As$_2$O$_3$ induces apoptosis in human hepatocellular carcinoma HepG2 cells through a ROS-mediated mitochondrial pathway and activation of caspases

Lin Jiang$^1$, Le Wang$^1$, Lei Chen$^2$, Guo-Hong Cai$^3$, Qin-You Ren$^1$, Jian-Zong Chen$^4$, Heng-Jun Shi$^1$, Yong-Hong Xie$^5$

Departments of $^1$Traditional Chinese Medicine, $^2$Neurosurgery, $^3$Respiratory, Tangdu Hospital, Fourth Military Medical University, Xi’an, Shaanxi 710038, PR China; $^4$Department of Anatomy, Histology and Embryology & K.K. Leung Brain Research Centre, Preclinical School of Medicine, Fourth Military Medical University, Xi’an 710032, PR China; $^5$Research Center of Traditional Chinese Medicine, Xijing Hospital, Fourth Military Medical University, Xi’an, Shaanxi 710032, PR China. *Equal contributors.

Received November 30, 2014; Accepted January 29, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: Arsenic trioxide (As$_2$O$_3$) has been shown to induce apoptosis in hepatocellular carcinoma cells. However, the molecular mechanism of As$_2$O$_3$-induced apoptosis in the hepatocellular carcinoma cells remains poorly understood. Here, we investigated the impact of As$_2$O$_3$ exposure on the human hepatocellular carcinoma cell line HepG2 and examined the underlying mechanism of cell death. As$_2$O$_3$ induced apoptosis of HepG2 cells in a dose- and time-dependent manner and caused a massive production of reactive oxygen species (ROS). The antioxidant N-acetylcysteine (NAC) was able to prevent As$_2$O$_3$-induced cell death, implying an involvement of ROS in the induction of As$_2$O$_3$-triggered apoptosis. Furthermore, As$_2$O$_3$ initiated apoptosis by triggering of the mitochondria apoptotic pathway as indicated by inhibited Bcl-2 expression, a collapse of the mitochondrial membrane potential (MMP), release of cytochrome c and activation of the caspase cascade. However, these As$_2$O$_3$-induced events can be prevented by NAC. Taken together, these findings suggest that the As$_2$O$_3$ induced apoptosis through a ROS-mediated mitochondrial pathway and activation of caspases.

Keywords: Arsenic trioxide, hepatocellular carcinoma, reactive oxygen species, mitochondrial pathway, caspase

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. It is caused by a malignant transformation of hepatocytes, the major cell type in the liver, and accounts for 80% to 90% of primary liver cancer. The annual incidence of HCC is more than 1,000,000 newly diagnosed cases, and less than 5% of patients survive beyond five years. Due to its high mortality, HCC is a major health problem worldwide [1, 2]. HCC treatment mainly consists of surgical resection, liver transplantation, and local ablative therapy. Systemic therapies, however, have not shown any survival benefit [2-4]. Moreover, prolonged treatment with chemotherapy drugs can lead to multidrug resistant tumor cells and these drugs have severe adverse effects [5, 6]. Therefore, novel and effective therapeutics with lower toxicity are urgently needed for patients with HCC.

The medicinal effect of As$_2$O$_3$ is of great significance, though, it is also well known for its toxicity. It has been used to treat various diseases in both ancient China and Western societies [7-9]. As$_2$O$_3$ treatment of Chinese patients with acute promyelocytic leukemia (APL) has shown some success [10, 11]. Subsequently, the effect of As$_2$O$_3$ on a variety of solid tumor cells such as hepatocellular carcinoma was investigated in vitro and vivo. Interestingly, the work of several labs suggested an important role for As$_2$O$_3$-induced apoptosis in the treatment of HCC cells [5, 9, 12]. However, the exact mechanism of action of As$_2$O$_3$-induced apoptosis in HCC cells remains unclear.

Here, we investigated the molecular mechanism of As$_2$O$_3$-induced apoptosis in HepG2 cells and examined mitochondrial apoptosis signaling pathways and ROS production in As$_2$O$_3$-
As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma

induced cell death. The results suggest that As$_2$O$_3$ induced apoptotic cells death in HepG2 cells occurs via a ROS-mediated mitochondrial pathway and activation of caspases.

**Materials and methods**

**Reagents and cell cultures**

As$_2$O$_3$ was obtained from Sigma-Aldrich Chemical company, 2',7'-Dichlorofluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), Annexin V-FITC Apoptosis Detection Kit, Hoechst 33258, Rhodamine-123 and 3-(4,5-dimethylthiazol-2-y-1)-2,5-diphenyltetrazolium bromide (MTT) were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Rabbit polyclonal antibodies against Bcl-2, Bax, cytochrome c, caspase-3, caspase-6, caspase-9, β-actin, were obtained from Abcam (Abcam, Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

As$_2$O$_3$ was prepared as a 10 mM stock solution in phosphate buffered and saline and stored at 4°C. The human hepatocellular carcinoma cell line HepG2 was obtained from the Chinese Academy of Sciences (Shanghai, China). All cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) which was supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), in a humidified atmosphere of 5% CO$_2$ at 37°C.

**Cell viability assays**

Cell viability was measured by MTT assay as previously described [13]. Briefly, 1×10$^4$ cells/well were plated in 96-well microplates and cultured for 24 h. The cells were subsequently treated with 2, 5, 15 μM As$_2$O$_3$ for 24, 48, and 72 h with or without pretreatment with 10 mM N-acetylcysteine (NAC) for 1 h, respectively. Cells were incubated with 20 μL MTT (5 mg/ml) for 4 h. Formazan crystals were dissolved in 150 μL DMSO, and the absorbance at 570 nm was measured in a microplate reader (Bio-Rad, USA). Each test was performed in triplicate.

**Apoptosis assay**

The percentage of apoptotic cells was measured by flow cytometry using an annexin V-FITC/PI kit (Beyotime) according to manufacturer’s instructions. Briefly, cells were treated for 24 h with the As$_2$O$_3$ concentrations mentioned above with or without 10 mM NAC pretreatment. The cells (1×10$^6$) were washed twice with ice-cold PBS and collected. Apoptosis rates were measured by double staining with annexin V-FITC and propidium iodide (PI) in binding buffer using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA).

The nuclear morphology of HepG2 cells was analyzed by staining with Hoechst 33258 according to manufacturer’s instructions. Briefly, the As$_2$O$_3$-treated cells were washed with PBS, fixed with 4% formaldehyde for 30 min, then washed with PBS three times, and incubated with Hoechst 33258 (3 μg/ml) for 30 min at room temperature in the dark. Then they were washed three times with PBS and the nuclear morphology was examined using a fluorescence microscope (Olympus, Japan).

**ROS production determinates**

The level of intracellular ROS was quantified using a Reactive Oxygen Species Assay Kit. DCFH-DA, a fluorescent probe, is oxidized by reactive oxygen species in viable cells to 2',7-dichlorofluorescein (DCF). Using the designated treatment, the same densities of cells were harvested by cell counting. The cells were then incubated with 100 μM DCFH-DA (dis-
As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma

solved in DMSO) for 30 min at 37°C. After washing three times with PBS, the relative levels of fluorescence were quantified by a multi-detection microplate reader (485 nm excitation and 535 nm emission).

Analysis of mitochondrial membrane potential ($\Delta$ψm)

The mitochondrial membrane potential was measured by the incorporation of the cationic fluorescent dye rhodamine 123, which preferentially partitions into active mitochondria based on the highly negative mitochondrial membrane potential. Depolarization of mitochondrial membrane potential results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence. After incubation in the normal medium with or without treatment, the cells were transferred to a serum-free medium containing 10 μM rhodamine 123 and incubated for 30 min at 37°C. The cells were then collected and the fluorescence intensity was analyzed by flow cytometry.

Western blot analysis

As$_2$O$_3$-treated HepG2 cells were harvested, washed twice with PBS, and lysed in 200 μl lysis buffer for 5 min on the ice. The cell lysates were centrifuged at 12,000×g for 5 min at 4°C. Protein concentration in the supernatant were measured using a Bradford Protein Assay. Samples with equal amounts of protein were separated through a 12% SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, UK) using a semi-dry transfer system (Bio-Rad). The membrane was blocked with 5% non-fat milk in TBST for 2 h, incubated with primary antibodies for 2 h at room temperature (diluted 1:2000 in TBST), washed and incubated with secondary antibodies (diluted 1:5000) in TBST at room temperature for 1 h, respectively. Lastly, the proteins were detected using enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia).

Statistical analysis

All experiments were performed at least in triplicate, and data were expressed as mean ± S.E.M. Statistical comparisons were performed using one-way analysis of variance (ANOVA) and Student’s t-test. P<0.05 was considered statistically significant.

Results

Effect of As$_2$O$_3$ on HepG2 cell viability

Cell counts were performed manually by workers blinded to the treatment schedule as described. To identify the therapeutic potential of As$_2$O$_3$, HepG2 cells were cultured with a concentration of 2, 5, 15 μM As$_2$O$_3$ for 24, 48, and 72 h, respectively. As$_2$O$_3$ inhibited the viability of HepG2 cells. Cell viability significantly decrease when the with concentration was 2, 5,
As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma

As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma

Figure 3. Fluorescence images show the nucleic changes of HepG2 cells incubated in As$_2$O$_3$ with or without NAC. Cells were stained with the DNA-binding fluorochrome Hoechst 33258. (A) shows normal culture medium nucleic morphology, (B-D) respectively show cells cultured in 2 μM, 5 μM and 15 μM As$_2$O$_3$ for 24 h. In addition, cells were pretreated with 10 mM NAC (E) for 1 h and then incubated in As$_2$O$_3$ (15 μM) for an additional 24 h. (F) Histograms showing ratio of condensed nuclei to total nuclei. White arrows represent location of apoptosis cell. Scale bars represent 50 μm. *P<0.05 and **P<0.01 compared with control; #P<0.05 compared with 15 μM As$_2$O$_3$ alone.

or 15 μM of As$_2$O$_3$ while being treated for 24, 48, or 72 h respectively, and the decrease could be prevented by NAC at 24, 48, and 72 h post-treatment (Figure 1).

As$_2$O$_3$-induced ROS generation in HepG2 cells

To further investigate the role of As$_2$O$_3$ in ROS production, we measured production of intracellular ROS in As$_2$O$_3$-treated HepG2 cells by flow cytometric using DCFH-DA. Incubation of HepG2 cell with 2 μM As$_2$O$_3$ induced a significant increase (124.7%) in intracellular ROS content. At As$_2$O$_3$ concentration lead to a ROS burst (282.5% compared to untreated cells, P<0.01), however, the increase was completely inhibited by ROS scavenger NAC (102.3%) (Supplementary Figure 1).

As$_2$O$_3$-induced apoptosis in HepG2 cells

To evaluate whether As$_2$O$_3$ induced apoptosis is related to ROS production in HepG2 cells, annexin V-FITC/PI double staining was performed. After treatment of HepG2 cells with As$_2$O$_3$ at concentration of 2, 5, and 15 μM, respectively, for 24 h with or without pretreatment with 10 mM NAC for 1 h, the percentage of apoptotic cells increased in an arsenic-dose-dependent manner (Figure 2B-D). However, this increase was significantly decreased by pretreatment with the antioxidant NAC (Figure 2E). Characteristic apoptotic morphological changes such as condensation of chromatin and nuclear fragmentation were found in arsenic-treated cells by Hoechst33258 staining (Figure 3B-D). A decreased amount of apoptotic cells were seen by Hoechst33258 staining after pretreatment with NAC (Figure 3E).

As$_2$O$_3$ induces apoptosis via a ROS-mediated mitochondrial pathway

To demonstrate whether As$_2$O$_3$-treatment leads to a collapse of the mitochondrial membrane potential (Δψm) in HepG2 cells, the change of Δψm was evaluated by flow cytometry using rhodamin-123, which can accumulate rapidly and selectively within the mitochondria depending on mitochondrial membrane potential. As$_2$O$_3$ induced a disruption of the Δψm as evidenced by a decline in the proportion of cells with high fluorescence intensity (Supplementary Figure 2). The mean rhodamin-123 fluorescence intensity of HepG2 cells decreased from about 114.8 (control) to 66.2 and 43.4 after 24 h treatment with 5 μM and 15 μM As$_2$O$_3$, respectively (P<0.05). Moreover, the disruption of Δψm was partially prevented by pretreatment with NAC (mean fluorescence intensity 86.6, P<0.05).
As$\textsubscript{2}$O$\textsubscript{3}$ induces apoptosis of hepatocellular carcinoma

Western blot analysis. The levels of activated caspase-3, caspase-6 and caspase-9 increased in a dose-dependent manner (Supplementary Figure 3).

Discussion

In the present study, we found that As$\textsubscript{2}$O$\textsubscript{3}$ induced apoptosis of HepG2 cells in a dose- and time-dependent manner and caused a massive production of ROS. As$\textsubscript{2}$O$\textsubscript{3}$ induced apoptosis in HepG2 cells by inhibiting Bcl-2 expression, a collapse of MMP, release of cytochrome c, and activation of the caspase cascade. These findings strongly suggest that the As$\textsubscript{2}$O$\textsubscript{3}$ induced apoptosis is mediated, at least in part, by controlling the ROS-mediated mitochondrial pathway and activation of caspases.

Compelling evidence suggests that most cancer cells are under oxidative stress associated with increased metabolic activity and production of ROS [14-17]. These biochemical characteristics make the cancer cells vulnerable to damage by additional oxidative stress, either through inhibiting ROS elimination, increasing their intracellular production or by adding exogenous ROS [18, 19]. The cell-damaging properties of ROS and the increased ROS production in cancer cells may be a potential therapeutic target, if it is possible to exploit the cell killing potential of ROS by using exogenous agents to increase intracellular ROS to a toxic level, or above the threshold that triggers cell death [20]. In the present study, a dramatic ROS burst was observed in As$\textsubscript{2}$O$\textsubscript{3}$-treated cells, and As$\textsubscript{2}$O$\textsubscript{3}$-induced cells apoptosis was prevented by pretreatment of the cells with the antioxidant NAC, suggesting that ROS generation is involved in As$\textsubscript{2}$O$\textsubscript{3}$-induced apoptosis in HepG2 cells.

Mitochondria play a key role in the regulation of apoptosis [21, 22]. Mitochondrial dysfunctions during apoptosis induction include the loss of mitochondrial membrane potential ($\Delta$ψm), permeability transition, and release of cytochrome c from mitochondria into the cytosol [23-25]. Here, we showed that As$\textsubscript{2}$O$\textsubscript{3}$ induced loss of mitochondrial membrane potential and release of cytochrome c into the cytosol in a dose-dependent manner in HepG2 cells. In addition, As$\textsubscript{2}$O$\textsubscript{3}$ significantly inhibited the expression of Bcl-2 and up-regulated the expression of Bax. However, pretreatment of the cells with NAC

Role of caspases in As$\textsubscript{2}$O$\textsubscript{3}$-induced apoptosis in HepG2 cells

The caspase cascade plays a key role in apoptosis induction. Therefore, we determined the levels of activated caspase-9, caspase-6, and caspase-3 in As$\textsubscript{2}$O$\textsubscript{3}$-treated HepG2 cells by
As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma

prevented these changes, suggesting a ROS-mediated mitochondrial apoptotic pathway in the induction of cell death by As$_2$O$_3$ in HepG2 cells.

Caspases are intracellular cysteine proteases that are key components in classic apoptosis. Caspase activation occurs in response to various types of mitochondrial damage and pro-apoptotic stimuli, which cause cytochrome c to be released into the cytosol, where it binds and activates Apaf-1, which in turn activates pro-caspase-9, caspase-9 cleaves procaspase-3, and downstream of caspase-3, caspase-6, and eventually lead to cell apoptosis [26-29]. Here, we showed that As$_2$O$_3$ treatment caused activation of caspase-9, -3, -6, suggesting that As$_2$O$_3$ induced cell death through a caspase-dependent mechanism.

In conclusion, As$_2$O$_3$ induced apoptosis in HepG2 cells at least in part through a ROS-mediated mitochondrial pathway and activation of caspases. These results also provide important new insights into the possible molecular mechanism of As$_2$O$_3$ treatment for HCC.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hengjun Shi, Department of Traditional Chinese Medicine, Tangdu Hospital, Fourth Military Medical University Xi’an 710038, China. Tel: +86 029 84777927; Fax: +86 029 84777927; E-mail: shihengjunfmmu@126.com; Dr. Yonghong Xie, Department of Respiratory, Tangdu Hospital, Fourth Military Medical University, Xi’an 710038, China. Tel: +86 029 84777691; Fax: +86 029 84777691; E-mail: xieyonghongfmmu@163.com

References

As$_2$O$_3$ induces apoptosis of hepatocellular carcinoma


Supplementary Figure 1. Effect of As$_2$O$_3$ on cellular ROS production. Cells were treated with indicated concentration of As$_2$O$_3$ for 24 h in the presence or absence of 10 mM NAC pretreatment for 1 h, and then ROS level was determined. Results were expressed as mean DCF fluorescence. Data represent means ± SE of three independent experiments, *P<0.05 and **P<0.01 compared with control; #P<0.05 compared with 15 μM As$_2$O$_3$ alone.

Supplementary Figure 2. Effect of As$_2$O$_3$ on HepG2 mitochondrial. Rhodamine 123 staining followed by flow cytometry analysis was performed to determined mitochondrial membrane potential. Results were expressed as mean Rh123 fluorescence. Data represent means ± SE of three independent experiments *P<0.05 and **P<0.01 compared with control; *P<0.05 compared with 15 μM As$_2$O$_3$ alone.

Supplementary Figure 3. Effect of As$_2$O$_3$ on caspase in HepG2 cells. HepG2 cells were treated with 0-15 μM As$_2$O$_3$ for 24 h. Expression of the cleaved caspase-3, caspase-6, and caspase-9 were detected by Western blotting analysis. The quantitative analysis of cleaved caspase-3, caspase-6, caspase-9, respectively. β-actin was used as internal control. Data represent as means ± SE of three independent experiments. *P<0.05 and **P<0.01 compared with control.