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Endoplasmic reticulum stress mediates the arsenic trioxide-induced apoptosis in human hepatocellular carcinoma cells

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ABSTRACT

Arsenic trioxide has been proven to trigger apoptosis in human hepatocellular carcinoma cells. Endoplasmic reticulum stress has been known to be involved in apoptosis through the induction of CCAAT/enhancer-binding protein homologous protein. However, it is unknown whether endoplasmic reticulum stress mediates arsenic trioxide-induced apoptosis in human hepatocellular carcinoma cells. Our data showed that arsenic trioxide significantly induced apoptosis in human hepatocellular carcinoma cells. Furthermore, arsenic trioxide triggered endoplasmic reticulum stress, as indicated by endoplasmic reticulum dilation, upregulation of glucose-regulated protein 78 and CCAAT/enhancer-binding protein homologous protein. We further found that 4-phenylbutyric acid, an inhibitor of endoplasmic reticulum stress, alleviated arsenic trioxide-induced expression of CCAAT/enhancer-binding protein homologous protein. More important, knockdown of CCAAT/enhancer-binding protein homologous protein by siRNA or inhibition of endoplasmic reticulum stress by 4-phenylbutyric acid alleviated apoptosis induced by arsenic trioxide. Consequently, our results suggested that arsenic trioxide could induce endoplasmic reticulum stress-mediated apoptosis in hepatocellular carcinoma cells, and that CCAAT/enhancer-binding protein homologous protein might play an important role in this process.

1. Introduction

Hepatocellular carcinoma (HCC) is a primary cancer of the liver. Despite improved diagnostic and treatment strategies, the majority of patients died within 1 year of diagnosis (El-Serag, 2011). Moreover, most patients commonly evolved resistance to conventional radiation and chemotherapy (El-Serag et al., 2006) and were often present at stages too late for surgical intervention (Bruix et al., 2004). Therefore, the requirement for ascertaining more efficacious therapies is urgent.

Arsenic trioxide (As_2O_3), an inorganic compound of trivalent arsenic, has attracted great attention for remarkable curative effects on leukemia and other solid tumors (Au et al., 2011; Sun et al., 2011; Tomuleasa et al., 2010). However, clinical response of solid tumors to single-agent As_2O_3 has been poor (Dilda and Hogg, 2007; Lin et al., 2007). Thereby, better understanding of the mechanisms of As_2O_3 will facilitate identification of potential targets for combination therapies of cancer.

The anti-tumor effect of As_2O_3 involves complex mechanisms that are incompletely elucidated. In many cases, induction of apoptosis may represent an important activity of As_2O_3 (Baysan et al., 2007; Lin et al., 2005; Liu et al., 2003; Mandegary et al., 2010). Recently, it has become clear that endoplasmic reticulum (ER) can be involved in apoptotic signaling pathways (Oyadomari and Mori, 2004; Szegezdi et al., 2006). The ER, a multifunctional cellular organelle, is primarily responsible for protein folding, lipid biosynthesis, and calcium homeostasis (Kim et al., 2008b; Ron

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and Walter, 2007). Multiple physiological and pathological conditions that affect protein folding and/or calcium homeostasis can interfere with ER function and cause imbalance of ER homeostasis (Minamino et al., 2010; Zhao and Ackerman, 2006). In response to such conditions, ER triggers an adaptive response termed the unfolded protein response (UPR) that attempt to restore ER homeostasis. The ER stress response is mediated by sensors located at the ER membrane. Under unstressed conditions, these sensors normally are occupied by glucose regulated protein 78 (GRP78) (Kohno, 2007). GRP78, also referred to as the immunoglobulin binding protein (BiP), is one of the initial components of the ER stress response (Kim et al., 2008b). On ER stress, these sensors are released from GRP78, permitting transduction of downstream signals and thereby initiating the ER stress response to ameliorate the accumulation of unfolded protein in ER (Ron and Walter, 2007). However, when ER homeostasis cannot be restored, excessive or prolonged ER stress may result in apoptosis (Minamino et al., 2010; Zhao and Ackerman, 2006).

The transcription factor C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is reported as a molecule involved in ER stress-induced apoptosis (Kim et al., 2008b; Oyadomari and Mori, 2004). CHOP is ubiquitously expressed at very low levels in non-stressed conditions, but markedly increases in response to ER stress (Nishitoh, 2012). The overexpression of CHOP has been reported to lead to apoptosis in several cell lines, whereas CHOP-deficient cells exhibit resistance to ER stress-induced apoptosis (Gotoh et al., 2002; Kim et al., 2008b; Oyadomari et al., 2002). Recent studies indicated that the suppression of the pro-survival protein B-cell CLL/lymphoma 2 (Bcl-2) and the induction of the pro-apoptotic proteins BCL2 interacting mediator of cell death (BIM) and p53 upregulated modulator of apoptosis (PUMA) as well as death receptor-5 (DR5) had been implicated in CHOP mediated apoptosis (Ghosh et al., 2012; Puthalakath et al., 2007; Yamaguchi and Wang, 2004). Therefore, the downstream targets of CHOP that have been implicated in apoptosis are complex.

Increasing evidence showed that As_2O_3 inhibited proliferation and triggered apoptosis in human HCC cells by several pathways, including the intrinsic mitochondria mediated apoptotic pathway (Alarifi et al., 2013; Siu et al., 2002; Tan et al., 2005; Xu et al., 2004). Recent reports indicated that As_2O_3 treatment might induce apoptosis through the activation of ER stress (Binet et al., 2011; Chen et al., 2012; Yen et al., 2012). However, it is unknown whether ER stress mediates As_2O_3-induced apoptosis in HCC cells. In the present study, we found that ER stress was involved in As_2O_3-induced apoptosis of human HCC cells. Then we further demonstrated that CHOP was an important factor for apoptosis in As_2O_3-exposed cells.

2. Materials and methods

2.1. Cell culture.

The human HCC cell lines HepG2 and SMMC-7721 (Heilongjiang cancer institute, China) were cultured in RPMI-1640 medium containing 10% Fetal Bovine Serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were routinely sub-cultured every 2–3 days and cell samples were all in the logarithmic growth phase.

2.2. Reagents and antibodies

Arsenic trioxide (As_2O_3), 4-phenylbutyric acid (4-PBA), trypsin, propidium iodide (PI) were purchased from Sigma Chemical Co. Antibodies against GRP78, pro-caspase 3, cleaved-caspase 3, pro-PARP, cleaved-PARP, CHOP, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish Peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology.

2.3. Apoptosis measurement by Annexin V-FITC Staining

Apoptosis was measured with the Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed twice with cold PBS, and then resuspended in 100 μl 1× binding buffer at a concentration of 1×10^6 cells/ml. 5 μl of annexin V-FITC and 5 μl of PI were added. Thereafter, these cells were incubated for 15 min at room temperature in the dark, and then 400 μl of 1× binding buffer was added to each tube. The cells were analyzed by FACSCanto flow cytometer within 1 h. The data were analyzed with Cell Quest software.

2.4. Transmission electron microscopy

Morphological changes of the HCC cells with As_2O_3 treatment were detected by transmission electron microscopy. After cultured with 8 μmol/L As_2O_3 for 24 h, HCC cells were fixed with a solution containing 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH = 7.3, for 1 h. After fixation, the samples were post-fixed in 1% OsO4 in the same buffer for 30 min. Ultra-thin sections were then analyzed under a transmission electron microscope at 80 kV.

2.5. Gene silencing using small interfering RNAs

Cells were seeded into six-well plates in growth RIPM-1640 medium without antibiotics to achieve a monolayer density of 30% to 40%. Six hours later, cells were transfected with CHOP siRNA or control siRNA using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) at a final concentration of 100 nmol/L, according to the manufacturer's protocol. After 48 h, the cells were then treated with or without 8 μmol/L arsenic trioxide for 24 h. The efficiency of gene silencing was assessed by western blotting.

2.6. Western blotting

Cells were lysed in a lysis buffer (Beyotime, Jiangsu, China) and subjected to SDS-polyacrylamide gel electrophoresis (30 μg of protein/lane). Proteins were then transferred to PVDF membrane. The membrane was blocked, incubated with primary antibodies overnight and secondary antibody for one hour at room temperature. Finally, the membrane was developed with chemiluminescent reagents (EZ-ECL, Beit-Haemek, Israel).

2.7. Quantitative RT-PCR.

Total RNA was extracted from treated cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Then first-strand cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Subsequently, quantitative polymerase chain reaction (qPCR) was performed in triplicates on the ABI7500 FAST (Applied Biosystems, Foster City, CA) with FastStart Universal SYBR Green Master (Roche) and primers (150 nM), and the amplified specific single product was validated by melt curve. The following gene-specific primer pairs were used: CHOP: (F) 5′-atggcagctgagtcattgcctttc-3′, (R) 5′-agaagcagggtcaagagtggtgaa-3′; BIM: (F) 5′-gcaggacaagtgaatggg-3′, (R) 5′-atcagaaggagggcatcg-3′;