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Sterigmatocystin-induced oxidative DNA damage in human liver-derived cell line through lysosomal damage

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ABSTRACT

Sterigmatocystin (STC) is a carcinogenic and mutagenic mycotoxin produced by fungi of many Aspergillus species. As a precursor of the aflatoxins, STC is a risk factor of liver cancer. The objective of this study is to investigate STC-induced DNA damage and underlying mechanisms. The single cell gel electrophoresis (SCGE) assay was applied to assess DNA damage induced by STC. To clarify the underlying mechanisms, we measured the intracellular generation of reactive oxygen species (ROS) using dichlorofluorescein diacetate as a fluorochrome. The level of oxidative DNA damage was evaluated by immunocytochemical analysis of 8-hydroxydeoxyguanosine (8-OHdG) and the acridine orange (AO) was used to measure the changes of lysosomal membrane stability. A significant dose-dependent increase of DNA strand breaks was found after treatment with STC (3 and 6 μM) respectively for 1 h. Increases in ROS level and the expression of 8-OHdG were also observed. A statistically significant increase in AO fluorescence intensity was observed in cells treated with STC (1.5, 3 and 6 μM) for 1 h. The DNA strand breaks induced by STC were almost prevented in cells pretreated with NH_4Cl (10 mM) and NAC (10 mM) for 1 h. Our results thus indicated that STC exerts genotoxic effects on HepG2 cells, most likely through oxidative stress and lysosomal leakage.

1. Introduction

Mycotoxins are secondary metabolites of filamentous fungi which cause a wide range of acute and chronic systemic consequences. Sterigmatocystin (STC) is one of the most prevalent toxins in the environment which has been detected in human food, animal feed and even in interior environment, such as carpet and building materials (Engelhart et al., 2002; Tuomi et al., 2000; Nielsen et al., 1999). STC producing fungi were frequently isolated from different foodstuffs, and STC can be detected in foodstuffs like corn, grains, soybeans, bread, cheese, spices, coffee beans, pistachio nuts, animal feed and silage (Versilovskis and De Saeger, 2010). Aspergillus *versicolor* is the most efficient producer of STC, which acts as a precursor in the biosynthesis of aflatoxin (Yabe and Nakajima, 2005; Keller et al., 1997, 2000; Ehrlich et al., 2005; Wilkinson et al., 2004). STC has been recognized as a 2B carcinogen (possible human carcinogen) by the International Agency for Research on Cancer (Kocic-Tanackov et al., 2012). It is less toxic compared to AFB1 for rodents and monkeys, but appears to be slightly toxic in zebra fish. It has been reported that after feeding a diet containing STC for 30 days, tissue examination in mice showed liver cell damage and necrosis phenomenon (Sivakumar et al., 2001). Chronic symptoms include induction of hepatomas in rats, pulmonary tumors in mice, renal lesions and alterations of liver and kidneys of African green monkeys. STC is considered to be involved in the etiology of chronic liver disease of people living in Africa (Kusunoki et al., 2011), and induces tumors in the lung and liver by inhibiting the synthesis of nucleic acids (Kusunoki et al., 2011; Noda and Ueno, 1981; Fujii et al., 1976;Essigmann et al., 1980).

Oxidative stress occurs when the balance between the production and scavenging of reactive oxygen species that can induce

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lipid peroxidation, DNA fragmentation, and protein oxidation is interupted (Nencini et al., 2007). Reactive oxygen species (ROS) is a byproduct of metabolism of oxygen produced by the cells (Devasagayam et al., 2004). Elevated ROS levels create oxidative stress in cells and chronic exposure to this stress can induce permanent genome changes (Nordberg and Arnér, 2001; Cooke et al., 2003). Besides, ROS induce lysosomal protease leakage, which lead to DNA damage (Ogawa et al., 2004).

The liver plays a pivotal role in the regulation of various physiological processes in the body, and it is the most important organ involved in the detoxification of various drugs (Sharma et al., 2011). HepG2 cells are similar in many functional respects to liver cells. When being used in vitro studies on genetic toxicity of exogenous materials, the exogenous activation system is out of reflection (Yao and Zhong, 2005). Therefore, HepG2 cells are considered as an ideal cell line when detecting hepatotoxicity and genotoxicity.

Despite its potential toxicity and carcinogenic properties in animals, the importance of STC in human health vulnerability is still unknown. In the present study, we used HepG2 cell line as an in vitro model to study the molecular mechanism of genetic toxicity of STC. It is expected that our research results could evaluate STC's risk to the necessary guidance for its detection and prevention, which could complement the genetic toxicology research data.

2. Materials and methods

2.1. Reagents

Sterigmatocystin (CAS NO.S3255 MSDS), Normal-melting point agarose (NMP) and low-melting point agarose (LMP),2,7-dichlorofluorescein diacetate (DCFH-DA), acridine orange (AO), ammonium chloride (NH_4Cl), N-acetyl-L-cysteine (NAC) were obtained from Sigma, Germany. Monoclonal 8-OHdG antibody was purchased from JaICA (Fukuroi, Japan). Secondary anti-mouse immunoglobulin G (IgG) and Diaminobenzidine (DAB) kit were purchased Maixin-Bio (FuJian, China). Minimum essential Eagle's medium (MEM), fetal bovine serum, antibiotics (penicillin and streptomycin), and trypsin–EDTA solution was supplied by Invitrogen (Carlsbad, CA). 8-OHdG mouse monoclonal antibody (SANTA CRUZ, USA).

2.2. Cell culture

The human hepatoma cell line HepG2 [American Type Culture Collection (ATCC) HB-8065] was obtained from Peking Union Medical College (Beijing, China). HepG2 cells were grown in minimal essential medium (MEM), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 IU/ml penicillin and 100 μg/ml streptomycin by incubating at 37 °C under 5% CO2. In each experiment, HepG2 cells were treated with different concentrations of STC in the culture medium mentioned above.

2.3. Preparation of chemical

STC powder was dissolved in DMSO, and stored at 22 °C as stock solutions at the concentration of 1.5 mM. The fresh dilutions of STC at indicated concentrations were made in medium before each experiment. The final concentration of DMSO in the culture medium was 0.1% (v/v). Thus, 0.1% DMSO was used as the control for comparison.

2.4. MTT assay

MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide) assay is routinely used to evaluate the cell viability and has been described previously (Fischer et al., 2003). HepG2 cells were plated in a 96-well microtiter plate at a density of 1.5×10^5 cells/well at 37 °C in an atmosphere of 5% CO_2 – 95% air mixture. The cells were treated with STC at a final concentration of (0, 0.375, 0.75, 1.5, 3, 6 and 12 μM) for 24 h. Washed and incubated with an MTT solution (5 mg/ml) for 2 h at 37 °C. The formazan crystals formed were dissolved in DMSO at 37 °C for 1 h in the dark, and the absorbance was read at 595 nm in a microplate reader (BIO-RAD Model 3550) and analyzed to calculate the percentage of cell viability.

2.5. Single-cell gel electrophoresis assay

DNA damage caused by the genotoxic biomaterials was detected by the SCGE assay of Singh and Stephens (1997) and Tice et al. (2000) with slight modification. HepG2 cells were suspended in 2.0 ml of MEM and incubated with STC (0, 1.5, 3 and 6 μM, respectively) at 37 °C for 1 h in the presence or absence of 10 mM NAC (pretreated for 1 h). Trypan blue (50 μg/ml) or Hoechst 33,342 (8 μg/ml) were used to determine the cell viability and the molecular damage responsively. Only cells with more than 90% viability and no apoptosis could be determined for DNA migration (An et al., 2007). Therefore, 20 μl of cell suspension was mixed with 160 μl of 1.0% LMA and spread onto frosted slides premiered with 1.5% NMA. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate and 1% Triton X-100) at 4 °C for 40 min. The slides were then placed in alkaline solution (1 mM Na2EDTA and 300 mM NaOH, pH 13.0) for 20 min and allowed the DNA to unwind before electrophoresis (20 V, 200 mA) for 30 min. Cells were neutralized three times with 0.4 M Tris (pH 7.5) and stained with 50 μl Ethidium Bromide (EB) (20 μg/ml). The images were taken under a fluorescence microscope with an excitation filter of 549 nm and barrier filter of 590 nm. All steps were carried on in the dark to prevent additional DNA damage. One hundred cells from each sample were selected randomly and analyzed by the free Comet Score Software. By using Autocomet software 3 parameters (tail length, %DNA in tail, tail moment), the genotoxicity of biomaterials was quantified.

2.6. Measurement of intracellular ROS

ROS measurements were performed by means of a dichlorofluorescein assay (Rakkestad et al., 2010). The 2,7-dichlorofluorescin diacetate (DCFH-DA), a fluorescent probe, diffuses through the cell membrane, which is hydrolyzed to non-fluorescent 2,7-dichlorofluorescin (DCFH). ROS can cause oxidation of DCFH and form fluorescent product, DCF. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellular. H_2O_2 is the principle ROS responsible for the oxidation of DCFH-DA to DCF. Cells (5×10^5 cells/ml) in logarithmic growth phase were exposed to STC for 1 h, then add DCFH-DA to the cell culture at a final concentration of 5 μM, incubate for 1 h at 37 °C in darkness. Detect the DCF fluorescence intensity with emission wavelength at 530 nm and excitation wavelength at 485 nm by fluorescence spectrophotometer (HITACHI650-60, Tokyo, Japan).

2.7. Assessment of lysosomal membrane stability

Lysosomal membrane stability was determined with a modified method (Li et al., 2008). HepG2 cells were treated with STC (0, 1.5, 3 and 6 μM) for 1 h in the presence or absence of 10 mM NH_4Cl (pretreated for 1 h). After that, the cells were washed twice with PBS (pH 7.8) and incubated with acridine orange (AO) at a final concentration of 5 μM in the dark at 37 °C for 15 min. Cells were washed and the fluorescence intensity of the cell suspensions