Coordination of JNK1 and JNK2 Is Critical for GADD45α Induction and Its Mediated Cell Apoptosis in Arsenite Responses*

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Arsenite is a well documented environmental pathogen, whereas it has also been applied as medication to treat various neoplasmas. The pathogenic and therapeutic effects of arsenite are associated with cellular apoptotic responses. However, the molecular mechanisms of arsenite-induced apoptosis are not very well understood. Our previous study has shown that arsenite exposure is able to activate JNKs, which subsequently mediates the apoptotic outcome. The present study further revealed that the coordination of JNK1 and JNK2 was critical for the arsenite-induced expression of GADD45α (growth arrest and DNA damage 45α), which in turn mediated the cellular apoptosis. The arsenite-induced apoptosis and GADD45α expression were significantly impaired in mouse embryonic fibroblasts deficient in either jnk1 (JNK1−/−) or jnk2 (JNK2−/−). Knockdown of GADD45α by its specific small interfering RNA also dramatically reduced the apoptotic responses, and upregulation of GADD45α in either JNK1−/− or JNK2−/− mouse embryonic fibroblasts partially reactivated the cell death. Furthermore, we found that the regulation of GADD45α by JNK1 and JNK2 was achieved through mediating the activation of c-Jun, since in the JNK1−/− and JNK2−/− cells the c-Jun activation was impaired, and upregulation of the dominant negative mutant of c-Jun (TAM67) in wild type cells could also block GADD45α induction as well as cellular apoptosis. Our results demonstrate that the coordination of JNK1 and JNK2 is critical for c-Jun/GADD45α-mediated cellular apoptosis induced by arsenite.

Arsenite occurs naturally in the earth’s crust and is widely distributed in the environment (1). Human exposure to arsenite occurs mainly by ingestion of drinking water contaminated with arsenite from naturally occurring sources or through the inhalation of contaminated dusts in occupational settings (2). As an environmental pathogen, arsenite causes a series of pathophysiological alterations, including immunosuppression and carcinogenesis (3). Paradoxically, arsenite has also been applied as chemotherapeutic reagents to treat various neoplasmas (4). Further insights into the pathogenic and therapeutic effects of arsenite show that it seems to be associated with the apoptotic induction in both normal and tumor cells (5, 6). The inhibition of antiapoptotic Bcl-2 and activation of proapoptotic mitogen-activated protein kinases has recently been shown to participate in the arsenite-induced apoptotic process in various cell models (7–12). These observations suggest that the alternation of the cascades of cellular survival/proapoptotic signaling pathways may be critical for cell apoptotic responses triggered by arsenite. However, the detailed molecular mechanisms still remain to be elucidated.

In this study, we applied wild type (WT), JNK1−/−, and JNK2−/−, three immortalized mouse embryonic fibroblasts (MEFs) to evaluate the roles of JNK1 and JNK2 in arsenite-induced apoptosis. It was found that the arsenite-induced apoptosis required both JNK1 and JNK2, since the deficiency of either impaired the apoptotic responses. Interestingly, GADD45α (growth arrest and DNA damage 45α) was detected to be a downstream target gene transcriptionally regulated by either JNK1 or JNK2, and it functioned as the critical mediator for the JNK1- and JNK2-associated apoptosis by arsenite. In addition, the regulation of GADD45α by JNK1 and JNK2 was, at least in part, dependent on c-Jun activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Immortalized WT, JNK1−/− (13), and JNK2−/− (14) MEFs as well as their stable transfectants were maintained at 37 °C in 5% CO2 incubator with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 25 μg/ml gentamicin. The cultures were dissociated with trypsin and transferred to new 75-cm2 culture flasks (Fisher) twice a week. Fetal bovine serum was purchased from Nova-Tech (Grand Island, NE), and the rest of the cell culture reagents were obtained from Sigma.

Plasmids and Transfection—HA-tagged murine jnk1 and jnk2 full-length cDNAs were subcloned into pcDNA3 expression vector, confirmed by DNA sequencing, and then named as HA-JNK1/pcDNA3 and HA-JNK2/pcDNA3, respectively (15, 16). A pcDNA3.1 plasmid containing c-Jun dominant negative mutant (pcDNA3.1/His-TAM67) was kindly provided by Dr. Tim G. Bowden (College of Pharmacy, University of Arizona, Tucson, AZ) and Dr. Matthew Young (Center for Cancer...
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Research, NCI-Frederick) (17). An expression construct containing HA-tagged full-length cDNA of murine gadd45α (HA-GADD45α) was described previously (18). HA-tagged JNKK2-JNK1 fusion protein expression vector was a generous gift from Dr. Han-Ming Shen (Department of Community, Occupational, and Family Medicine, Faculty of Medicine, National University of Singapore). Transfection experiments were performed with Lipofectamine2000 (Invitrogen) according to the instructions from the manufacturer. For the transfection of pcDNA3.1/His-TAM67 into WT MEFs, 5 µg of plasmids were used, and the stable transfectants were generated by G418 selection (500 µg/ml). For the transfection of HA-JNK1/pcDNA3, HA-JNK2/pcDNA3, or HA-GADD45α in JNK1−/− and JNK2−/− MEFs, 5 µg of the individual plasmids were co-transfected with 0.8 µg of the hygromycin-resistant plasmid, respectively. The stable transfectants were established by hygromycin selections (50 µg/ml for JNK1−/− cells and 400 µg/ml for JNK2−/− cells).

The GADD45α siRNA expression plasmids were made by using the GeneSuppressorTM system (Imgenex Co., San Diego, CA). The two siRNA target sequences for gadd45α were as follows: 5′-GTC TCT AGC AGG CAA CCT C-3′ (siRNA1) and 5′-GCT GCT CAA CGT AGG ATC CT-3′ (siRNA2). Constructs containing the reversed target sequences were used as a negative control. The GADD45α siRNA1 and siRNA2 were co-transfected into WT MEFs, and the stable transfectants were established by G418 selection.

Flow Cytometry—To analyze the apoptotic cells with propidium iodide staining, WT, JNK1−/−, and JNK2−/− cells were plated in 6-well plates with a density of 2 × 105 cells/well and cultured in normal 10% serum medium until 70–80% confluence. After exposure to 20 µM sodium arsenite (Fisher) for 24 h, the cells were collected by centrifugation and fixed in ice-cold 80% ethanol at −20 °C overnight. The fixed cells were stained in the buffer containing 100 mM sodium citrate, 0.1% Triton X-100, 0.2 mg/ml RNase A, and 50 µg/ml propidium iodide at 4 °C for 1 h and then analyzed by an Epics XL fluorescence-activated cell sorter (Beckman Coulter, Miami, FL) as described in our previous publication (19).

To determine the cell mitochondrial membrane potential by fluorochrome DiOC6 staining, the WT cells were seeded in 6-well plates. After exposure to 20 µM arsenite for 24 h, the cells were applied with 10 µg/ml DiOC6 dye (dissolved in Me2SO and diluted with phosphate-buffered saline) for 30 min. The stained cells were then washed twice with phosphate-buffered saline, harvested, and analyzed by flow cytometry (20).

To detect the activated caspase-3, the APO ACTIVE 3TM kit (Cell Technology Inc., Minneapolis, MN) was applied as previously described (21). Briefly, the cells were harvested by centrifugation 24 h after arsenite exposure, fixed at room temperature for 30 min, labeled with anti-activated caspase-3 antibody for 1 h, and then incubated with fluorescein isothiocyanate-conjugated secondary antibody in the dark for 30 min. The cells labeled with anti-activated caspase-3 antibody were detected by flow cytometry.

Polymerase Chain Reaction (PCR) for jnk1 and jnk2 Gene Knock-out Identification—To identify jnk1 and jnk2 gene deficiencies in JNK1−/− and JNK2−/− cells, genomic DNA was isolated from the cells, and the deleted targets of jnk1 and jnk2 sequences were amplified by PCR using two pairs of primers designed from the jnk1 and jnk2 genomic sequences according to previous publications with some modifications (13, 14). The primer sequences for jnk1 were 5′-CGT CTG GTG GAA GGA GAG AG-3′ (sense primer) and 5′-TAA TAA CGG GGG TGG AGG AT-3′ (antisense primer) (13). The primer sequences for jnk2 were 5′-TCT GAC GTC CTG GGC TGG AC-3′ (sense primer) and 5′-GCA GCA GCA CTC ATG AGC T-3′ (antisense primer) (14). The PCR products were separated on 2% agarose gels and stained with ethidium bromide, and the images were scanned using a UV light.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Twelve hours after exposure to arsenite, cells were collected, and total RNA was extracted from the cells with TRIzol reagent (Invitrogen). Total cDNA was synthesized by ThermoScriptTM RT-PCR system (Invitrogen). The amount of GADD45α mRNA was measured by semiquantitative RT-PCR using a pair of primers (5′-ATG ACT TTT GAG GAA ATC TCG-3′ and 5′-CAC TGA TCC ATG TAG CGA CT-3′). The control mouse 3-actin mRNA was also detected by RT-PCR using the primers (5′-GAC GAT GAT ATT GCC GCA CT-3′ and 5′-GAT ACC ACC CT T GCT CTG AG-3′). The PCR products were separated on 2% agarose gels and stained with ethidium bromide, and the images were scanned with a UV light.

Western Blotting—MEFs and their transfected cells were plated in the 6-well plates and cultured in normal 10% serum medium until 70–80% confluence. After exposure to arsenite for various doses and time periods as indicated in the figure legends, the cells were washed once with ice-cold phosphate-buffered saline and collected with SDS-sample buffer (22). The cell extracts were sonicated, denatured by heating at 100 °C for 5 min, and quantified with a Dc protein assay kit (Bio-Rad). Equal amounts of protein were resolved on 10% SDS-polyacrylamide gels. The proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked, and probed with one of the polyclonal antibodies against phospho-specific c-Jun and JNK1/2, nonphosphorylated c-Jun and JNK1/2, HA (Cell Signaling Technology, Beverly, MA), poly(ADP) polymerase (PARP), caspase-3, GADD45α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or β-actin (Sigma). Primary antibody-bound proteins were detected by using an alkaline phosphatase-linked secondary antibody and an ECF Western blotting system (Amersham Biosciences) (23).

RESULTS

Arsenite Induces Apoptosis in MEFs—To study the effect of arsenite in cell apoptosis, MEFs were incubated with 20 µM arsenite for 24 h. The key step of the apoptotic process is chromosome DNA degradation. The degraded DNA fragments are then encapsulated in the apoptotic bodies that can be detected by flow cytometry as sub-G0/G1 phase cells (24). As shown in Fig. 1A, exposure of MEFs to arsenite resulted in significant accumulation of sub-G0/G1 phase cells by 55.50% compared with 1.12% in the cells without arsenite exposure, indicating that arsenite caused the cellular DNA fragmentation in the MEFs. Next we examined whether arsenite-induced DNA fragmentation was associated with caspase-3

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activation, the key executor of apoptosis, by using an APO ACTIVE 3™ kit and Western blotting assay. Arsenite exposure led to over 8-fold increases of the activated caspase-3 compared with that of medium control cells detected by flow cytometry (36.87% versus 4.53%) (Fig. 1B). Western blotting data further confirmed the presence of the activated caspase-3 (17-kDa band) (Fig. 1C). Moreover, PARP, an intracellular substrate of the activated caspase-3, was also detected cleaved from 116-kDa to 85-kDa fragments after arsenite treatment (Fig. 1C). The MEFs were also exposed to 5 μM arsenite for 24 h; however, no obvious cell death was observed, as indicated by the absence of caspase-3 activation and PARP cleavage (Fig. 1C), so 5 μM of arsenite treatment was used as a negative control in the following studies. It may be noticed that the cytotoxicity of arsenite to cells is dependent on the forms of arsenite used (25, 26) and cell types as well as species (27, 28). For example, the cytotoxicity as well as cellular biological effects of arsenic trioxide and sodium arsenite are quite different when they are compared at the same concentration, which has been observed from the findings of various laboratories (5, 10). Mitochondria potential reduction has been reported to be critical for the apoptotic induction through the mitochondria pathway (29, 30); therefore, the mitochondrial potential was measured by fluorochrome DiOC₆ staining. As shown in Fig. 1D, DiOC₆ incorporation was 35% less in the arsenite-treated cells compared with that of the medium control cells (53.73% versus 89.25%), indicating that arsenite exposure reduced the mitochondrial membrane potential. These data strongly indicate that arsenite is able to induce apoptosis in MEFs.

Coordination of JNK1 and JNK2 Is Required for the Arsenite-induced Apoptosis—The proapoptotic role of JNKs has been proposed recently in different cellular conditions (8, 10, 12, 31), but their precise molecular mechanisms still remain unclear. To gain further insight into the JNK1- and/or JNK2-dependent proapoptotic signal pathway, we compared the apoptosis induction by arsenite in WT, JNK1−/−, and JNK2−/− MEFs. Before performing the comparison experiments, the jnk1 and jnk2 gene deficiencies in their knock-out MEFs were initially confirmed by both Western blotting and PCR (Fig. 2A). Interestingly, both JNK1−/− and JNK2−/− cells were resistant to arsenite-induced apoptosis, as indicated by the absence of the increased sub-G₀/G₁ phase cells after the arsenite treatment for 24 h in the two knock-out cell lines (7.88% in JNK1−/− cells and 0.70% in JNK2−/− cells) compared with that in WT cells (53.49%) (Fig. 2B). Similarly, neither caspase-3 activation nor PARP cleavage was observed in the arsenite-treated JNK1−/− and JNK2−/− MEFs (Fig. 2C). Even when the arsenite treatment time was prolonged to 48 h, there was still no obvious cell death observed in the two knock-out cell lines (Fig. 2D). All of these data suggested that JNK1 and JNK2 were both required for arsenite-induced apoptosis, and a deficiency of either one did impair the apoptotic response. However, the cell death induced by other environmental carcinogens, such as nickel chloride, was not different among JNK1−/−, JNK2−/−, and WT MEFs, as indicated by morphological changes (Fig. 2E) or caspase-3 activation and PARP cleavage (Fig. 2F). This demonstrates that the escape from apoptosis due to the deficiency of either jnk1 or jnk2 is a relatively specific feature in the cells, at least in MEFs, when exposed to arsenite.

FIGURE 1. Arsenite induces apoptosis in MEFs. WT MEFs (2 × 10⁵) were seeded into each well of 6-well plates and cultured until the cell density reached 70–80% confluence. The cells were then exposed to 5 and/or 20 μM of sodium arsenite for 24 h. The chromosome DNA fragmentation indicated by the proportion of sub-G₀/G₁ phase cells was determined using propidium iodide staining and detected by flow cytometry (A). Caspase-3 activation was detected using an APO ACTIVE 3™ kit (B) as well as Western blotting (C). PARP cleavage was detected by Western blotting (C). The mitochondrial membrane potential was determined using fluorochrome dye DiOC₆ and detected by flow cytometry (D).
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A

WT  JNK1−/−  JNK2−/−  JNK2
β-Actin  JNK1  jnk1  JNK1

WT  JNK1−/−  JNK2−/−  jnk2  β-Actin

C

WT  JNK1−/−  JNK2−/−
Arsenite (µM)
0  20  5  0  20  5  0  20  5

procaspase-3 (32kDa)
activated caspase-3 (17kDa)
PARP (116kDa)
Cleaved PARP (85kDa)
β-Actin

D

WT  JNK1−/−  JNK2−/−
Arsenite (µM)
0  20  5  0  20  5  0  20  5

PARP (116kDa)
Cleaved PARP (85kDa)
β-Actin

E

Medium Control  NiCl₂ 2 mM

WT

JNK1−/−

JNK2−/−

F

WT  JNK1−/−  JNK2−/−
NiCl₂ (mM)
0  2  0  2  0  2

procaspase-3 (32kDa)
Activated caspase-3 (17kDa)
PARP (116kDa)
Cleaved PARP (85kDa)
β-Actin
To further confirm the essential requirement of JNK1 and JNK2 in arsenite-induced apoptosis, we stably reconstituted the HA-tagged JNK1 or JNK2 into their deficient MEFs (Fig. 3A). As expected, the restored expression of either JNK1 or JNK2 in each knock-out cell line mostly returned their abilities as WT MEFs to undergo apoptosis as indicated by caspase-3 activation and PARP cleavage (Fig. 3B). It need be noted here that the stable transfectants of JNK1 or JNK2 reconstituted cells were established as a mass pool, which might include a proportion of unsuccessfully transfected cells, so the exogenous JNK1 or JNK2 was expressed much lower as compared with those in WT cells (Fig. 3A).

Expression of GADD45α Is Regulated by the c-Jun-dependent Pathway—GADD45α was originally identified as a gene transcribed in response to DNA damage by UV irradiation (32) and has been reported to be involved in many biological functions, such as suppressing cell growth (33), participating in DNA damage repair (34), regulating cell cycle G2/M checkpoint (35), and mediating apoptotic signal pathways (36, 37). To determine whether GADD45α is the downstream target gene of JNK1 and JNK2 in responses to arsenite exposure, the GADD45α induction was detected in WT and the two JNK knock-out MEFs. As shown in Fig. 4A, treatment with 20 μM arsenite obviously induced GADD45α protein expression in WT MEFs at all of the time points tested.

It was more important to observe that this induction was blocked in either JNK1−/− or JNK2−/− MEFs, indicating that JNK1 and JNK2 were both critical for the GADD45α up-regulation in response to arsenite. This notion was further confirmed by the reconstitution experiments, which showed that restoring JNK1 or JNK2 led to an obvious GADD45α induction in their respective knock-out cells, although not as strong as those in the WT MEFs (Fig. 4B), which was consistent with the exogenous JNK1 or JNK2 expression levels of the reconstituted cells (Fig. 3A).

To determine the mechanisms of GADD45α induction by JNK1 and JNK2, we also tested the changes of the gadd45α mRNA level. The RT-PCR data showed that arsenite-induced gadd45α mRNA expression was readily observed in the WT cells; however, it was reduced due to jnk1 deficiency and totally blocked in jnk2-deficient cells (Fig. 5A), coinciding with the basal levels of JNKS protein expression in these two knock-out cell lines (Fig. 2A) as well as their sensitivities to arsenite-associated cell death (Fig. 2B), indicating that JNK1 and JNK2 were required for the arsenite-associated GADD45α mRNA induction.

Transcription factor c-Jun is a well known downstream target of JNKs (38). To evaluate the relevance of c-Jun for JNK1- and JNK2-mediated GADD45α induction, the activation of JNK and c-Jun in WT, JNK1−/−, and JNK2−/− MEFs were ana-
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Arsenite exposure led to phosphorylation of both JNK1 and JNK2 in WT cells. In JNK1−/− cells, the obvious phosphorylation of JNK2 was observed; however, in JNK2−/− cells, the phosphorylated JNK1 was comparably weaker, which may be due to the lower expression level of basal JNK1 protein in JNK2−/− MEFs (Fig. 5B). c-Jun activation was indicated by phosphorylations of c-Jun at Ser63 and Ser73, the critical residues for c-Jun activation (39). Compared with the full phosphorylation of c-Jun up to 8 h of arsenite exposure in WT cells, jnk1 deficiency resulted in relative low activation of c-Jun, and jnk2 deficiency led to an even more obvious attenuation of c-Jun activation (Fig. 5B), which was consistent with the phosphorylated JNK levels in JNK1−/− and JNK2−/− cells as well as the GADD45α mRNA induction in these cell lines (Fig. 5A), suggesting that the cascade activation of JNKS/c-Jun regulated the induction of GADD45α. To test whether blocking c-Jun activation was responsible for impairing GADD45α induction, a plasmid containing a dominant negative mutant of c-Jun (TAM67) was stably transfected into WT MEFs. As expected, TAM67 totally blocked c-Jun phosphorylations (Fig. 5C) and dramatically reduced GADD45α induction (Fig. 5D), indicating that the activation of c-Jun was essential for GADD45α induction by arsenite. These results provide strong evidence that blocking GADD45α induction upon deficiency of jnk1 or jnk2 is, at least in part, due to repressing the activation of c-Jun. This may suggest that the JNK1/2/c-Jun signaling pathway is one of the signaling pathways involved in GADD45α induction in cell response to arsenite exposure. However, the JNK1/2/c-Jun pathway is not the only mediator for GADD45α induction by arsenite. This notion was supported by our findings that only activating the JNK1/c-Jun pathway by transfection with a plasmid expressing HA-tagged JNK2 in JNKS fusion protein in WT cells or by transfection of c-Jun expression vector in JNK1−/− cells (Figs. 5, E and G) was not able to elevate GADD45α protein expression (Fig. 5, F and H). Moreover, overexpression of exogenous c-Jun in the JNK1−/− cell (Fig. 5G) could not restore the arsenite-induced apoptotic response (Fig. 5, H and I), indicating that some other pathway(s) may also be critical for GADD45α induction as well as apoptotic promotion in cell response to arsenite exposure.

JNK1- and JNK2-mediated GADD45α Induction Is Essential for Arsenite-associated Apoptosis—To elucidate the importance of GADD45α in the arsenite-induced apoptosis, we applied siRNA technology to inhibit endogenous GADD45α expression to determine whether blocking GADD45α expression could affect cellular apoptotic responses. The two different GADD45α siRNA constructs were co-transfected into WT MEFs, and the siRNA efficiency was shown in Fig. 6A. Arsenite-induced apoptotic related cellular morphological alterations, such as cell shrinkage, bubble, and detachment, were partially impaired by knockdown of endogenous GADD45α (Fig. 6B). The interference with apoptosis by the GADD45α siRNA was further confirmed by the absence of caspase-3 activation and PARP cleavage (Fig. 6C), suggesting that GADD45α is critical for arsenite-induced apoptosis. The relevance of GADD45α in JNK1- and JNK2-mediated apoptosis was directly tested by stably expressing GADD45α in JNK1−/− and JNK2−/− MEFs (Fig. 7A). Overexpression of exogenous GADD45α partially sensitized the arsenite-induced apoptosis in both JNK1−/− and JNK2−/− MEFs, indicated by the apoptotic morphological alterations and caspase-3 activation as well as PARP cleavage (Fig. 7, B and C). Therefore, our data provide strong evidence that blocking GADD45α induction in either JNK1−/− or JNK2−/− MEFs can protect cells from the arsenite-associated apoptosis. These results further imply that JNK1- and JNK2-dependent cell death stimulated by arsenite, for a large part, is mediated by up-regulated GADD45α expression.

DISCUSSION

In this study, we have demonstrated that the coordination of JNK1 and JNK2 is required for the arsenite-induced apoptosis in MEFs. Moreover, GADD45α is identified as the downstream target of JNK1/2 signaling, and up-regulation of GADD45α is critical for the JNK1/2-mediated apoptosis, at least in part, dependent on the activation of c-Jun. To our knowledge, this is the first time to describe the cascade activation of the JNK1/2/c-Jun/GADD45α signaling pathway in the arsenite-induced apoptosis.

Apoptosis is one of the arsenite-caused multiple stress responses in mammalian cells (40–42). Arsenite exposure causes programmed cell death in both normal healthy cells (7, 43) and malignant transformed cells (44–46), which accounts for its pathogenic (47) as well as therapeutic properties (4). However, the molecular events in the apoptotic responses of cells exposed to arsenite remain unclear. Initially, we apply WT
FIGURE 5. GADD45α induction is regulated by JNK1 and JNK2 signaling pathway through the activation of c-Jun. WT, JNK1−/−, and JNK2−/− MEFs (2×10^5) were seeded into each well of 6-well plates and cultured until the cell density reached 70–80% confluence. The cells were then exposed to 20 μM sodium arsenite. Total RNA was extracted 12 h post-arsenite exposure by TRIZol reagent, and cDNAs were synthesized by ThermoScript™ RT-PCR system. The cDNAs were used as a template for the detection of GADD45α expression by PCR (A), c-Jun phosphorylation and basal expression, as well as JNKs phosphorylation were detected by Western blotting after the cells were exposed to 20 μM sodium arsenite for the indicated time periods (B) or after the WT cells and stable transfectants with TAM67 (WT/TAM67) were treated with 5 or 20 μM arsenite for 6 h (C). For the detection of GADD45α expression, WT and WT/TAM67 cells were treated with 10 or 20 μM arsenite for 24 h, and the cells were extracted with SDS-sample buffer and detected by Western blotting (D). WT MEFs and stable transfectants expressing HA-JNKK2-JNK1 fusion protein were subjected to Western blotting for detection of HA and phospho-specific and total JNKs, as well as GADD45α protein expression, and arsenite treatment was used as positive control for GADD45α induction (E and F). Stable transfectants of JNK1−/− cells with c-Jun expression vector was identified with specific antibody against c-Jun (G). The indicated types of cells were treated with sodium arsenite of 20 μM for 12 and 24 h. The apoptotic responses were compared by Western blotting and morphological changes (H and I).
MEFs as a model to replicate arsenite-induced apoptosis. Activation of caspase-3, which functions as a final common pathway in the apoptotic machinery (48), and the cleavage of critical cellular substrate, PARP, are observed in MEFs after arsenite exposure (Fig. 1, B and C), which is consistent with previous reports obtained in other laboratories (49). Therefore, we use caspase-3 activation as well as subsequent PARP cleavage as the indices of apoptotic response in the following studies.

JNK signaling pathway activation has been implicated in apoptosis induction in different cell types (7, 8, 50–52). The most convincing evidence comes from the observation that the JNK1 and JNK2 double knock-out cells are resistant to the apoptosis induced by UV irradiation, indicating that JNK activation is associated with UV-induced apoptosis (52). Our previous report also demonstrates that sodium arsenite also causes the cell apoptosis in the mouse epidermal cell line JB6 P+ Cl 41, and more importantly, the expression of dominant negative JNK1 in Cl41 cells abrogates the apoptotic response (8). Using primary cultured rat cerebellar neurons, Namgung and Xia (7) report that JNK3, the brain-specific expressed JNK isoform, is associated with sodium arsenite-induced apoptosis, whereas JNK1 and JNK2 are not stimulated by arsenite in this cell type, although they exhibit high basal activity, indicating that the activation of JNKs is essential for arsenite-induced apoptosis in different types of cells. The present study further reveals that the coordination of JNK1 and JNK2 is required for the apoptotic responses caused by arsenite in MEFs, because knock-out of either jnk1 or jnk2 totally blocks arsenite-induced DNA fragmentation, caspase-3 activation, and PARP cleavage (Fig. 2, B–D). JNK2−/− cells (cell death 0.70%) seem even more resistant to arsenite-induced apoptosis compared with JNK1−/− cells (cell death 7.88%), which is coincident with the basal JNK1/2 expression levels of these two cell lines (Fig. 2, A and B). It should be mentioned that the JNK1 level in JNK2−/− was somewhat lower than that in WT cells, which might be due to the interaction of JNK1 and JNK2 isoforms. This notion has been supported by the findings from other laboratories that knocking down JNK2 expression by its specific siRNA also reduces the JNK1 protein expression (53–55). The proapoptotic role of the coordination of JNK1 and JNK2 is further confirmed by restoring either JNK1 or JNK2 in their deficient MEFs (Fig. 3B). However, the deficiency of either jnk does not affect the other environmental carcinogen stimuli, such as nickel chloride-induced apoptosis (Fig. 2, E and F). It seems that the JNK-independent apoptotic outcome caused by nickel might be due to the activation and/or alteration of the different proapoptotic and/or antiapoptotic signaling cascades.

GADD45 plays a prominent role in the control of cell growth, cell cycle checkpoint, and nucleotide excision repair (56–58). Several lines of evidence directly implicate that GADD45α, one of the three GADD45 isoforms, is important in the apoptotic process (59). The knock-out of the gadd45α gene significantly impairs apoptosis and is susceptible to ionizing radiation, UVB, and 3,12-dimethylbenzanthracene-induced tumors in mouse models (59, 60). The present study has also indicated that arsenite-induced apoptosis is mediated by the up-regulation of GADD45α. Elimination of endogenous GADD45α by siRNA partially impairs the apoptosis response to arsenite (Fig. 6, B and C). Interestingly, the deficiency of either jnk1 or jnk2 obviously blocks GADD45α induction at both the protein and
FIGURE 7. GADD45α overexpression in JNK1−/− and JNK2−/− MEFs increases in their apoptotic response to arsenite exposure. HA-GADD45α-expressing plasmid was transfected into JNK1−/− and JNK2−/− MEFs, and the stable transfectants were generated by hygromycin selection and identified by Western blotting with specific antibodies against GADD45α and HA (A). WT, JNK1−/−, and JNK2−/− as well as their stable transfectants were seeded into each well of 6-well plates and treated with 20 or 5 μM sodium arsenite for 24 h. The apoptotic responses were compared by morphological changes (B) and Western blotting (C).
mRNA levels (Figs. 4A and 5A). The mRNA induction of gadd45α in JNK2−/− cells is even more clearly inhibited compared with that in JNK1−/− cells, which is consistent with the basal JNK protein expression levels as well as the sensitivities of these two cells to arsenite-associated apoptosis (Fig. 2, A and B). Reconstitution of JNK1 or JNK2, respectively, restores GADD45α induction partially (Fig. 4B) due to the limited expression levels of the exogenous JNK1 or JNK2 in the transfectant masses (Fig. 3A). These results suggest that the impairment of apoptotic response in JNK1−/− and JNK2−/− cells is most likely associated with the levels of GADD45α protein expression. To confirm the hypothesis, exogenous GADD45α is transfected into the two knock-out cell lines. Expression of the exogenous GADD45α partially sensitizes the apoptotic responses in either jnk1- or jnk2-deficient MEFs (Fig. 7, B and C). Our data demonstrate that the proapoptotic function of either JNK1 or JNK2 is mediated by the GADD45α induction, which is consistent with the report that the expression of a JNK1 dominant negative mutant substantially abrogates the UV irradiation-associated GADD45 promoter induction (61). Yin et al. (62) also find that in MCF7 breast carcinoma cells, troglitazone-induced GADD45 up-regulation is achieved through JNK signaling, because inhibiting JNK activity by a JNK inhibitor (SP600125) significantly suppresses gadd45 mRNA expression. In the human bronchial epithelial cell line (BEAS-2B), Chen et al. (63) determine that the inhibition of NFκB activation by the stable expression of a kinase-mutated form of IkB (IKKβ-KM) causes increased and prolonged GADD45 induction by arsenite; however, transfection of IKKβ-KM cells with a dominant negative mutant of SEK1 (SEK1-KM), which partially reduces JNK activation, suppresses the arsenite-induced GADD45 expression. Our present study further reveals that the coordination of JNK1 and JNK2 is required for the GADD45α induction, through which JNK1 and JNK2 promote arsenite-associated cell death. To our knowledge, this is the first time it is shown that the coordination of JNK1 and JNK2 affects the arsenite-associated cell fate through the regulation of GADD45α expression.

Interestingly, Harkin et al. (37) report that BRCA1 triggers apoptosis through up-regulation of GADD45 and subsequent JNK activation, in which case GADD45 functions as the upstream activator of JNKs. Conversely, it is also reported that the activation of JNKs is negatively regulated by another GADD45 isoform, GADD45β, which binds to MKK7 directly and blocks its catalytic activity, thereby suppressing activation of JNK pathways (64, 65). It is to be noted here that our laboratory’s most recent findings show that in the same MEF cell model with the same dose of arsenite treatment, GADD45α in turn up-regulates JNK pathway activation through activating MKK4,3 another mitogen-activated protein kinase kinase that is reported to be required for JNK activation. Therefore, it is likely that GADD45α and the JNK pathway form a feedback loop, and the activation of one component can positively affect the other. Combined with the other observations of the negative effect of GADD45β on JNK pathway activation, it seems that different GADD45 isoforms possess different or sometimes contradictory functions on JNK signaling pathway activation.

Since c-Jun is a well documented downstream target of JNK signaling, we chose to examine whether the coordination of JNK1 and JNK2 regulates GADD45α expression through c-Jun activation. The promoter region of c-Jun contains the TRE and the JNK pathway forms a feedback loop, and the activation of one component can positively affect the other. Combined with the other observations of the negative effect of GADD45β on JNK pathway activation, it seems that different GADD45 isoforms possess different or sometimes contradictory functions on JNK signaling pathway activation.

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Expression. To confirm the hypothesis, exogenous GADD45α induction partially (Fig. 4B) due to the limited expression levels of the exogenous JNK1 or JNK2 in the transfectant masses (Fig. 3A). These results suggest that the impairment of apoptotic response in JNK1−/− and JNK2−/− cells is most likely associated with the levels of GADD45α protein expression. To confirm the hypothesis, exogenous GADD45α is transfected into the two knock-out cell lines. Expression of the exogenous GADD45α partially sensitizes the apoptotic responses in either jnk1- or jnk2-deficient MEFs (Fig. 7, B and C). Our data demonstrate that the proapoptotic function of either JNK1 or JNK2 is mediated by the GADD45α induction, which is consistent with the report that the expression of a JNK1 dominant negative mutant substantially abrogates the UV irradiation-associated GADD45 promoter induction (61). Yin et al. (62) also find that in MCF7 breast carcinoma cells, troglitazone-induced GADD45 up-regulation is achieved through JNK signaling, because inhibiting JNK activity by a JNK inhibitor (SP600125) significantly suppresses gadd45 mRNA expression. In the human bronchial epithelial cell line (BEAS-2B), Chen et al. (63) determine that the inhibition of NFκB activation by the stable expression of a kinase-mutated form of IkB (IKKβ-KM) causes increased and prolonged GADD45 induction by arsenite; however, transfection of IKKβ-KM cells with a dominant negative mutant of SEK1 (SEK1-KM), which partially reduces JNK activation, suppresses the arsenite-induced GADD45 expression. Our present study further reveals that the coordination of JNK1 and JNK2 is required for the GADD45α induction, through which JNK1 and JNK2 promote arsenite-associated cell death. To our knowledge, this is the first time it is shown that the coordination of JNK1 and JNK2 affects the arsenite-associated cell fate through the regulation of GADD45α expression.

Interestingly, Harkin et al. (37) report that BRCA1 triggers apoptosis through up-regulation of GADD45 and subsequent JNK activation, in which case GADD45 functions as the upstream activator of JNKs. Conversely, it is also reported that the activation of JNKs is negatively regulated by another GADD45 isoform, GADD45β, which binds to MKK7 directly and blocks its catalytic activity, thereby suppressing activation of JNK pathways (64, 65). It is to be noted here that our laboratory’s most recent findings show that in the same MEF cell model with the same dose of arsenite treatment, GADD45α in turn up-regulates JNK pathway activation through activating MKK4,3 another mitogen-activated protein kinase kinase that is reported to be required for JNK activation. Therefore, it is likely that GADD45α and the JNK pathway form a feedback loop, and the activation of one component can positively affect the other. Combined with the other observations of the negative effect of GADD45β on JNK pathway activation, it seems that different GADD45 isoforms possess different or sometimes contradictory functions on JNK signaling pathway activation.

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