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Aflatoxin G_1 reduces the molecular expression of HLA-I, TAP-1 and LMP-2 of adult esophageal epithelial cells *in vitro*

Zengning Li[a,*], Jinfeng Cui[b], Xianghong Zhang[b,*], Weijun Kang[c]

[a] *Department of nutrition, The First Attached Hospital, Hebei Medical University, Shijiazhuang, China*
[b] *Department of Pathology, Hebei Medical University, Shijiazhuang, China*
[c] *School of Public Heath, Hebei Medical University, Shijiazhuang, China*

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ABSTRACT

Aflatoxins (AF) are the mycotoxin with the carcinogenic effect produced by the fungi like *Aspergillus flavus*, including AFB_1, B_2, G_1, G_2, M_1, and so on. The detection rate of Aflatoxin G_1 (AFG_1) is very high in the diet of residents in the high incidence area of the gastric carcinoma and esophageal carcinoma in China, which is the main polluting mycotoxin of the local grain. However, there were no available data regarding the gastric toxicity of AFG_1 up to now. In the present study, The toxicity of AFG_1 *in vitro*-cultured human primary adult esophageal epithelial cells was explored by employing the methods of Western blot, semi-quantitative RT-PCR and observing the effect of AFG_1 on the expression of the HLA-I molecules of the human esophageal epithelial cells and the antigen processing relative genes TAP-1 and LMP-2. The results showed that AFG_1 treatment could decrease both the protein expression of HLA-ABC of the cell surface and the mRNA expression of HLA-A and HLA-B. Moreover, In the AFG_1 treatment groups of different densities, both the TAP-1 mRNA and protein expression level of the human esophageal epithelial cells decreased. Furthermore, the LMP-2 protein expression level of the human esophageal epithelial cells decreased, while the mRNA expression level was not obviously changed by AFG_1 treatment. It is indicated that the decrease of the HLA-I expression of the cell surface was probably dependent on the decrease of the TAP-1 and LMP-2 expression to a great extent. The decrease of LMP-2 expression resulted in the decrease of HLA-I expression and stability in the cell membrane and finally led to the defect in the antigen presentation, which hindered its identification by the T lymphocytes, made it escape the immune surveillance, and then caused the tumor genesis.

1. Introduction

Aflatoxin G_1 (AFG_1) is one of the members of aflatoxins, the toxic metabolites produced by *Aspergillus flavus*, etc. (Razzaghi-Abyaneh et al., 2007). AFG_1 is commonly found at corn and other cereals grains used worldwide in animal feeds and human foods. In 1993, aflatoxins (naturally occurring mixtures of) were classified as "carcinogenic to humans (group 1)" by the International Agency for Research on Cancer (IARC, 1993). Studies showed that AFG_1 was the most frequently detected contaminating mycotoxins in the foodstuffs of the high incidence areas of esophageal cancer in Taihang mountain area in north China (Zhang et al., 1998).

The carcinogenicity of AFG_1 in experimental animals is also well established. Our previous studies showed that AFG_1 could induce hyperplastic lesions and adenocarcinoma of lung in NIH mice by intragastric administration for 24 weeks (Huang et al., 2004). Moreover, epidemiological investigation was reported that the high incidence rate of esophageal cancer in Cixian of China has been correlated with AFG_1 (Wang et al., 1999).

Although sufficient evidence in experimental animals suggests that AFG_1 is carcinogen, the underlying mechanism of AFG_1-mediated carcinogenesis has not been fully elucidated. As we known, the immune function was one of major factors which affected the development of carcinoma. And the monitoring function of immune system was negative in carcinogenesis for a lot of reasons. HLA class I antigen presentation pathway plays an important role in immunosurveillance against virally infected and malignantly transformed cells. The endogenous antigens are degraded by the proteasome, of which LMP-2 and LMP-7 were both units (Mehta et al., 2008). Subsequently, the generation of peptide fragments is transported into the endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP) complex (Ogino et al., 2006). These molecular defects which involved in antigen processing pathway are frequently found in many different types of human tumors, such as head and neck squamous cell

* Corresponding authors. Tel.: +86 311 85917009.
E-mail addresses: Zengningli@hebmu.edu.cn (Z. Li), zhangxh@hebmu.edu.cn (X. Zhang).

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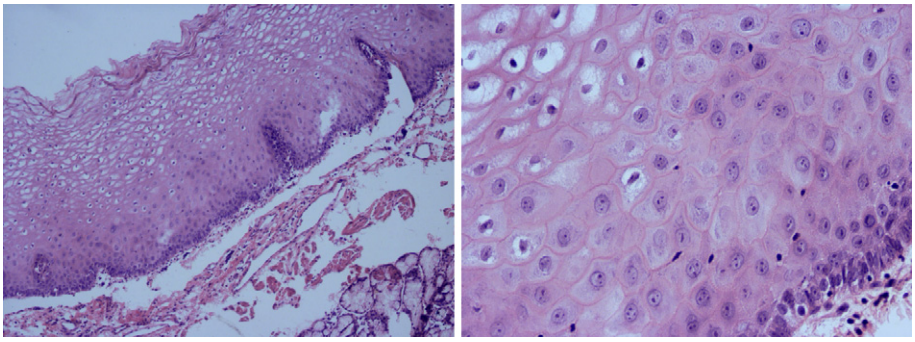

Fig. 1. Normal esophageal mucosa. Left: HE 200×; right: HE 400×.

carcinoma (Meissner et al., 2005), small-cell lung carcinoma (Doyle et al., 1985), hepatocellular carcinoma (Paterson et al., 1988), colon adenocarcinoma (Van den Ingh et al., 1987). These findings let us to consider how AFG_1 is carcinogenic to esophageal epithelial cells. It is hypothesised that AFG_1 might affect the function of immune system by inhibiting the process of antigen presentation. We cultured human primary esophageal epithelial cells *in vitro* to evaluate the effect of AFG_1 treatment on the antigen presentation molecule. The toxicity of AFG_1 *in vitro*-cultured human primary esophageal epithelial cells was explored by employing the methods of Western Blot, semi-quantitative RT-PCR and observing the effect of AFG_1 on the expression of the HLA-I molecules and the antigen processing relative genes TAP-1 and LMP-2. In the present study, we demonstrated that AFG_1 could reduce the expression of HLA class I antigen via the decreased expression of LMP-2 and TAP-1 expression.

2. Materials and methods

2.1. Aflatoxin G_1 and reagents

Highly purified Aflatoxin G_1 (>99% purity, benzene free) was purchased from the Sigma Company (America). Nutrient mixture F-12 and RPMI medium 1640 were purchased from the Gibco Company (CA, USA), insulin from the Sigma Company, epidermis growth factor (EGF) from the TOYOBO Company (Japan). The esophageal squamous epithelial tissues were taken from the operation samples of esophageal carcinoma from March 2008 to September 2008 at The Fourth Hospital of Hebei Medical University. The primary antibodies used for Western blot analysis were mouse anti-human HLA-ABC antibody (Biolegend, USA), mouse anti-human TAP-1 polyclonal antibody (Biotechnology Company, CA Germany), mouse anti-human LMP-2 polyclonal antibody (Santa Cruz Company, CA, America). ECL kits were purchased from the Lianke Biological Company (China). The RT-PCR reagents, such as guanidinium thiocyanate, AMV reverse enzyme, Rnasin, Oligo $(dT)_{15}$, Taq DNA Polymerase, were purchased from the Promega Company (USA).

2.2. Cell culture and treatment

Esophageal samples were taken within 3 h after the esophageal carcinoma operation as the materials. Selecting the esophageal mucosa of approximately 2 cm × 3 cm, which was 3 cm away from the tumor edge, the cells were placed in RPIM-1640 medium supplemented with 200 U/ml penicillin and 200 U/ml streptomycin, then were taken back to the laboratory immediately. Part of the sample was fixed with 10% formalin solution and conducted the routine HE staining. The pathomorphology observation results showed that all the selected tissues were the esophageal normal squamous epithelial tissues, with no lesion like hyperplasia, tumorigenesis, and so on (Fig. 1). The other part of the sample was used for the culture of cells. The cells were routinely cultured in DMEM/F12 1:1 mixture medium supplemented with 200 U/ml penicillin, 200 U/ml streptomycin, and 15% fetal bovine serum (FBS), 5 μg/L EGF and 5 mg/L insulin under 5% CO2/95% air. The cells were then treated with solvent (DMSO, final solvent concentration 0.2%) alone or with various concentrations of AFG_1 (50, 100, 1000 and 2000 μg/L) for 24 h.

2.3. Protein extraction and measurement

Cells were harvested after AFG_1 treatment for 24 h and washed with ice-cold PBS. Total protein was extracted using a lysis buffer containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin and 0.5 mM DTT. The samples were centrifuged at 12,000 rpm for 30 min at 4 °C. Protein concentration was determined using a standard Coomassie brilliant blue (CBB) total protein assay kit (Jiancheng bioengineering, Nanjing, China).

2.4. Western blot analysis

30–50 μg of protein per sample was loaded onto a discontinuous 4% stacking and 15% running SDS polyacrylamide gel (SDS-PAGE). Electrophoresis was performed at 100 V for 2 h. Proteins were then transferred to PVDF membrane at 4 °C. Subsequently, the membranes were blocked with 5% skim milk and incubated with specific antibody (1:200) at 4 °C overnight. The signals were detected with horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody at 1:5000 dilution at 37 °C and visualized by ECL chemiluminescent detection system. Band density was quantified using Snygene-Image Systems and normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.5. Reverse transcription-PCR

After incubation for 24 h at 37 °C with or without AFG_1, total RNA of the human esophageal epithelial cells were extracted using the one-step isothiocyanate method, and the concentration of RNA was measured with the spectrophotometer.

The first strand cDNAs was synthesized using AMV reverse transcriptase Kit (Promega, Madison, WI). The reaction was performed at 42 °C for 90 min in a total volume of 25 μl reaction mixture containing 2 μg of total RNA, 5 μl of 5× AMV buffer, 0.5 μl of oligo primer (0.5 μg/ml), 0.4 μl of AMV (5 U/μl), 2.5 μl of dNTP mixture (10 mM), and 0.5 μl of RNase inhibitor (30 U/μl).

The expression of HLA-ABC, TAP-1 and LMP-2 at mRNA level in human esophageal epithelial cells were determined using the RT-PCR technique on GeneAmp PCR system 9600. Gene-specific primers used in the present study (Table 1) were designed using the sequences accessible in the NCBI Reference Sequence and the software Primer Premier 5.0.

cDNA was amplified in 25 μl reaction mix containing 5 μl of Taq buffer, 0.35 μl of dNTP mixture (10 mM), 0.15 μl of Taq DNA Polymerase (5 U/μl), 1 μl of primer I (1 μM) and 1 μl of primer II (1 μM). The PCR reactions began with a denaturation step of 5 min at 94 °C and then cycled 30 times at 94 °C/(30 s), 55 ± 5 °C/(30 s) and 72 °C/(30 s), with a final extension at 72 °C/(10 min). After amplification, each sample was analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The fragment for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA gene was used as an internal control to evaluate the relative expressions of mRNA amount and results were calculated after standardization at GAPDH mRNA content.

Table 1
Primers for RT-PCR amplification.

Primers	Sequence	Length
HLA-A-sense HLA-A-antisense:	5′-CCTACGACGGCAAGGATTACA-3′ 5′-ACATCACGGCAG CGACCA-3′	573 bp
HLA-B-sense HLA-B-antisense	5′-CTACGACGGCAAGGATTAC-3′ 5′-GGTGGACTGGGAAGACG-3′	493 bp
HLA-C-sense HLA-C-antisense	5′-GCAGTTCGTGCGGTTCG-3′ 5′-GTCTC CTTCCCGTTCTCC-3′	442 bp
TAP-1-sense TAP-1-antisense	5′-GCTCAG CCGATACCTTCA-3′ 5′-CCACTTTCAGCAGCATACC-3′	246 bp
LMP-2-sense LMP-2-antisense	5′-GTTGTGATGGGTTCT-3′ 5′-GAGCAA TAGCGTCTGTG-3′	448 bp
GAPDH-sense GAPDH-antisense	5′-GGAAGGTGAA GGTCGGAG-3′ 5′-CCTGGAAGATGGTGATGGG-3′	231 bp