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Journal of Cellular Biochemistry 109:1264–1273 (2010)

Journal of Cellular Biochemistry

GADD45α Mediates Arsenite-Induced Cell Apoptotic Effect in Human Hepatoma Cells Via JNKs/AP-1-Dependent Pathway

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ABSTRACT

Arsenite (As(III)), an effective chemotherapeutic agent for the acute promyelocytic leukemia (APL) and multiple myeloma (MM), might be also a promise for the therapy of other cancers, including the solid tumors. However, the molecular bases of arsenite-induced cytotoxicity in the tumor cells have not been fully defined. In this study, we have disclosed that arsenite effectively induces the apoptotic response in the HepG2 human hepatoma cells by triggering GADD45α induction and the subsequent activation of JNKs/AP-1 cell death pathway. However, signaling events relating to GADD45α/JNKs/AP-1 pathway activation have not been observed in HL7702 human diploid hepatic cells under the same arsenite exposure condition. Our results thus have illustrated the selective pro-apoptotic role of arsenite in the hepatoma cells by activating GADD45α-dependent cell death pathway whereas with little effect on the normal hepatic cells. The approaches to up-regulate GADD45α levels might be helpful in improving the chemotherapeutic action of arsenite on certain solid tumors including hepatoma. J. Cell. Biochem. 109: 1264–1273, 2010.

KEY WORDS: GADD45α; ARSENITE; HEPATOMA; APOPTOSIS

Arsenic trioxide (As_2O_3, arsenite) is a major environmental contaminant in drinking water worldwide. Chronic exposure to low dose of arsenite is associated with the increased incidence of a variety of human cancers. Arsenite is also a FDA-approved tumor therapeutic agent as a result of its ability to selectively induce apoptosis of the tumor cells under moderate to high doses of stimulation [Litzow, 2008]. It has been found that arsenite is effective in the treatment of patients with the acute promyelocytic leukemia (APL) and other leukemia cancers, such as multiple myeloma (MM) [Chen et al., 1997; Rousselot et al., 1999; Zhang et al., 2001]. In addition, arsenite can also induce apoptosis of the solid tumor cells derived from the different tissues [Zhang et al., 1999, 2003; Ora et al., 2000; Uslu et al., 2000; Kito et al., 2002; Li et al., 2003; Taylor et al., 2006]. Therefore, the potential clinical application of arsenite for treating solid tumors is also under consideration. However, the molecular mechanisms of arsenite in playing the anti-tumor role without the risk of side effects are too complicated to be completely discovered [Litzow, 2008]. Some of the pilot studies have showed that reactive oxygen species (ROS) generation, stress-related MAPK pathways activation, and the suppression of STAT3-dependent cell survival signals may facilitate arsenite-induced cell death events [Darnell, 2002; Levy and Darnell, 2002; Ivanov and Hei, 2004; Pei et al., 2008; Ruiz-Ramos et al., 2009].

The growth arrest and DNA damage-inducible (GADD) 45α is a stress-responsive protein and involved in numerous biological process including cell cycle control, senescence, apoptosis, and nucleotide excision repair [Zhan, 2005; Gao et al., 2009]. Our previous publications as well as data from other groups have disclosed that the strong induction of GADD45α by arsenite plays an important role in mediating the apoptotic response in the mouse embryonic fibroblast cells (MEFs) and the human bronchial epidermal cells [Chen et al., 2001; Song et al., 2006; Zhang et al., 2006]. These results from the normal diploid cells thus implied that GADD45α might also be critical for tumor cell death induced by arsenite exposure. However, some recent reports have challenged the tumor suppressor role of GADD45α by demonstrating the correlation of GADD45α expression to the survival and even progression of certain tumor cells [Yamasawa et al., 2002; Gupta et al., 2006; Fayolle et al., 2008]. Therefore, GADD45α seems to be a

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30871277, 30970594; Grant sponsor: Beijing Natural Science Foundation; Grant numbers: 5092022, 5102035.

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Received 1 October 2009; Accepted 31 December 2009 • DOI 10.1002/jcb.22509 • © 2010 Wiley-Liss, Inc.
Published online 23 February 2010 in Wiley InterScience (www.interscience.wiley.com).

double-edged sword in both tumor suppression and progression [Gao et al., 2009].

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors and is hard for prevention and thoroughly healing [Gish and Baron, 2008]. Previous studies have proved that arsenite is able to induce apoptosis in the rat and human HCC cells [Kito et al., 2002; Zhang et al., 2003], the results of which suggested the potential effectiveness of arsenite in HCC therapy. Here, we showed that moderate to high dose of arsenite stimulation induced apoptosis in the HepG2 human hepatoma cells whereas had little effect on the normal diploid hepatic cells HL7702. The following investigations on the different sensitivities of HepG2 and HL7702 cells to arsenite exposure disclosed the critical role of GADD45α induction in mediating the apoptotic response in HepG2 cells. Moreover, the function of GADD45α was delivered by activating the JNKs/AP-1 signaling pathway, an event which was absent in the HL7702 cells under the same arsenite treatment conditions. Therefore, we have identified the tumor suppressor role of GADD45α in mediating arsenite-induced apoptotic response in the human hepatoma cells, which will be valuable to arsenite-related cancer therapy.

MATERIALS AND METHODS

CELL LINES, ANTIBODIES, PLASMIDS, AND OTHER REAGENTS

The human hepatoma cell line HepG2 was obtained from ATCC (Manassas, VA). The human normal diploid hepatic cell line HL7702 were purchased from Shanghai Institutes for Biological Sciences and provided by Dr. Hui Zhong (Beijing Institute of Biotechnology). The antibodies against phospho-JNKs, JNKs, phospho-c-Jun, and c-Jun were purchased from Cell Signaling Technology (Beverly, MA). The antibodies against GADD45α, Fra-1, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmid containing DN-c-Jun cDNA (TAM67) and the AP-1 luciferase reporter plasmid were described previously [Song et al., 2008]. Arsenic trioxide and the JNKs inhibitor SP600125 were purchased from Sigma (St. Louis, MO).

CELL CULTURE AND TRANSFECTION

The HepG2 and HL7702 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS). All of the stable and transient transfections were performed with Lipofectamine 2000 reagents (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions. For stable transfection, cultures were subjected to G418 drug selection and cells surviving from the selection were pooled as a mass. These stable transfectants were cultured in the selective drug-free medium for at least two passages before subjecting to the according experiments.

WESTERN BLOT ASSAY

Whole cell extracts were prepared with the cell lysis buffer (10 mM Tris–HCl, pH 7.4, 1% SDS, and 1 mM Na_3VO_4). Protein concentrations were determined by the Bio-Rad protein quantification assay kit. Proteins were resolved by SDS–PAGE, probed with the indicated primary antibodies, and then incubated with the HRP-conjugated second antibody. Signals were detected by enhanced chemiluminescence (ECL) Western blotting system.

LUCIFERASE REPORTER ASSAY

Luciferase reporter assay was performed with the Dual-Luciferase Reporter Assay System (Promega, Medison, MI). Briefly, cells were transient transfected with the AP-1-luciferase reporter construct and the Renilla luciferase vector. After 36 h of transfection, the cells were seeded into 96-well plates (1×10^4 per well) and then subjected to arsenite stimulation. Cellular lysates were prepared at the indicated time points and the luciferase activities were determined by Perkin Elmer 1420 Multilabel Counter (Perkin Elmer Inc., Waltham, MA). The results were expressed as relative AP-1 induction which normalized to the control cells without any treatment as described previously [Song et al., 2008].

CONSTRUCTION OF GADD45α AND Fra-1 shRNA EXPRESSION PLASMIDS

The small heparin RNAs (shRNAs) targeting the human GADD45α gene and *Fra-1* gene were designed with the aid of the siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA_finder.html; Ambion Inc., Austin, TX), and the corresponding expression plasmids were constructed using the GeneSuppressor system (Imgenex Co., San Diego, CA). The target sequences of human GADD45α shRNA were 5′-aacgtggtgttgtgcctgctg-3′ and 5′-cgacatcaacatcctgcgcgt-3′. The target sequence of human Fra-1 shRNA was 5′-caccatgagtggcagtcag-3′ [Vial and Marshall, 2003]. The construct containing the GFP sequence (5′-gagagaccacatggtccttct-3′) was used as the control shRNA. The established constructs were transfected into the HepG2 cells for stable expression.

CELL PROLIFERATION ASSAY

Cell proliferation was determined by Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) assay according to the manufacturer's protocol. Briefly, CCK-8 was added to individual cultures and incubated for 1–2 h at 37°C and then the optical density was determined with Perkin Elmer 1420 Multilabel Counter (Perkin Elmer Inc., Waltham, MA). The data were normalized to untreated cultures. The quantitative results were averaged from three independent experiments.

CELL CYCLE DISTRIBUTION AND APOPTOSIS ASSAY

Cell cycle distribution and cell death induced by arsenite exposure were determined by flow cytometric analysis following propidium iodide (PI) staining of the nuclei as described previously [Song et al., 2006, 2008].

RESULTS

ARSENITE EXPOSURE SPECIFICALLY INDUCES GROWTH ARREST AND APOPTOSIS OF THE HEPATOMA CELLS WHEREAS HAS LITTLE EFFECT ON THE NORMAL DIPLOID HEPATIC CELLS

To evaluate the biological effects of arsenite on the human hepatoma cells, we first compared the changes on the cell cycle distribution of HepG2 cells before and after arsenite stimulation. As shown in Figure 1A, arsenite treatment induced a dose-dependent growth arrest response at both G2/M and S phase boundaries 24 h after exposure. In line with this response, the proliferation of HepG2 cells was effectively inhibited by arsenite; under moderate to high

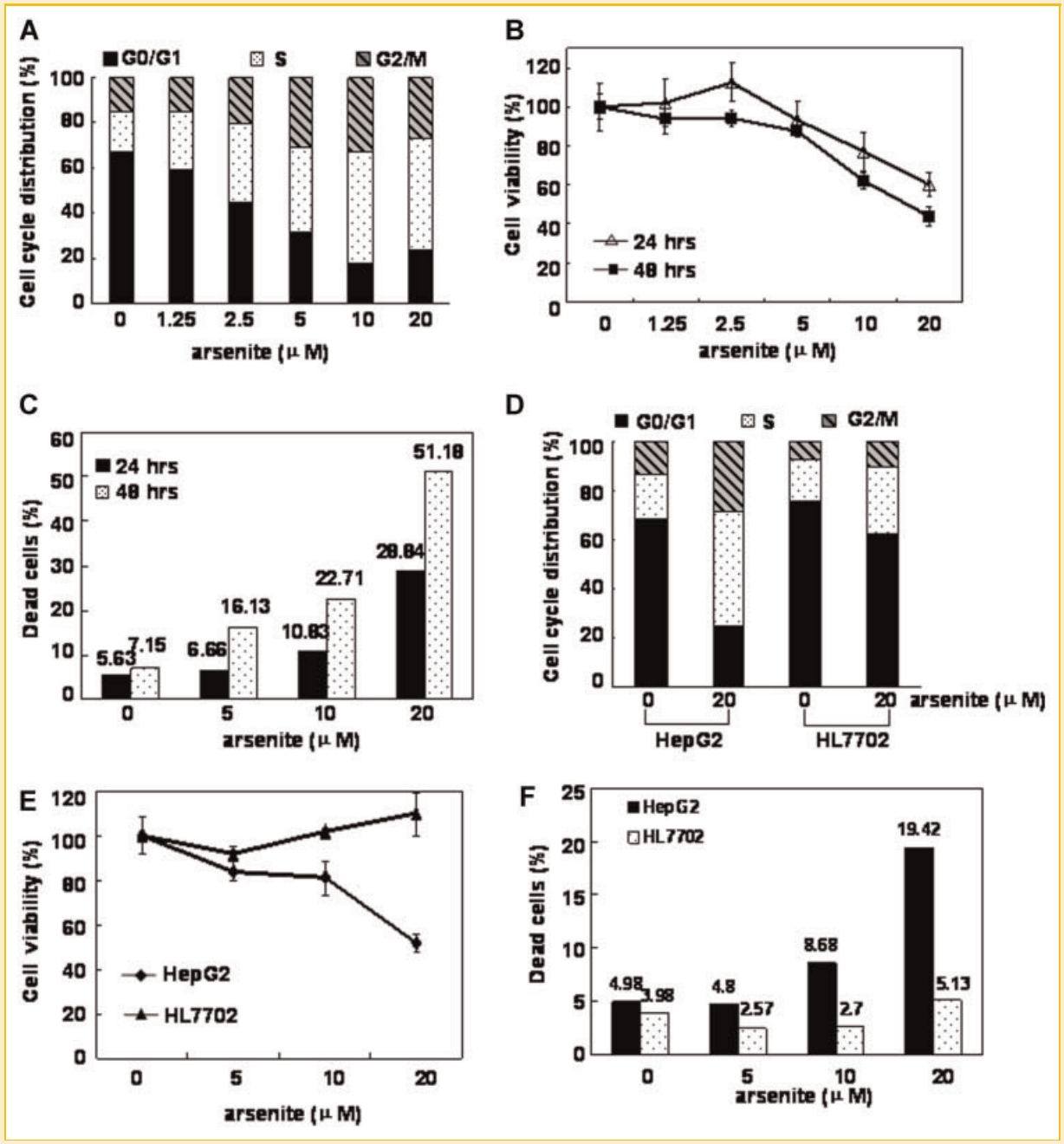

Fig. 1. HepG2 cells are more sensitive to arsenite exposure than HL7702 cells. A: HepG2 cells were treated with different doses of arsenite and the cell cycle distribution was detected by flow cytometric analysis 24 h after stimulation. Data from one representative experiment is shown. B: HepG2 cells were treated with different doses of arsenite and cell proliferation was detected by CCK-8 assay at the time points indicated. Results are presented as mean ± SD from three independent experiments. C: HepG2 cells were treated with different doses of arsenite and then the cell death incidence was detected by flow cytometric analysis following PI staining of the nuclei at the time points indicated. D: HepG2 and HL7702 cells were treated with 20 μM of arsenite and the cell cycle distribution was analyzed as described in (A) 24 h after exposure. E: HepG2 and HL7702 cells were treated with different doses of of arsenite and cell proliferation was detected as described in (B) 48 h after exposure. F: HepG2 and HL7702 cells were treated with different doses of arsenite and the cell death incidence was detected as described in (C) 24 h after exposure.

concentration of arsenite stimulation (10–20 μM), only 40–60% of HepG2 cells remained viable 48 h after the treatment (Fig. 1B). Moreover, arsenite exposure was also able to induce a remarkable dose- and time-dependent increase of cell death in the HepG2 cells (Fig. 1C). Similar effect of arsenite was further confirmed in two additional human hepatoma cell lines (data not shown). These results together indicate that arsenite could inhibit the proliferation, trigger the growth arrest and apoptosis of the human hepatoma cells.

To address whether arsenite could exert a specific killing effect on the hepatoma cells, we compared the response of HepG2 and the normal human diploid hepatic cells HL7702 under the same aresnite stimulation conditions in the following study. As shown in

Figure 1D–F, HL7702 cells exhibited obvious insensitivity to arsenite exposure compared with HepG2 cells, evidenced by no obvious alteration of the cell proliferation rate, cell cycle distribution, and the percentage of apoptotic cells under the same doses of arsenite stimulation. These data together indicate that arsenite exposure can specifically induce growth arrest and apoptosis of the hepatoma cells whereas has little effect on normal diploid hepatic cells. The finding that diploid hepatic cells tolerate more toxicity of arsenite than the hepatoma cells may provide evidence of arsenite functioning as the effective tumor therapeutic agent with high specificity.

GADD45α IS THE CRITICAL MEDIATOR OF THE APOPTOTIC RESPONSE IN THE HepG2 CELLS INDUCED BY ARSENITE STIMULATION

Our previous studies have demonstrated that GADD45α played a key role in the arsenite-induced apoptotic response in mouse embryonic fibroblast cells [Song et al., 2006]. However, some recent reports have disclosed the correlation of GADD45α expression to the survival and even progression of certain human tumor cells [Yamasawa et al., 2002; Gupta et al., 2006; Fayolle et al., 2008]. Therefore, it is interesting to address whether GADD45α can exert a conservatively pro-apoptotic function in the arsenite-treated hepatoma cells or antagonize the apoptotic response and therefore contribute to the hepatoma cell survival under arsenite exposure. To address this question, we next detected the expression levels of GADD45α in the HepG2 cells under arsenite stimulation. As shown in Figure 2A, by treatment of moderate to high doses of arsenite, a strong and persistent induction of GADD45α expression was observed in the HepG2 cells, correlating with cell growth arrest and apoptosis under the same condition (Fig. 1A–C). To further reveal the significance of GADD45α induction in the HepG2 cells, we designed a pair of shRNAs that targeted two different regions on the GADD45α mRNA, and then the stable HepG2/shRNA transfectants were established. The effectiveness of these two shRNAs on the attenuation of arsenite-induced GADD45α expression was confirmed by Western blot assay (Fig. 2B). Under the same conditions, the apoptotic response in HepG2 cells was also significantly suppressed by both two GADD45α shRNA compared with the control GFP shRNA (Fig. 2C). These data together indicate that the

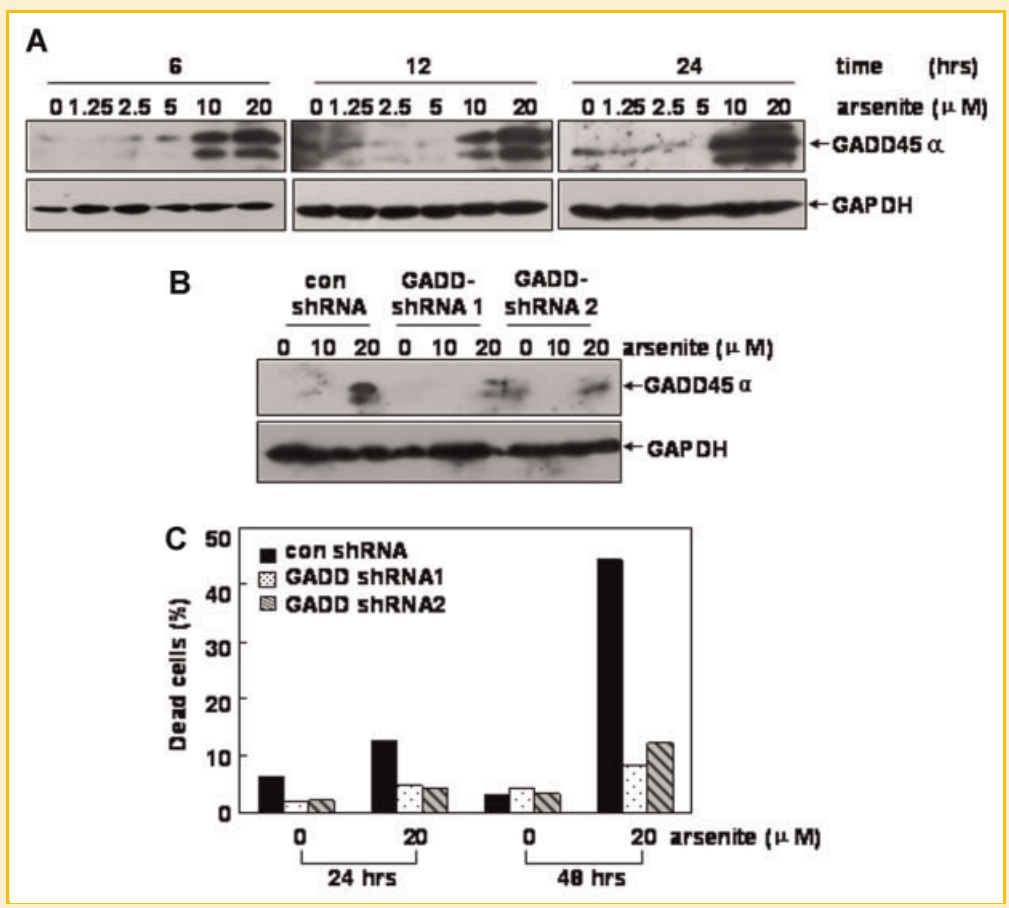

Fig. 2. Induced expression of GADD45α is critical for mediating arsenite-induced cell death response. A: HepG2 cells were treated with different doses of arsenite and the expression of GADD45α was detected by Western blot assay at the time points indicated. B: HepG2 cells were transfected with GADD45α-specific shRNA constructs or the control GFP shRNA construct and then the stable transfectants were established. The transfectants were treated with different doses of arsenite and then the expression of GADD45α was detected 12 h after exposure. C: HepG2 cells with the stable transfection of GADD45α shRNA constructs or the control shRNA construct were subjected to arsenite exposure (20 μM) and then the cell death incidence was detected at the indicated time points.

induced expression of GADD45α by arsenite plays a critical role in triggering the apoptosis of HepG2 cells under the arsenite stress condition.

JNKs FUNCTION AS THE DOWNSTREAM TARGET OF GADD45α IN ARSENITE RESPONSE IN THE HepG2 CELLS

One of the well-accepted mechanisms of GADD45α in mediation of the stress response is delivered by MEKK4-depedent JNKs activation [Miyake et al., 2007]. Therefore, we proposed that JNKs might also function as the downstream target of GADD45α and mediate arsenite-induced response in the human hepatoma cells. To address this possibility, we first detected the activation status of JNKs in response to arsenite stimulation in the HepG2 cells. As expected, persistent JNK phosporylation (from 6 to 24 h) was observed in HepG2 cells by the treatment of 10–20 μM of arsenite (Fig. 3A), reminiscent of the GADD45α induction under the same doses of arsenite stimulation (Fig. 2A). These data thus indicate the potential role of JNKs involved in the GADD45α-mediated apoptotic response induced by arsenite. Therefore, in the following study, we compared the activation status of JNK activation in the presence or absence of GADD45α induction in the arsenite-treated HepG2 cells. As shown in Figure 3B, when GADD45α induction was specifically suppressed by ectopic overexpression of GADD45α shRNA, the induced activation of JNKs was also dramatically inhibited under the same conditions. This result indicates that JNKs function as the downstream target of GADD45α in the HepG2 cells under arsenite stimulation. Next, we further confirmed the requirement of JNK activation for mediating arsenite-induced apoptotic response in the HepG2 cells by using the specific JNK inhibitor II, SP600125. As shown in Figure 3C and D, pretreatment of the HepG2 cells with SP600125 nearly abolished JNK activation, accompanied by a substantial decrease of cell apoptosis under the same arsenite

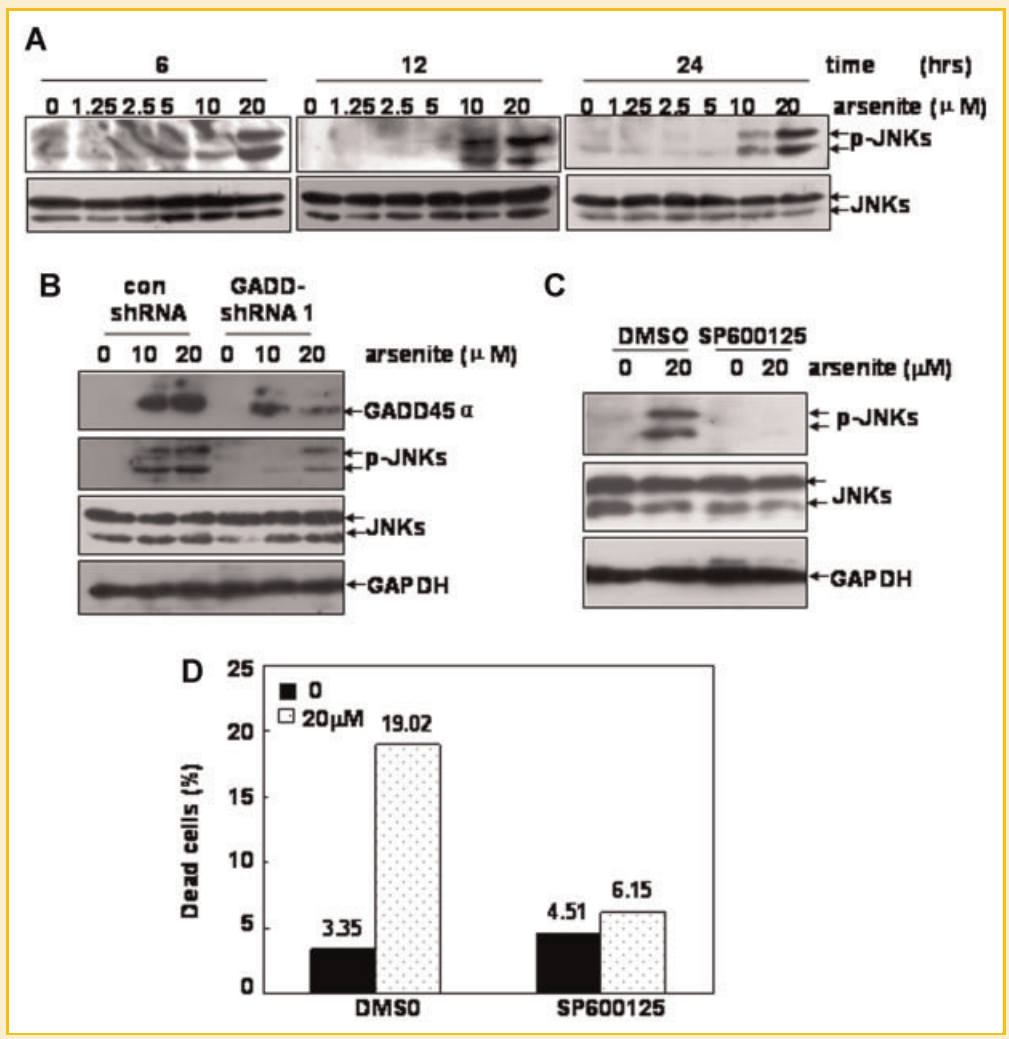

Fig. 3. JNK is the downstream target of GADD45α to mediate arsenite-induced response in the HepG2 cells. A: HepG2 cells were treated with different doses of arsenite and the induction of JNK activation was detected by Western blot assay at the time points indicated. B: HepG2 cells with the stable transfection of GADD45α shRNA constructs or the control shRNA construct were subjected to different doses of arsenite exposure and then the induction of JNK activation was detected 12 h after exposure. C: HepG2 cells were pretreated with SP600125 (20 μM) or its vehicle (DMSO) for 2 h and then subjected to arsenite exposure (20 μM). JNK activation status was detected by Western blot assay 12 h after arsenite treatment. D: HepG2 cells were treated as described in (C) and then cell death incidence was determined 24 h after arsenite exposure.

treatment conditions. Therefore, we conclude that GADD45α mediates arsenite-induced apoptotic response in the HepG2 cells by triggering JNK activation.

AP-1 IS THE DOWNSTREAM EFFECTOR OF JNKs IN MEDIATING ARSENITE-INDUCED APOPTOTIC RESPONSE IN THE HepG2 CELLS

The transcriptional factor AP-1 is one of the most important downstream targets of JNKs under the stress conditions, which are composed of Jun (c-Jun, Jun B, and Jun D), Fos (c-Fos, Fos B, Fra-1, Fra-2) and ATF (ATF-2, ATF-3, B-ATF) protein subfamilies. The diverse combination of the AP-1 components gives rise to multiple AP-1 dimeric pairs with different biological functions [Ashida et al., 2005]. Data from our previous studies have disclosed that c-Jun, Fra-1, and ATF-2 are three major AP-1 components in the MEFs to mediate the arsenite response [Song et al., 2008]. To disclose whether these AP-1 components and AP-1 transactivation also exert the critical roles in the arsenite-induced apoptotic response in the hepatoma cells, we next transfected the AP-1 luciferase reporter plasmid into the HepG2 cells and then the induced AP-1 transactivation was detected under arsenite exposure. As shown in Figure 4A, persistent increase of AP-1 transactivity was observed in the HepG2 cells induced by different doses of arsenite exposure. Under moderate to high concentrations of arsenite (10–20 μM) stimulation, we simultaneously detected a remarkable phosphorylation of c-Jun and induction of Fra-1 expression in the HepG2 cells being accompanied with significant AP-1 transactivation, which was consistent with the previous data, obtained from the MEF cells. However, although induction of ATF2 activation also has been proved as the one of the critical events followed by JNKs activation in the arsenite-treated MEF cells [Song et al., 2008], we did not observe similar phenomena in HepG2 cells under arsenite treatment (data not shown). Furthermore, pretreatment of HepG2 cells with SP600125 dramatically suppressed AP-1 transactivation (Fig. 4C) as well the induction of c-Jun and Fra-1 phosphrylation/expression (Fig. 4D). Therefore, these data together indicate that c-Jun and Fra-1 are the major AP-1 components that are involved in JNKs-dependent cell apoptotic response in the arsenite-treated HepG2 cells.

Usually, Jun proteins can homodimerize or heterodimerize with ATF or Fos family proteins; whereas Fos proteins do not form stable homodimers but prefer forming heterodimers with Jun proteins that are more stable than Jun:Jun dimmers [Ferrer et al., 2000; Ashida

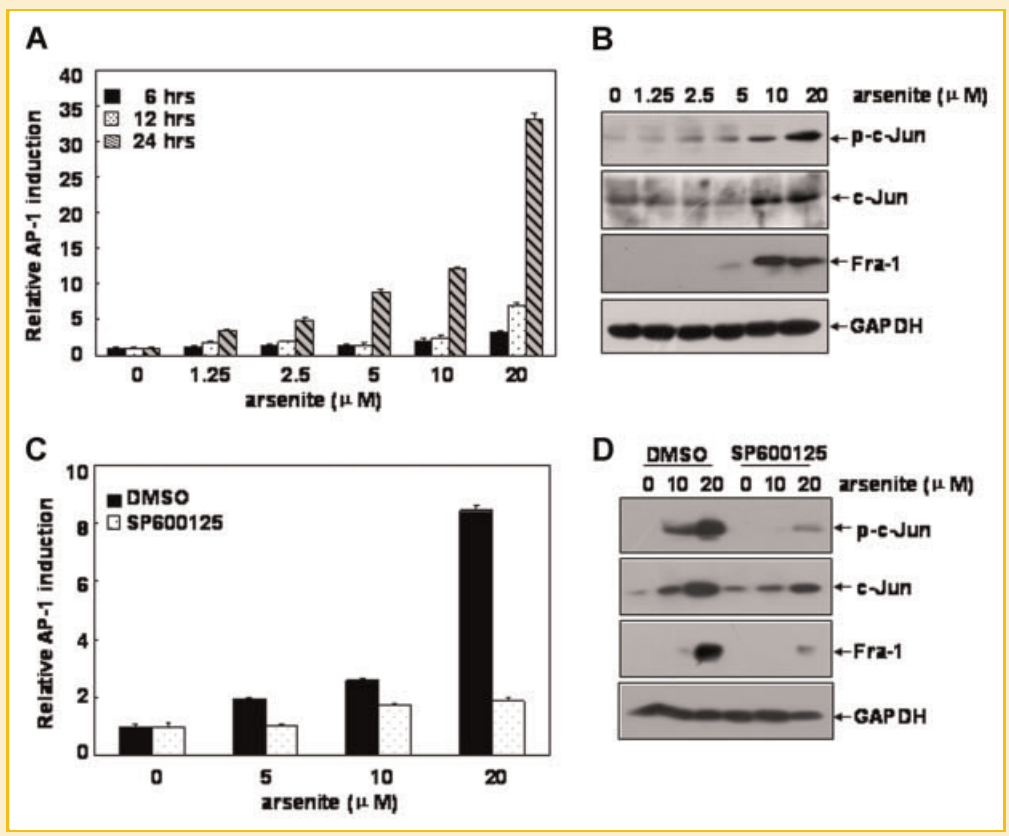

Fig. 4. AP-1 functions as the downstream mediator of JNKs in the arsenite-induced cell death response in the HepG2 cells. A: HepG2 cells were transfected with the AP-1 luciferase reporter plasmid and then treated with different doses of arsenite 36 h after transfection. Transactivation of AP-1 was detected at the time points indicated and the results were presented as the relative AP-1 induction which normalized to the control cells without any treatment. B: HepG2 cells were treated with different doses of arsenite for 12 h and the induced activation or expression of c-Jun and Fra-1 were detected by Western blot assay. C: HepG2 cells were transfected with the AP-1 luciferase reporter plasmid. Thirty-six hours after transfection, the cells were pretreated with SP600125 or DMSO for 2 h, and then subjected to different doses of arsenite exposure. AP-1 luciferase activities were then detected 12 h after arsenite exposure. D: HepG2 cells were pretreated with SP600125 or DMSO for 2 h and then subjected to different doses of arsenite exposure. The induced activation or expression of c-Jun and Fra-1 were detected by Western blot assay 12 h after arsenite exposure.

et al., 2005]. Therefore, we proposed that the functional AP-1 induced by arsenite stimulation in the HepG2 cells might consist of the c-Jun:c-Jun homodimer or the c-Jun:Fra-1 heterodimer. To further assess the role of AP-1 transactivation in arsenite-induced apoptotic response in the HepG2 cells, we next transfected a dominant negative c-Jun mutant (TAM67) into the HepG2 cells and the stable transfectant was then established. The efficiency of TAM67 in blocking arsenite-induced c-Jun activation was confirmed by Western blotting assay (Fig. 5A). As expected, overexpression of TAM67 significantly inhibited arsenite-induced AP-1 transactivation compared with the vector control transfected cells (Fig. 5A). Under the same conditions, arsenite-induced cell death incidence was also partially attenuated in the TAM67-transfected cells (Fig. 5B). These data thus have confirmed the critical role of c-Jun in mediating AP-1 transactivation and cell apoptotic response in the arsenite-treated HepG2 cells. In the following study, we also examined the contribution of Fra-1 induction to AP-1 transactivation and cell apoptotic response in the arsenite-treated HepG2 cells by ectopic overexpression of Fra-1 shRNA. As shown in Figure 5C and D, When Fra-1 induction was specifically reduced by Fra-1 shRNA transfection, AP-1 transactivation as well as cell apoptotic response induced by arsenite were partially inhibited under the same conditions. Taken together, these data indicate that AP-1 functions as the downstream effector of JNKs in mediating the arsenite-induced apoptotic response in the HepG2 cells.

THE SIGNALING EVENT OF GADD45α/JNKs/AP-1 CELL DEATH PATHWAY ACTIVATION IN THE ARSENITE-TREATED HepG2 CELLS WAS ABSENT IN THE NORMAL HEPATIC HL7702 CELLS

These data have demonstrated that GADD45α plays a critical role in the mediation of arsenite-induced cell apoptotic effect in the HepG2 cells via activation of the JNKs/AP-1 pathway. To address whether GADD45α/JNKs/AP-1 signaling cascade activation contributes to the different sensitivities of the hepatoma and normal hepatic cells to the arsenite stimulation, we next compared the activation status of this pathway in the HepG2 and HL7702 cells under the same doses of arsenite treatment. As shown in Figure 6A, all of the signaling events observed in the arsenite-treated HepG2 cells, including induced expression of GADD45α, JNKs activation as well as c-Jun and Fra-1 phoshorylation/induction, were totally absent in the

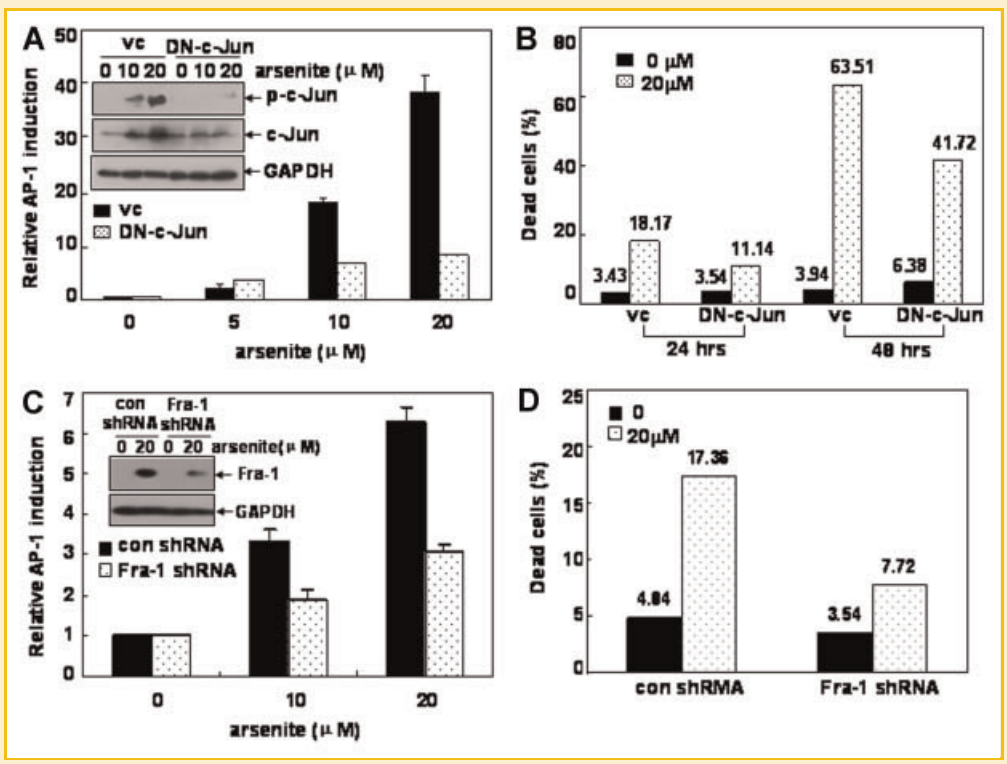

Fig. 5. Suppressing AP-1 transactivation reduces apoptotic response in the arsenite-treated HepG2 cells. A: HepG2 cells were transfected with the dominant-negative mutant of c-Jun (TAM67) or its vector control and then the stable transfectants were selected. The transfectants were treated with different doses of arsenite for 12 h and the activation of c-Jun was detected by Western blot assay. Then, AP-1 luciferase reporter plasmid was transiently introduced into the HepG2 cells with the TAM67 or the control vector stable transfection. The cells were treated with different doses of arsenite 36 h after transfection and AP-1 transactivation was detected 12 h after exposure. B: HepG2 cells with the TAM67 stable transfection or the control cells were left untreated or treated with arsenite (20 μM) and then the cell death incidence was analyzed at the indicated time points. Data from one representative experiment is shown. C: HepG2 cells were transfected with the Fra-1 shRNA or the control GFP shRNA. After 36 h of transfection, the cells were treated with different doses of arsenite and the induction of Fra-1 was detected 12 h after arsenite exposure. Then, HepG2 cells were co-transfected with the AP-1 luciferase reporter plasmid and the Fra-1 shRNA or the control GFP shRNA. AP-1 luciferase activities were detected as described in Figure 4A. D: HepG2 cells transfected with the Fra-1 shRNA or the control GFP shRNA were left untreated or treated with arsenite (20 μM) and then the cell death incidence was analyzed 24 h after arsenite exposure. Data from one representative experiment is shown.

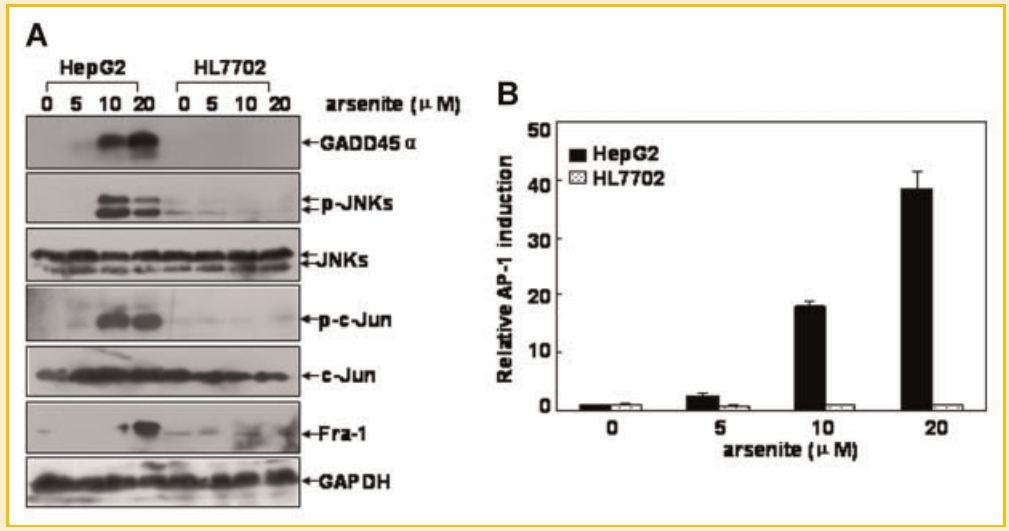

Fig. 6. GADD45α-dependent JNKs/AP-1 cell death pathway is absent in the normal hepatic cell HL7702 under arsenite exposure. A: HepG2 and HL7702 cells were treated with different doses of arsenite for 12 h and the induced activation or expression of JNKs, c-Jun, Fra-1, and GADD45α were detected by Western blot assay. B: HepG2 and HL7702 cells were transiently transfected with the AP-1 luciferase reporter plasmid and then treated with different doses of arsenite for 36 h after transfection. Transactivation of AP-1 was detected 24 h later and the results were presented as the relative AP-1 induction which normalized to the control cells without any treatment.

HL7702 cells under the same conditions. Moreover, arsenite exposure failed to induce AP-1 transactivation in the HL7702 cells, either (Fig. 6B). Therefore, we conclude that GADD45α/JNKs/AP-1 pathway plays an important role in dictating the apoptosis of the hepatoma cells under arsenite exposure; whereas the deficiency of this pathway activation renders insensitivity of the normal hepatic cells to the chemotherapeutic action of arsenite.

DISCUSSION

Arsenite has a long history in treating leukemia, which might be also effective in the therapy of other cancers. Understanding the mechanism associated with the sensitivity of the various tumor cells to arsneite-induced apoptosis is valuable in improving the clinical usage of this chemotherapeutic agent [Litzow, 2008]. In this study, we focused on investigating the validity of arsenite in treating solid tumors by using the hepatoma cell lines as the model. Our data have provided the evidence that arsenite exposure is able to effectively induce apoptosis in the HepG2 (Fig. 1) and other two human hepatoma cell lines (SMMC7721 and LM6, data not shown), the results of which are consistent with the previous studies obtained from the rat and human hepatocellular carcinoma cells [Kito et al., 2002; Zhang et al., 2003]. However, the cytotoxity of arsenite was neither observed in the normal diploid hepatic cell line HL7702 (Fig. 1) nor LO_2 (data not shown) under the same conditions. Thus, the specific killing effect of arsenite on the hepatoma cells without side impact on the normal hepatic cells might provide the important molecular bases to enable arsenite to be potentially subjected to the treatment of human hepatoma.

GADD45α is a stress-responsive protein which can be induced by a wide range of DNA-damaging agents or growth arrest signals. The induced expression of this protein has been proved to be involved in a variety of biological functions including apoptosis [Zhan, 2005]. Our previous investigation on the molecular mechanism related to the pro-apoptotic effect of arsenite has demonstrated that GADD45α induction is the critical signaling event for mediating arsenite-induced apoptotic response in the mouse fibroblast cells [Song et al., 2006; Zhang et al., 2006]. The data obtained from this study further confirmed the function of GADD45α in mediating human hepatoma cell apoptosis in response to arsenite stimulation (Fig. 2). In addition, we also observed the cytotoxicity of arsenite in the other two human hepatoma cell lines, human cervical cancer cell line and the breast cancer cell line, the effects of which were also mediated by GADD45α accumulation (data not shown). Therefore, GADD45α seems to exert a conservative role in the cell death response in the arsenite-treated tumor cells. Most importantly, the induced expression of GADD45α was absent in HL7702 (Fig. 2B) and LO_2 cells (data not shown), which conferred the resistance to the arsenite-induced apoptotic effect in these two hepatic cells. From these data, we conclude that GADD45α plays a key role in delivering the pro-apoptotic effect of arsenite selectively in the hepatoma cells. The approaches to sensitize human hepatoma to arsenite-induced apoptosis through up-regulating GADD45α induction might be promising in HCC therapy.

One of the mechanisms of GADD45α in delivering the stress signals is through the activation of MEKK4-depedent MAPK pathways [Miyake et al., 2007]. The critical role of JNKs in mediating arsenite-induced apoptotic responses in the epithermal and fibroblastic cells has been demonstrated in the previous reports [Huang et al., 1999; Song et al., 2006; Zhang et al., 2006]. Data in this study have disclosed that JNKs also function as the major MAPK family members under the downstream of GADD45α in mediating arsenite response in the HepG2 cells, evidenced by the identical kinetics of JNK activation and GADD45α induction in response to

the same doses of arsenite exposure as well as the suppression of JNK activation in the GADD45α shRNA-transfected HepG2 cells (Fig. 3). In fact, we have also observed the GADD45α-regulated p38K and ERK activation in the HepG2 cells under high doses of arsenite stimulation. However, in contrast to the persistent GADD45α induction and JNKs activation, only transient phosphorylation of p38K and ERKs can be observed in arsenite-treated HepG2 cells (data not shown). Therefore, the contribution of p38K and ERKs to the arsenite-induced cytotoxic effect needs further investigation. Usually, JNKs mediate cell apoptotic effect via both the intrinsic and extrinsic cell death pathways [Dhanasekaran and Reddy, 2008]. Data in this study have proved that dysfunction of c-Jun and Fra-1-dependent AP-1 transactivation functions as the effector to execute cell death dictation in the arsenite-treated HepG2 cells (Fig. 4A,B). However, owing to partial inhibition of cell apoptotic effect in either DN-c-Jun or Fra-1 shRNA-transfected HepG2 cells compared with the control group, it is reasonable to speculate that signaling events other than AP-1-delivered extrinsic cell death pathway might be also involved in arsenite-induced apoptotic response in the HepG2 cells. The previous studies on the sensitivity of APL cells to aresnite exposure have reported the apoptotic response relating to the generation of hydrogen peroxide (H_2O_2) and ROS, which might deliver the cell damage effect by deregulating the mitochondrial activity [Ruiz-Ramos et al., 2009]. Therefore, whether dysfunction of mitochondria also contributes to GADD45α-mediated apoptotic response in the hepatoma cells is currently under investigation.

In summary, we have for the first time disclosed the selective efficacy of arsenite in triggering apoptosis in the human hepatoma while not in the normal hepatic cells. The specific activation of GADD45α-dependent JNKs/AP-1 pathway is the critical signaling event to mediate the anti-tumor effect of arsenite on the hepatoma cells. Further elucidation of the mechanism involving GADD45α-dependent cytotoxicity might be valuable in sensitizing the hepotoma to undergo apoptosis induced by arsenite treatment.

REFERENCES

Ashida R, Tominaga K, Sasaki E, Watanabe T, Fujiwara Y, Oshitani N, Higuchi K, Mitsuyama S, Iwao H, Arakawa T. 2005. AP-1 and colorectal cancer. Inflammopharmacology 13:113–125.

Chen GQ, Shi XG, Tang W, Xiong SM, Zhu J, Cai X, Han ZG, Ni JH, Shi GY, Jia PM, Liu MM, He KL, Niu C, Ma J, Zhang P, Zhang TD, Paul P, Naoe T, Kitamura K, Miller W, Waxman S, Wang ZY, de The H, Chen SJ, Chen Z. 1997. Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL): I. As_2O_3 exerts dose-dependent dual effects on APL cells. Blood 89:3345–3353.

Chen F, Lu Y, Zhang Z, Vallyathan V, Ding M, Castranova V, Shi X. 2001. Opposite effect of NF-kappa B and c-Jun N-terminal kinase on p53-independent GADD45 induction by arsenite. J Biol Chem 276:11414–11419.

Darnell JE, Jr. 2002. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2:740–749.

Dhanasekaran DN, Reddy EP. 2008. JNK signaling in apoptosis. Oncogene 27:6245–6251.

Fayolle C, Pourchet J, de Fromentel CC, Puisieux A, Dore JF, Voeltzel T. 2008. Gadd45a activation protects melanoma cells from ultraviolet B-induced apoptosis. J Invest Dermatol 128:196–202.

Ferrer I, Pozas E, Planas AM. 2000. c-Jun/AP-1 (N) expression and apoptosis. Neuroscience 96:447–448.

Gao M, Guo N, Song L. 2009. Diverse roles of GADD45α in stress signaling. Curr Protein Pept Sci 10:388–394.

Gish RG, Baron A. 2008. Hepatocellular carcinoma (HCC): Current and evolving therapies. IDrugs 11:198–203.

Gupta M, Gupta SK, Hoffman B, Liebermann DA. 2006. Gadd45a and Gadd45b protect hematopoietic cells from UV-induced apoptosis via distinct signaling pathways, including p38 activation and JNK inhibition. J Biol Chem 281:17552–17558.

Huang C, Ma WY, Li J, Dong Z. 1999. Arsenic induces apoptosis through a c-Jun NH2-terminal kinase-dependent, p53-independent pathway. Cancer Res 59:3053–3058.

Ivanov VN, Hei TK. 2004. Arsenite sensitizes human melanomas to apoptosis via tumor necrosis factor alpha-mediated pathway. J Biol Chem 279:22747–22758.

Kito M, Akao Y, Ohishi N, Yagi K, Nozawa Y. 2002. Arsenic trioxide-induced apoptosis and its enhancement by buthionine sulfoximine in hepatocellular carcinoma cell lines. Biochem Biophys Res Commun 291:861–867.

Levy DE, Darnell JE, Jr. 2002. Stats: Transcriptional control and biological impact. Nat Rev Mol Cell Biol 3:651–662.

Li X, Ding X, Adrian TE. 2003. Arsenic trioxide induces apoptosis in pancreatic cancer cells via changes in cell cycle, caspase activation, and GADD expression. Pancreas 27:174–179.

Litzow MR. 2008. Arsenic trioxide. Expert Opin Pharmacother 9:1773–1785.

Miyake Z, Takekawa M, Ge Q, Saito H. 2007. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans autophosphorylation of the MTK1 kinase domain. Mol Cell Biol 27:2765–2776.

Ora I, Bondesson L, Jonsson C, Ljungberg J, Porn-Ares I, Garwicz S, Pahlman S. 2000. Arsenic trioxide inhibits neuroblastoma growth in vivo and promotes apoptotic cell death in vitro. Biochem Biophys Res Commun 277:179–185.

Pei B, Wang S, Guo X, Wang J, Yang G, Hang H, Wu L. 2008. Arsenite-induced germline apoptosis through a MAPK-dependent, p53-independent pathway in *Caenorhabditis elegans*. Chem Res Toxicol 21:1530–1535.

Rousselot P, Labaume S, Marolleau JP, Larghero J, Noguera MH, Brouet JC, Fermand JP. 1999. Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. Cancer Res 59:1041–1048.

Ruiz-Ramos R, Lopez-Carrillo L, Rios-Perez AD, De Vizcaya-Ruiz A, Cebrian ME. 2009. Sodium arsenite induces ROS generation, DNA oxidative damage, HO-1 and c-Myc proteins, NF-kappaB activation and cell proliferation in human breast cancer MCF-7 cells. Mutat Res 674:109–115.

Song L, Li J, Zhang D, Liu ZG, Ye J, Zhan Q, Shen HM, Whiteman M, Huang C. 2006. IKKbeta programs to turn on the GADD45alpha-MKK4-JNK apoptotic cascade specifically via p50 NF-kappaB in arsenite response. J Cell Biol 175:607–617.

Song L, Li J, Hu M, Huang C. 2008. Both IKKalpha and IKKbeta are implicated in the arsenite-induced AP-1 transactivation correlating with cell apoptosis through NF-kappaB activity-independent manner. Exp Cell Res 314:2187–2198.

Taylor BF, McNeely SC, Miller HL, Lehmann GM, McCabe MJ, Jr., States JC. 2006. p53 suppression of arsenite-induced mitotic catastrophe is mediated by p21CIP1/WAF1. J Pharmacol Exp Ther 318:142–151.

Uslu R, Sanli UA, Sezgin C, Karabulut B, Terzioglu E, Omay SB, Goker E. 2000. Arsenic trioxide-mediated cytotoxicity and apoptosis in prostate and ovarian carcinoma cell lines. Clin Cancer Res 6:4957–4964.

Vial E, Marshall CJ. 2003. Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells. J Cell Sci 116:4957–4963.

Yamasawa K, Nio Y, Dong M, Yamaguchi K, Itakura M. 2002. Clinicopathological significance of abnormalities in Gadd45 expression and its relationship to p53 in human pancreatic cancer. Clin Cancer Res 8:2563–2569.

Zhan Q. 2005. Gadd45a, a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage. Mutat Res 569:133–143.

Zhang TC, Cao EH, Li JF, Ma W, Qin JF. 1999. Induction of apoptosis and inhibition of human gastric cancer MGC-803 cell growth by arsenic trioxide. Eur J Cancer 35:1258–1263.

Zhang TD, Chen GQ, Wang ZG, Wang ZY, Chen SJ, Chen Z. 2001. Arsenic trioxide, a therapeutic agent for APL. Oncogene 20:7146–7153.

Zhang T, Wang SS, Hong L, Wang XL, Qi QH. 2003. Arsenic trioxide induces apoptosis of rat hepatocellular carcinoma cells in vivo. J Exp Clin Cancer Res 22:61–68.

Zhang D, Song L, Li J, Wu K, Huang C. 2006. Coordination of JNK1 and JNK2 is critical for GADD45alpha induction and its mediated cell apoptosis in arsenite responses. J Biol Chem 281:34113–34123.