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Studies on the Mechanism of Arsenic Trioxide-Induced Apoptosis in $HepG_2$ Human Hepatocellular Carcinoma Cells

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OBJECTIVE To study the anti-tumor effect of arsenic trioxide on the $HepG_2$ human hepatocellular carcinoma cell line, and to explore its mechanism of action.
METHODS The MTT assay was used to determine the inhibitory effect of As_2O_3 on $HepG_2$ cells at various As_2O_3 concentrations. The expression of p-JNK, caspase-3 and PARP was detected by Western blots.
RESULTS As_2O_3 markedly inhibited the growth of the $HepG_2$ cells and induced apoptosis. The results of Western blot analysis showed that the As_2O_3-induced apoptosis was accompanied by caspase-3 and PARP activation. p-JNK was detected at 10 min following As_2O_3 treatment, and preceded to peak at 20 min, and decreased by 30 min. The total protein content did not obviously change. The activation of JNK occurred prior to cell apoptosis. SP600125, a JNK inhibitor, suppressed the As_2O_3-induced activation of caspase-3 and PARP cleavage.
CONCLUSION As_2O_3 inhibits the proliferation of human $HepG_2$ hepatocellular carcinoma cells by inducing apoptosis in vitro. As_2O_3-induced apoptosis is accessed through the caspase-3 pathway. The JNK signal-transduction pathway and caspase-3 are involved upstream in the As_2O_3-induced $HepG_2$ apoptotic response.

KEYWORDS: arsenic trioxide, hepatic cancer, MTT assay, Western blot.

Introduction

Arsentic trioxide (As_2O_3) is the major active component of the traditional Chinese medicine, arsenicum sublimatum. Chinese researchers have achieved outstanding clinical results in treating acute promyelocytic leukemia (ALP) using As_2O_3[1,2]. Recent studies have shown that As_2O_3 has a significant inhibitory effect when used to treat solid tumors such as esophageal carcinoma, gastric cancer, colon carcinoma and especially hepatoma[3,4]. Several reports have indicated that As_2O_3 can induce hepatic cell apoptosis[5,6]. However further research is needed to elucidate the mechanism of action of As_2O_3, especially related to the signal-transduction pathway. In our study we used the $HepG_2$ human hepatic cell line in an in vitro model to examine the effect of As_2O_3on protein expression of c-Jun amino-terminal kinase (JNK), caspase-3 and poly (ADP-ribose) polymerase (PARP) in relation to possible mechanisms of As_2O_3-induced apoptosis.

Materials and Methods

Reagents

RPMI 1640 was purchased from the Huamei Biotechnology Co.

BSA and MTT were obtained from the Gibco Co., and dimethyl sulfoxide (DMSO) was purchased from Sigma. Rabbit anti-phospho-SAPK/JNK antibody, rabbit anti-PARP polyclonal antibody, rabbit anti-cleaved caspase-3 antibody and SP600125 were acquired from the Jingmei Biotechnology Co., and injectable As_2O_3 was purchased from the Yida Medicine Co., Harbin, China.

Cell line and cell culture

The $HepG_2$ human hepatoma cell line was provided by the First Affiliated Hospital of CMU (Shenyang, China). The cells were cultured with RPMI 1640 medium supplemented with 100 ml/L calf serum, 1×10^5 U/L penicillin and streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2 in air.

MTT assay

The $HepG_2$ cells were seeded in 96-well plates at a density of 1×10^4/ml and incubated for 0~72 h with different concentrations of As_2O_3, followed by incubation with 5 mg/mL of MTT for 4 h. Then the supernatant was removed following centrifugation, and 100 μl of DMSO was added. Absorbance at 570 nm (A_{570}) was measured with an enzyme-labeling instrument. The relative cell-proliferative inhibition rate (IR)= (1-average A_{570} of the experimental group/average A_{570} of the control group)×100%.

Western blot analysis

As previously described[7], cells were treated by low-serum starvation, after which they were collected, washed 3 times with PBS, lysed in cell lysate and then centrifuged at 12,000 r/min for 20 min at 4℃. Following determination of the protein in the supernatant by the Bradford method, protein samples were electrophoresed on 100 g/L or 60 g/L SDS-PAGE at 100 mA for 3 h, and finally transferred onto PVDF membranes. The PVDF membranes were treated with BSA (10 g/L), followed by incubation with the first antibody (1:1000 dilution) for 2 h. The corresponding secondary antibody (1:3,000 dilution) was added and incubated at room temperature for 1 h. After washing the membrane, the results were observed by ECL chemiluminescence with β-actin as an internal reference.

JNK-signal transduction was blocked by pre-incubating the $HepG_2$ cells with 20 μmol/L SP600125 for 1h, then As_2O_3 was added. Western blots were used to detect the expression of all of the proteins as described above.

Statistical analysis

Analysis of the data was performed with a SPSS 12.0 software package. The differences among the groups were compared by one way ANOVA and Student's *t* test. The level of significance was set at $P\leq0.05$.

Results

Inhibition of $HepG_2$ proliferation by As_2O_3

The results from the MTT assay showed that various levels of As_2O_3 markedly inhibited $HepG_2$ cellular proliferation (Fig.1A), in a concentration and time-dependent manner. The inhibitory ratio showed a linear correlation with the incubation time ($P<0.01$). With 10 μmol/L of As_2O_3, the inhibitory ratios at 24, 48 and 72 h were 29.6, 71.2 and 81.4%, respectively. This concentration of As_2O_3 caused the greatest inhibition of $HepG_2$ cellular proliferation (Fig.1B). Therefore, we used 10 μmol/L of As_2O_3 in subsequent experiments.

As_2O_3-induced activation of caspase-3

Western blot results showed that the caspase-3 precursor and its substrate PARP were activated after 24 h of treatment with As_2O_3. A cleaved caspase-3 band (17kDa) and cleaved PARP (85kDa) band were detected at 24 h. The cleavage increased with the time of incubation (Fig.2).

As_2O_3 activation of JNK

Western blots showed that JNK was activated by 10 min of As_2O_3 treatment, and preceded to peak at 20 min followed by a decrease at 30 min during induced apoptosis. The total protein content did not obviously change (Fig.3). The activation of JNK occurred prior to cell apoptosis.

The influence of SP600125 on JNK

The result of Western blots showed that pre-incubating $HepG_2$ cells for 1 h with 20 μmol/L SP600125 followed by addition of As_2O_3 for 48 h, inhibited the expression of p-JNK. Meanwhile the activation of caspase-3 and PARP were also markedly inhibited, suggesting that the JNK-signal pathway participated in the As_2O_3-induced $HepG_2$ apoptosis (Fig.4A). The results of the MTT assay showed that pre-incubation with SP600125 weakened the effect of As_2O_3-induced $HepG_2$ apoptosis (Fig.4B).

Discussion

The first anti-tumor effects of As_2O_3 were discovered in the treatment of APL. Since then, studies have focused on the treatment of solid tumors, especially inhibition of hepatoma cells[8], and it has been shown that As_2O_3 can inhibit hepatoma cellular proliferation[9]. We used the MTT assay to examine the in-

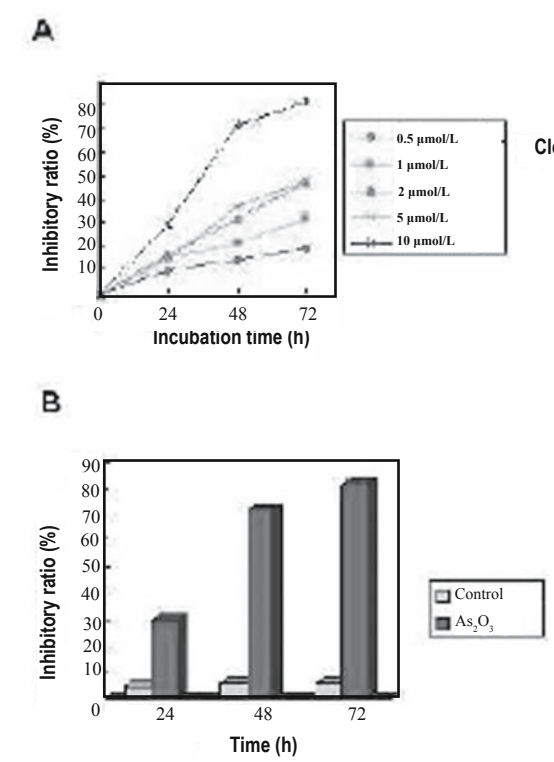

Fig.1. (A) The effect of various concentrations of As_2O_3 on the proliferation of $HepG_2$ Cells. (B) The effect of 10 μmol/L with the time of incubation.

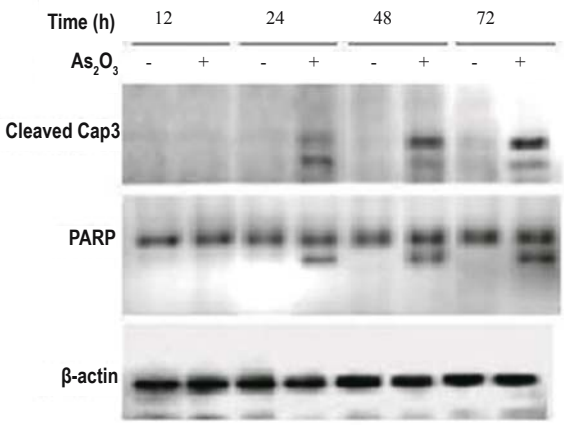

Fig.2.Western blot analysis of caspase-3 and its substrate PARP activation by treatment of $HepG_2$ cells at 24, 48 and 72 h. Activated cleaved caspase-3 (17 kDa) and cleaved PARP (85 kDa) was detected at 24 h.The effect increased with time of incubation.

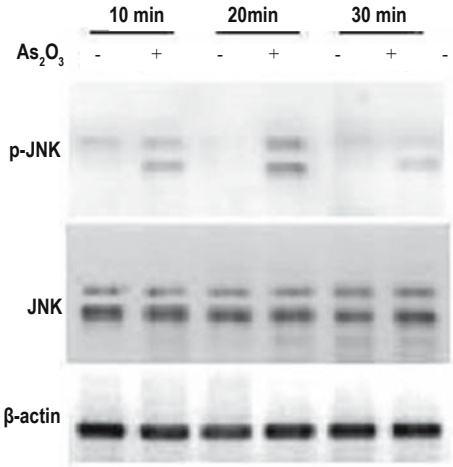

Fig.3.The increase in p-JNK protein expression by10 min after As_2O_3 treatment of the $HepG_2$ cells determined by Western blots. The expression peaked at 20 min and decreased by 30 min. Total JNK protein content showed no obvious change.

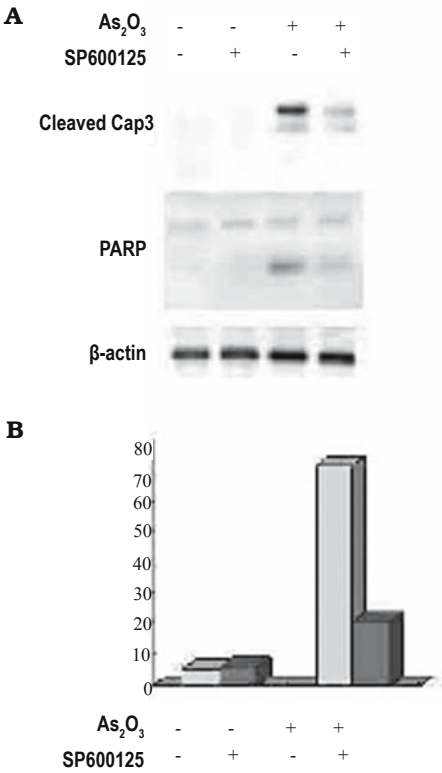

Fig.4. Pre-incubation of $HepG_2$ cells with SP600125 for 1 h decreased the activation of caspase-3 and PARP and reduced the inhibitory effect of As_2O_3 treatment on cell proliferation.

fluence of different concentrations of As_2O_3 on the $HepG_2$ hepatoma cells. The results showed that the inhibition by As_2O_3 was both time and concentration dependent, displaying an in vitro anti-tumor effect [10].

Caspase-3 is an important member of the ICE/CED-3 family, and plays a significant role in the regulation of apoptosis. It exists as a cytoplasmic non-active precursor, and when activated, promotes apoptosis along with other members of the ICE family[11,12]. Since PARP is the substrate for caspase-3, it is known as a "death substrate" for caspase-3 as its activation leads to apoptosis and cell death[13]. Therefore, activation of caspase-3 is a useful marker for initiation of apoptosis. Our result showed that addition of As_2O_3 to the cultured $HepG_2$ cells resulted in activation of caspase-3 and PARP at, and after 24 h. Their activation increased with time to at least 72 h resulting in cellular apoptotic inhibition of proliferation[14]. Caspase-3, as the primary apoptotic factor is regulated by a variety of upstream signal-transduction pathways. We found that JNK was detected at 10 min following As_2O_3 addition, preceded to peak at 20 min and decreased from 30 min. The total protein expression did not obviously change. Thus, As_2O_3 activated the JNK protein[15] prior to cell apoptosis. By pre-incubating the $HepG_2$ cells with

SP600125, a specific JNK antagonist, the activation of caspase-3 and PARP was markedly suppressed, suggesting that As_2O_3 may activate caspase-3 to start apoptosis and proliferative inhibition through a JNK signal-transduction pathway. However, even though SP600125 can completely block the JNK pathway, it can not completely block activation of caspase-3 and the inhibitory effect of As_2O_3 on $HepG_2$ cell proliferation. There may be some other pathways involved in As_2O_3-induced $HepG_2$ apoptosis and inhibition of proliferation.

In summary, our study showed that As_2O_3 inhibited the proliferation of the $HepG_2$ hepatoma cells by inducing apoptosis in vitro via activation of caspase-3 through a JNK signal-transduction pathway.

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