**Arsenic Induces Apoptosis through a c-Jun NH$_2$-Terminal Kinase-dependent, p53-independent Pathway**

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**Abstract**

Arsenic has been used as an effective chemotherapy agent for some human cancers, such as acute promyelocytic leukemia. In this study, we found that arsenic induces activation of c-Jun NH$_2$-terminal kinases (JNKs) at a similar dose range for induction of apoptosis in JB6 cells. In addition, we found that arsenic did not induce p53-dependent transactivation. Similarly, there was no difference in apoptosis induction between cells with p53 +/− or p53 −/−. In contrast, arsenic-induced apoptosis was almost totally blocked by expression of a dominant-negative mutant of JNK$_3$. These results suggest that the activation of JNKs is involved in arsenic-induced apoptosis of JB6 cells. Taken together with previous findings that p53 mutations are involved in ~50% of all human cancers and nearly all chemotherapy agents kill cancer cells mainly by apoptotic induction, we suggest that arsenic may be a useful agent for the treatment of cancers with p53 mutation.

**Introduction**

Chronic exposure to inorganic arsenicals through contaminated drinking water, medical or other occupational exposure, is associated with increased risk of human cancers of the skin, lung, kidney, bladder, liver, and hematopoietic system (1, 2). Less severe dermatological effects, including hyperpigmentation and hyperkeratosis, appear on the palms and soles after arsenic exposure (3). Epidemiological studies in humans have suggested that the cancer risk (~1%) due to arsenic in the drinking water of the United States may be comparable with that of environmental tobacco smoke and radon in homes (4). Interestingly, arsenic-containing compounds have been used for treatment of cancer for hundreds of years in both Western and traditional Chinese medicine (5–10). Arsenite was routinely used to control elevated leukocyte counts in chronic myelogenous leukemia in the early 1900s (6, 7). Recently, arsenic trioxide (As$_2$O$_3$) was reported to induce complete remission in a high proportion of patients with refractory acute promyelocytic leukemia (3). Reports from in vitro studies using NB$_3$ cells demonstrated that arsenite-induced apoptosis correlated with down-regulation of Bcl-2 (8–10). Therefore, although arsenic is a known carcinogen, it is also an agent that may be useful as a chemotherapeutic agent in cancer treatment.

We have reported recently that arsenic exposure in a mouse epidermal cell line induces cell transformation at only low concentration (<25 µM) but not at higher concentration (>50 µM; Ref. 11). Interestingly, higher concentrations of arsenic are required for activation of JNKs, whereas activation of Erks occurs broadly at doses ranging from 3.2 to 200 µM (11). More importantly, only activation of Erks and not JNKs is required to induce cell transformation, as determined by using both dominant-negative Erk$_1$ and JNK$_1$ (11). These findings, taken together with the observation that arsenite exposure can induce apoptosis without differentiation in both t-RA-sensitive and t-RA-resistant promyelocytes (12–14), raise intriguing questions about the role of activation of JNKs in cancer cell apoptosis. Therefore, this study was initiated to examine the role of JNKs and p53 in arsenic-induced apoptosis.

**Materials and Methods**

**Reagents.** Both arsenic and arsenate were obtained from Sigma Chemical Co.; Klenow FragEL kit was from Oncogene Research Products, Calbiochem; EGF was from Collaborative Research; luciferase assay substrate was from Promega; FBS, MEM, DMEM, RPMI 1640, and BME were from BioWhittaker; Phosphoplus MAPK antibody kit and SAPK/JNK assay kit were purchased from New England Biolabs.

**Cell Culture.** JB6 P$_3$ mouse epidermal cell line CI 41 and its stable p53 luciferase reporter plasmid transfect, CI 41 p53 cells, as well as dominant-negative JNK$_3$ and its vector stable transfectants, CI 41 DN JNK$_3$ mass 1 and CI 41 CMV-neo mass 2, were cultured in monolayers at 37°C, under 5% CO$_2$ using Eagle’s minimal essential medium containing 5% FCS, 2 mM L-glutamine, and 25 µg of gentamicin per ml (11, 15). Normal embryo fibroblasts (p53 +/+ ) or p53-deficient embryo fibroblasts (p53 −/− ) were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and 25 mg of gentamicin per ml (16, 17).

**Anchorage-independent Transformation Assay.** Inhibition of arsenic on EGF-induced cell transformation was investigated in JB6 CI 41 cells. Cells (1 × 10$^5$) were exposed to EGF (10 ng/ml) with or without different concentrations of arsenic in 0.33% BME agar containing 10% FBS over 3.5 ml of DMEM with 10% FBS, 2 mM L-glutamine, and 25 mg of gentamicin per ml (16, 17).

**Assay for p53-dependent Transcription Activity.** The CI 41 p53 cell line stably expresses a luciferase gene under the control of p53 binding sequences (PG13; Ref. 16, 19). The cells were trypsinized, and 8 × 10$^4$ viable cells in 100 µl of 5% FBS MEM were added into each well of a 96-well plate. The cells were incubated at 37°C in a humidified atmosphere of 5% CO$_2$. Twelve to 24 h later, cells were starved by culturing in 0.1% FBS MEM for 12 h. The cells were then exposed to UVC (60 J/m$^2$) or different concentrations of arsenic for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured with a luminometer (Monolight 2010). Results are expressed as relative p53-dependent transcription activity (16, 19).

**DNA Fragment End Labeling Assay.** The cells were cultured on microscope slides, treated with arsenic for 18–24 h, and then fixed. DNA fragment end labeling assays were then performed as described previously (20) with Klenow FragEL DNA fragmentation detection kit. A minimum of 200 cells was scored for the incidence of apoptosis.

**JNK Activity Assay.** JNK activity assay was carried out as described previously (11, 21). Briefly, JB6 CI 41 cells were starved for 48 h in 0.1% FBS MEM at 37°C in a 5% CO$_2$ atmosphere incubator. The cells were then exposed to arsenite at concentrations and times as indicated in the figure legends. The cells were then harvested and precipitated with 2 µg of NH$_2$-terminal e-Jun (1–89) fusion protein bound to glutathione-Sepharose beads overnight at 4°C. The beads were washed twice with 500 µl of lysis buffer with phenylmethylsulfonyl fluoride and twice with 500 µl of kinase buffer. The kinase reactions

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Inhibition of EGF-induced Cell Transformation by Arsenic. Clinical studies have reported that arsenite is effective and relatively safe in the treatment of acute promyelocytic leukemia (8–10). How-ever, the results from both epidemiological investigations and experimental animal models have shown that arsenic is a well-documented human carcinogen (1–4). In a late-stage tumor promotion cell culture model, a mouse epidermal JB6 cell line, we reported previously that low doses of arsenite (<25 μM) could induce cell transformation by itself (11). While studying the synergistic effect of arsenic and EGF on cell transformation efficiency, we found that relatively high doses of arsenic markedly inhibit EGF-induced cell transformation (Fig. 1). The inhibitory effects of arsenic appear to be dose dependent (Fig. 1).

Induction of Apoptosis by Arsenic in JB6 Cells. Evidence from both in vitro and in vivo studies have demonstrated that induction of apoptosis is involved in cancer treatment success by some chemical agents and drugs (8–10, 12, 14, 16). Because it has been reported that arsenite induces apoptosis in some leukemia cell lines (8–10, 12, 14), we analyzed apoptotic induction in JB6 cells (Fig. 2). Treatment of cells with arsenite or arsenate resulted in apoptosis by 44.5 and 61.5%, respectively (Fig. 2). These results revealed that induction of apoptosis by arsenic may be associated with its inhibitory effects on EGF-induced cell transformation.

p53 Is Not Involved in Apoptosis Induction by Arsenic. It is known that a number of factors and pathways can lead to apoptosis (16, 22–24). Many studies report that normal function of p53 is crucial for the induction of apoptosis in human and murine cells after DNA damage (16, 22, 25, 26). Apoptosis of thymocytes after irradiation was almost completely blocked in p53-deficient mice (27). To investigate the possible role of p53 in the induction of apoptosis by arsenic, we first investigated the induction of p53-dependent transcriptional activity by arsenic in JB6 cells. We found no increase in p53-dependent transcription activity in Cl 41 p53 cells treated with arsenic at doses ranging from 12.5 to 200 μM (Fig. 3g), whereas UVC radiation (60 J/m²) induced a >20-fold increase of p53-dependent transcription activity (Fig. 3g). These data suggested that p53 may not be involved in apoptosis induction by arsenic. To obtain direct evidence that p53 is not required for apoptosis induction by arsenic, we tested the effects of arsenic on two fibroblast cell lines, p53+/+ and p53−/−, derived from mouse embryos containing either wild-type p53 (p53+/+ or were p53 deficient (p53−/−; Ref. 16, 17). The results showed that treatment of cells with arsenite or arsenate for 20 h could cause apoptosis by 38.5 and 41.0% in p53+/+ cells or by 36.5 and 34.5% in p53−/− cells, respectively (Fig. 3, a–f). There was no difference in apoptosis induction between these two cell lines with arsenic exposure (Fig. 3, a–f). In contrast, the p53−/− fibroblast failed to respond to phenethyl isothiocyanate and resveratrol apoptosis stimulation as described in our previous reports (16, 20). Thus, these results strongly argue against the involvement of p53 in arsenic-induced apoptosis.

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**Fig. 2.** Induction of apoptosis by arsenic in JB6 cells. Subconfluent (80–90%) monolayer JB6 Cl 41 cells on microscope slides were treated with medium control (a), arsenite (As3+, 200 μM; b), or arsenate (As5+, 200 μM; c) for 15 h. Then, the cells were fixed on slides, and DNA fragment end labeling assays were performed as described in Klenow FragEL, DNA fragmentation detection kit by Oncogene.
Introduction of Dominant-Negative Mutant of JNK1 Blocks the Arsenic-induced Apoptosis. Because the activation of JNKs is suggested to play a critical role in apoptosis induction of epithelial and lymphoid cells in response to radiation, tumor necrosis factor-α or Fas ligand (23) and our findings indicate that activation of JNKs by arsenite is at the same dose range for its induction of apoptosis (11), we hypothesized that the activation of JNKs is required for apoptosis induced by arsenic. To test this hypothesis, we first analyzed the induction of the phosphorylation of JNKs and activity by arsenic at the same induction conditions in JB6 cells. The results showed that arsenite and arsenate markedly induced both phosphorylation and activity of JNKs (Fig. 4). To further investigate the role of the activation of JNKs in arsenic-induced apoptosis, we used the well-characterized cell line, a stable transfectant of JB6 cells with dominant-negative mutant of JNK1, to test its effects on arsenic-induced apoptosis (11). We found that arsenite-induced activation of JNKs was impaired in this cell line at all time points tested (11). Expression of dominant-negative mutant JNK1 blocked the apoptosis induction by arsenite (4%) or arsenate (7%) as compared with vector-transfected control cells (31.5 and 40.5% for arsenite and arsenate, respectively; Fig. 5). These data demonstrated that the pathway of JNKs plays an essential role in arsenic-induced apoptosis.

Discussion

The report presented here demonstrates that activation of JNKs, but not p53 protein, is the mediator of arsenic-induced apoptosis. This notion is supported by evidence from experiments using a p53-luciferase reporter stable transfectant, the p53 knockout cell line, as well as the dominant-negative mutant JNK1 stable transfectant. We
found that the activation of JNKs and induction of apoptosis occur only in the cells exposed to higher doses of arsenic (>50 μM), whereas arsenic did not induce p53-dependent transcription at all doses studied. Furthermore, expression of the dominant-negative mutant of JNK1 blocks arsenic-induced cell apoptosis, whereas there is no difference for apoptosis induction by arsenic between p53+/+ and p53−/− cells. These data, together with our previous findings that the activation of Erks is required in arsenic-induced cell transformation, not only assess the role of MAPK members in arsenic-induced apoptosis but also provide important information in the understanding of arsenic-induced biological activity, including the effect of arsenic on cancer and in carcinogenesis. In consideration of the fact that p53 mutations are very common in human cancer and that apoptosis plays a key role in most of the chemotherapeutic agents, arsenic may potentially be very useful for the treatment of certain human cancers, especially for “drug-resistant” cancers, the effectiveness of which are through p53-dependent apoptosis induced by most of the clinically used chemotherapy agents.

Apoptosis can be initiated by a wide variety of stimuli, including developmental signals, cellular stress, disruption of cell cycle, and different kinds of chemicals (16, 20, 22–24, 28). A number of key factors involved in the regulation and coordination of apoptosis have been identified. The tumor suppressor gene, p53, has been clearly linked to the pathways leading to apoptosis in human and murine cells in response to DNA damage. This notion is supported by the findings that p53 is the most commonly mutated tumor suppressor gene, and the lack of p53 suppression or function is associated with an increased risk of tumor formation (29–31). Transgenic mice expressing mutant p53 or p53 knockout mice with both alleles of p53 disrupted are prone to both spontaneous and induced tumors (30, 32, 33). Ectopic expression of wild-type p53 in murine myeloid leukemia cells induces rapid apoptosis (34). Thymocytes from p53−/− mice are resistant to ionizing radiation-induced apoptosis (27). p53 is also required for the removal of damaged keratinocytes after UV irradiation on the skin (35). Known target genes of p53 in apoptosis are BAX, Bcl-Xl, Fas1, FASL, IGF-Bp3, PGA608, and DR5 (22). Because it has been suggested that arsenic induces DNA damage, such as chromosome aberration and sister chromatid exchange, we tested the possible role of p53 in arsenic-induced apoptosis. Our results indicated that p53 is not involved in apoptosis induction by arsenic because arsenic does not induce p53-dependent transcriptional activation and still initiates apoptosis in p53−/− cells.

Recently, the pathways of JNKs were reported to be implicated in induction of apoptosis (23). The evidence for requirement of JNKs in apoptosis derives from studies where overexpression of JNKs results in apoptosis in some cells (23), and the antisense inhibition of the use
of dominant-negative constructs attenuates the apoptotic response (23). It was reported that in mice deficient in JNK\(_2\), which is restricted to the brain, there was a reduction in seizure activity and hippocampal apoptosis in response to the excitotoxic glutamate-receptor agonist kainic acid (36). However, there are numerous studies providing evidence that JNK activation is either antiapoptotic or nonapoptotic (23). The explanation for differences among different studies may be due to cell type specificity. However, consensus has not been established for either a strictly apoptotic or antiapoptotic role for the signaling of JNKs (23). For example, it has been reported that inhibition of the signaling of JNKs does not block Fas-mediated killing in Jurkat T cells (37, 38), whereas it was found that Fas-mediated apoptosis in neuroblastoma cells requires the pathway of JNKs (39). Similarly, in contrast to the evidence provided by the JNK\(_2\) knockout, data from the TRAF\(_2\) knockout mouse suggest that TRAF\(_2\) signaling patients with cancers with p53 inactivation.

The activation of TRAFs and apoptosis induced by arsenic induces apoptosis through JNK-dependent and p53-independent pathways. In this study, we demonstrated that arsenic-induced apoptosis is dependent on the JNKs pathway by using mouse epidermal JB6 cells stably transfected with dominant-negative mutant JNK\(_2\). Additional studies will focus on the role that ceramide generation plays in apoptosis induction by arsenic.

Nearly all chemotherapeutic drugs cause DNA damage and kill cancer cells, mainly by activating endogenous biochemical pathways for induction of tumor cell apoptosis (41, 42). It is known that p53-dependent apoptosis after DNA damage is mediated by the CD95 (APO-1/Fas) receptor (43). Anticancer agents, such as cisplatin, mitomycin, methotrexate, mitoxantrone, doxorubicin, and bleomycin, at concentrations present in the sera of patients during therapy led to an up-regulation of the CD95 receptor. The up-regulation of the CD95 receptor is observed only in cells with wild-type p53 expression and not in cells with p53 mutation or that are p53 deficient (41). Therefore, p53 inactivation is associated with resistance to anticancer chemotherapy. Because p53 is inactivated in the majority of human cancers, novel approaches to counteracting drug resistance is the goal in the development of new chemotherapeutic agents that are able to bypass the p53-dependent pathway and that can be specifically directed against p53-defective cancer cells (43). In this study, we found that arsenic induces apoptosis through JNK-dependent and p53-independent pathways. Thus, arsenic is a potential treatment for cancer patients who have developed drug resistance, especially for those patients with cancers with p53 inactivation.

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