$) and BEAS–2 B cells ($r = -0.91$, $P < 0.05$).

3.2. ST induces apoptosis in A549 and BEAS–2B cells in vitro

Based on the MTT results, we therefore selected 6, 12 and 24 μM of ST to explore its cytotoxic effects on BEAS–2B and A549 cells, which is consistent with the concentrations of the study about cell cycle arrest induced by ST(Huang et al., 2014). So, we next detected the apoptosis in A549 and BEAS–2 B cells after ST treatment (6, 12, 24 μM) by FCM analysis. As shown in Fig. 2A and 2B, after treatment with different concentrations of ST (6, 12, 24 μM), apoptosis was increased in a concentration-dependent manner in both A549 ($r = 0.831$ $P < 0.05$) and BEAS–2B cells ($r = 0.974$ $P < 0.05$).