Oncogene (2001) 20, 3585–3589

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Contrasting roles of NF-κB and JNK in arsenite-induced p53-independent expression of GADD45α

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Growth arrest and DNA damage-inducible protein 45α (GADD45α) is an important cell cycle checkpoint protein that arrests cells at G2/M phase by inhibiting the activity of G2-specific kinase, cyclin B/p34cdc2. We report here that arsenite induces GADD45α expression in a p53-independent fashion and that this GADD45α induction by arsenite is regulated by NF-κB and c-Jun-N-terminal kinase (JNK) oppositely. In human bronchial epithelial cells overexpressing a kinase-mutated form of IκB kinase β (IKKβ-KM), the activation of NF-κB was inhibited. However, the G2/M cell cycle arrest and expression of GADD45α was substantially enhanced in response to arsenite in these cells. Expression of a dominant-negative mutant of SEK1 that blocks JNK activation decreased arsenite-induced GADD45α expression. Analysis of GADD45α expression in both wild-type and p53−/− fibroblasts indicated that the induction of GADD45α by arsenite was independent of the status of p53 protein. *Oncogene* (2001) **20,** 3585–3589.

Keywords: arsenite; GADD45α; NF-κB; JNK; cell cycle; p53

Emerging evidence indicates that arsenite acts as a potent environmental and occupational carcinogen by inducing cell cycle disregulation, cytogenetic alterations, anchorage independence, aneuploidy, and gene amplification, characteristics that are commonly observed in most tumor cells (Gebel, 2000; Guha Mazumder *et al*., 1998; Huang and Lee, 1998; Kayajanian, 2000; Styblo *et al*., 1999). The exact molecular mechanisms that account for the carcinogenic effect of arsenite remain to be investigated. It has been hypothesized that some of the effects of arsenite are due to aberrant regulation of mitogen-activated protein (MAP) kinases and several transcription factors, such as AP-1 and nuclear factor κB (NF-κB) (Cavigelli *et al*., 1996; Hamilton *et al*., 1998). These transcription factors are important in the regulation of a number of early response genes involved in cell migration, cell cycle progression, apoptosis, and carcinogenesis.

In mammalian cells, cell cycle transition is under the control of a tightly regulated network of cell division kinases (cdks) and a number of surveillance mechanisms, the so-called checkpoints (Russell, 1998). GADD45 family is one of several known checkpoint proteins that arrests cells at G2/M phase by inhibiting the activity of cyclin B/p34cdc2 complex in response to stress (Jin *et al*., 2000a; Wang *et al*., 1999b; Yang *et al*., 2000). Currently, three GADD45 isoforms have been identified. The different isoforms, named GADD45α, GADD45β and GADD45γ, show similar structural characteristics and expression patterns in response to extracellular stimuli (Takekawa and Saito, 1998). GADD45α was originally identified as a gene whose expression is rapidly induced in response to unreplicated DNA or DNA damage in a p53-dependent manner (Kastan *et al*., 1992). However, there are several reports suggesting that some stress inducers induce GADD45α in the absence of active p53 (Jin *et al*., 2000b; O'Reilly *et al*., 2000). Both NF-κB and c-Jun-N-terminal kinase (JNK) are well-established stress sensors and have been indicated as important mediators regulating stress-induced cell apoptosis (Karin and Delhase, 1998). However, little is known regarding their roles in the regulation of cell cycle in response to stressers.

To determine the roles of NF-κB in arsenite-induced cellular responses, we established several stably transfected human bronchial epithelial cell lines (BEAS-2B) in which the activation pathway for the NF-κB has been specifically inhibited by over-expression of a kinase-mutated form of IKKβ (IKK-KM) along with a NF-κB-dependent luciferase reporter construct. As a control, some cells were transfected with a vector expressing a wild-type IKKβ, the major IκBα kinase activated in response to a variety of inflammatory stimuli. In agreement with previous reports (Chu *et al*., 1999; Geleziunas *et al*., 1998), stable expression of wild-type IKKβ did not alter basal or inducible IKK or NF-κB activation compared to the transfection of an empty vector (data not shown). Analysis of NF-κB-dependent luciferase activity indicates that arsenite treatment of IKKβ-expressing cells induces a dose-dependent increase of luciferase reporter gene activity (Figure 1a). Peak activation of NF-κB-

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Received 12 October 2000; revised 2 March 2001; accepted 7 March 2001

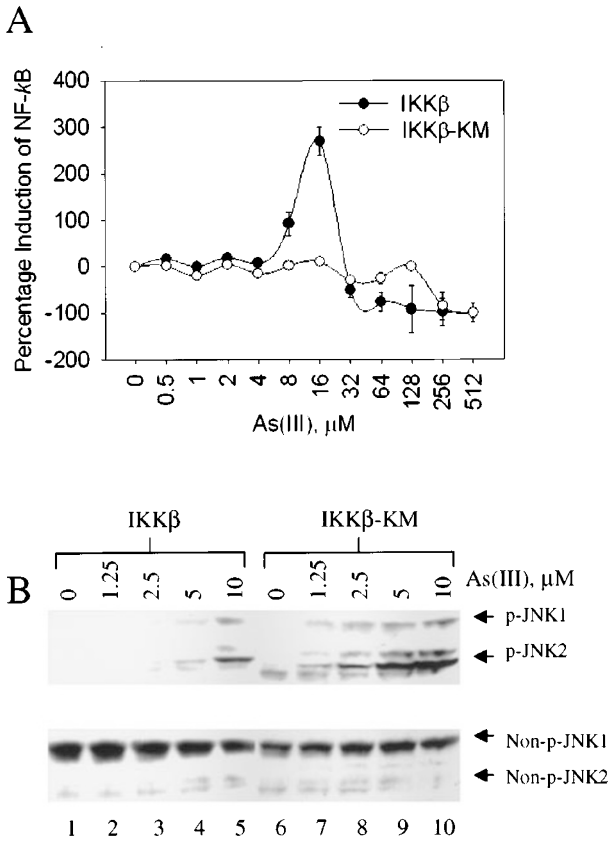

Figure 1 Inhibition of IKKβ blocked NF-κB activation and potentiated JNK activation induced by arsenite. (**a**) BEAS-2B cells stably transfected with a NF-κB-dependent luciferase reporter construct along with a vector expressing IKKβ or IKKβ-KM. Stably transfected cells were treated with various concentrations of arsenite for 12 h and then subjected to luciferase activity assay ($n=4$). (**b**) Both IKKβ cells and IKKβ-KM cells were treated with various concentrations of arsenite for 12 h. Total cellular proteins were subjected to the determination of JNK activation using antibody against phosphorylated JNK (upper panel). Non-phosphorylated total JNK protein was also determined as internal control

dependent reporter gene by arsenite occurred between 15 to 20 μM. In contrast, no induction of NF-κB-dependent luciferase activity was observed in the cells expressing IKKβ-KM in response to arsenite at any given concentrations. These results indicate that the IKKβ, a pivotal signaling component for NF-κB activation is defective in the cells expressing IKKβ-KM.

IKKβ gene knockout studies suggested that the activation pathways for the MAP kinases are normal in mice with IKKβ gene interruption (Li *et al*., 1999). Consistent with this notion, no impairment of Erk, JNK and p38 was observed in the cells stably expressing IKKβ-KM (Figure 1b and data not shown). In fact, we noticed a greater enhancement of JNK activation in IKKβ-KM cells than that in IKKβ cells (Figure 1b).

NF-κB has been considered as an important anti-apoptotic transcription factor under many circumstances (Chen *et al*., 1999). To determine whether NF-κB inhibition by expression of IKKβ-KM affects arsenite-induced cell growth, cell apoptosis and cell cycle profile were measured by flow cytometry. Both unsynchronized IKKβ-expressing cells and unsynchronized IKKβ-KM cells were stimulated with 10 μM arsenite for 48 h and then subjected to flow cytometry analysis. Under the basal condition, both IKKβ cells and IKKβ-KM cells had similar cell cycle distributions (Figure 2a). Arsenite treatment of IKKβ-KM cells caused a significant accumulation of the cells in G2/M phase with a concomitant disappearence of the G1 fraction. In contrast, in IKKβ cells, arsenite only induced a marginal change of G1 and G/M fractions. No sub-G1 fraction was detected in this assay in either type of cell stimulated with arsenite, indicating apoptosis did not occur.

The above results indicate that NF-κB inhibition by expression of IKKβ-KM potentiated G2/M phase cell cycle arrest. To further dissect the effects of NF-κB on the cell cycle alteration induced by arsenite, we analysed the expression of GADD45α, a recently identified inhibitor for cyclin B/p34cdc2 complex required for the G2/M phase transition (Jin *et al*., 2000a; Wang *et al*., 1999b; Yang *et al*., 2000). Figure 2b shows that after arsenite treatment, IKKβ-KM cells express more GADD45α protein than did IKKβ cells. The induction of GADD45α in both types of

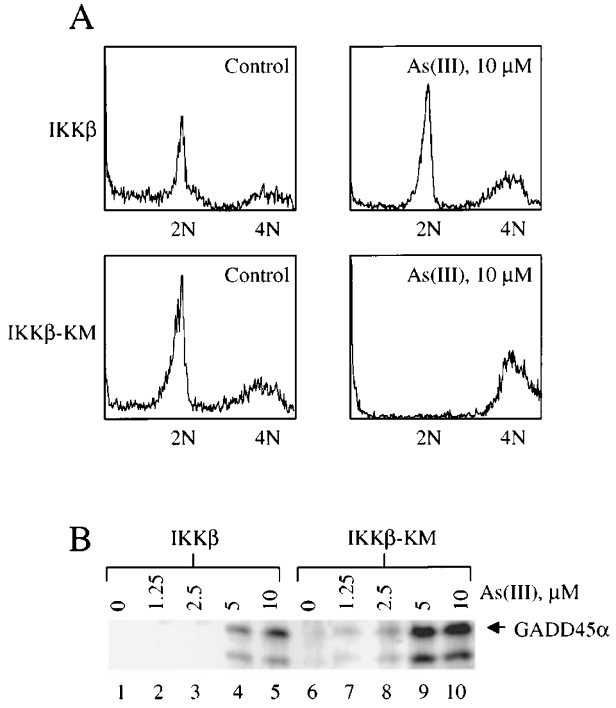

Figure 2 Inhibition of NF-κB potentiated arsenite-induced G2/M cell cycle arrest and GADD45α expression. (**a**) IKKβ cells (upper row) and IKKβ-KM cells (lower row) were either untreated or treated with 10 μM arsenite as indicated. Cell cycle profile was determined 48 h after exposure of the cells to arsenite. 2 N and 4 N DNA contents correspond to cells in G_1 and G_2/M, respectively. (**b**) IKKβ cells and IKKβ-KM cells were treated with different concentrations of arsenite for 12 h. Total cellular proteins were subjected to immunoblotting for the detection of GADD45α protein using 16% SDS–PAGE gels

cells occurs in a dose-dependent manner. Arsenite at both lower concentrations (1.25 and 2.5 μM) and higher concentrations (5 and 10 μM) induced a noticeable expression of GADD45α protein in IKKβ-KM cells (Figure 2b, lanes 7–10). In IKKβ cells, in contrast, only higher concentrations of arsenite induced appreciable expression of GADD45α (lanes 4 and 5).

Since we observed that NF-κB inhibition by expression of IKKβ-KM enhanced arsenite-induced JNK activation, we reasoned that the enhanced JNK activation in IKKβ-KM cells might be responsible for the elevated induction of GADD45α by arsenite. To determine whether JNK contributes to arsenite-induced GADD45α expression, IKKβ-KM cells were transiently transfected with a vector expressing a dominant-negative mutant of SEK1, an upstream kinase activating JNK. Compared to control vector (pcDNA) transfection (Figure 3, lanes 1–5), immunoblotting experiments showed a reduced activation of JNK in these IKKβ-KM cells transfected with mutated SEK1 (SEK1-KM) following arsenite treatment (Figure 3, top panel, lanes 6–10). The expression of SEK1-KM caused an appreciable suppression of GADD45α induction by arsenite (Figure 3, bottom panel, lanes 6–10).

GADD45α has been initially considered as a p53-target gene whose transcription/expression is under the control of the p53 activation (Hollander *et al*., 1993; Yu *et al*., 1999). The cells used in this study, however, were previously shown to express a functionally deficient p53 protein (Gerwin *et al*., 1992; Lehman *et al*., 1993). Furthermore, no change of the N-terminal serine 15 phosphorylation of p53 protein was noted in either IKKβ or IKKβ-KM cells in response to arsenite (Figure 4a). To address whether p53 is involved in arsenite-induced GADD45α expression, we next determined the induction of GADD45α by arsenite in

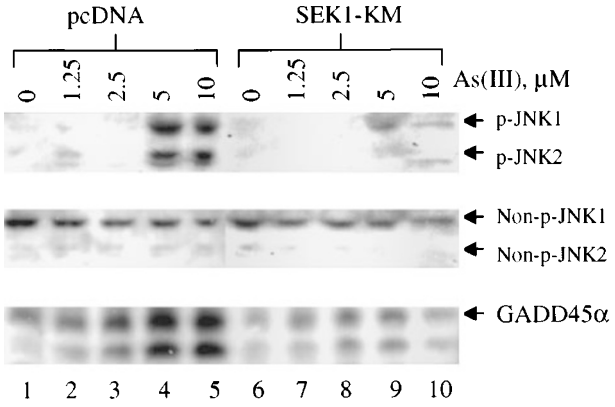

Figure 3 Inhibition of JNK activation reduced GADD45α induction by arsenite. IKKβ-KM expression cells were transiently transfected with a control vector pcDNA (lanes 1–5) or a vector expressing a kinase-mutated form of SEK1 to inhibit JNK activation pathway specifically (lanes 6–10). Cells were further treated with various doses of arsenite for an additional 12 h. Total cellular proteins were prepared and subjected to immunoblotting for the determination of phosphorylation of JNK1/2 (top panel), non-phosphorylated JNK1/2 (middle panel) and GADD45α expression (bottom panel)

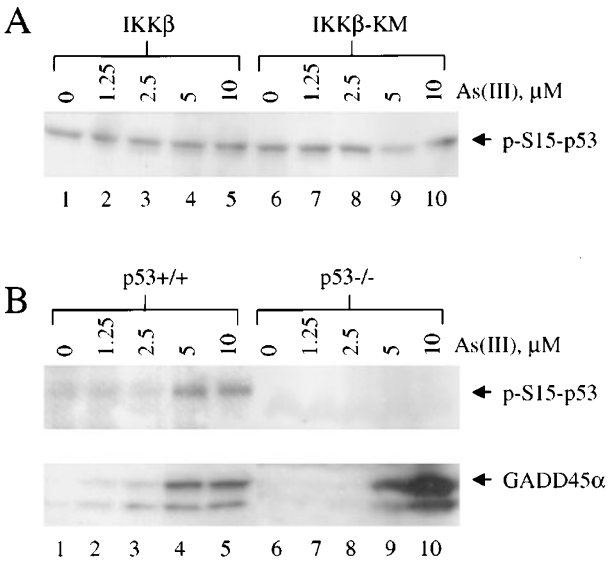

Figure 4 Arsenite-induced GADD45α expression was p53-independent. (**a**) BEAS-2B cells stably expressing IKKβ (lanes 1–5) or IKKβ-KM (lanes 6–10) were treated with various doses of arsenite for 12 h. Total proteins were prepared and subjected to immunoblotting using antibody against serine 15-phosphorylated p53 protein. (**b**) Mouse embryonic fibroblasts derived from both wild-type mice (p53+/+) (lanes 1–5) and p53 gene knockout mice (p53−/−) (lanes 6–10) were treated with various doses of arsenite for 12 h, respectively. Total cellular proteins were prepared and subjected to immunoblotting using antibodies against serine 15-phosphorylated p53 protein (upper panel) or GADD45α protein (lower panel)

mouse embryo fibroblast (MEF) cells derived from wild-type mice (p53+/+) and p53 gene knockout mice (p53−/−), respectively. Figure 4b showed that arsenite was able to induce the increase of serine 15 phosphorylation in p53+/+ cells (lanes 1–5) but not in p53−/− cells (lanes 6–10). Assessment of protein expression revealed no deficiency in GADD45α induction by arsenite in p53−/−cells. In fact, GADD45α induction by arsenite was actually higher in the p53−/− cells relative to p53+/+ cells (Figure 4b, bottom panel, compare lanes 9 and 10 with lanes 4 and 5. These results suggest that the induction of GADD45α by arsenite is p53-independent.

In this study we provide evidence that NF-κB and JNK oppositely regulate arsenite-induced expression of GADD45α, a cell cycle checkpoint protein inhibiting the activity of cyclin B/p34cdc2 complex and arresting cell cycle at G2/M phase (Jin *et al*., 2000a; Wang *et al*., 1999b; Yang *et al*., 2000). Inhibition of NF-κB by expression of a kinase-mutated IKKβ, IKKβ-KM, potentiated the G2/M phase cell cycle arrest and GADD45α expression in response to arsenite. Interruption of JNK signaling pathway by transfecting the cells with a kinase-mutated SEK1, on the other hand, decreased the induction of GADD45α by arsenite. In addition, using fibroblasts originated from wild-type and p53 gene knockout mice, we here demonstrated that arsenite-induced GADD45α is independent of the status of p53.

Mechanistic studies indicated a possible non-IKK signaling pathway mediating arsenite-induced NF-κB activation in several types of cells (Jaspers *et al*., 1999; Kapahi *et al*., 2000). Interruption of IKK activity by overexpression of IKKβ-KM, however, may either block the basal NF-κB activity or the auto-amplifying loop of NF-κB that requires IKKβ. The ability of arsenite on the activation of NF-κB appears to be dose- and cell type-dependent (Barchowsky *et al*., 1996; Hamilton *et al*., 1998). In the present study, we demonstrated that arsenite induced NF-κB-dependent reporter gene activity in a narrow dose-range (4–30 μM). Consistent with previous reports that higher concentrations of arsenite might interfere with the DNA binding of NF-κB (Roussel and Barchowsky, 2000), we observed an inhibitory effect of arsenite at concentrations of more than 30 μM on NF-κB reporter gene activity.

NF-κB has been considered as a positive transcriptional regulator for most NF-κB target genes. However, several recent reports have demonstrated that NF-κB might also serve as a negative regulator on its target genes (Du *et al*., 2000; Guttridge *et al*., 2000), such as MyoD and proteasome C3. Examination of the nucleotide sequence of human GADD45α promoter and introns showed several sequences homologous to consensus NF-κB-binding sites at the regions of promoter, intron 1 and intron 3. The intron 3 NF-κB-binding site (4529-GTAATTACCC-4538, GenBank L24498) is located at nucleotide position next to the BRCA1/ZBRK1 binding site (4497-GGGTTCA-GACTTT-4509), a site that is required for the p53-independent induction of GADD45α by BRCA1 (Harkin *et al*., 1999; Zheng *et al*., 2000). We are currently investigating if the binding of NF-κB on this site interferes with the transcriptional regulation of BRCA1/ZBRK1 on GADD45α.

The relationship between JNK and GADD45α is controversial. Studies by Takekawa and Saito (1998) indicated that GADD45α interacted and activated MEKK4, a MAPK kinase kinase activating JNK and p38. This observation, however, was contradicting by the two follow-up studies using gadd45α-gene knock-out embryonic fibroblasts or cells in which the GADD45α expression was diminished (Shaulian and Karin, 1999; Wang *et al*., 1999a). Using a dominant-negative SEK1 to block JNK activation induced by arsenite, we observed a reduced expression of GADD45α, indicating that JNK activation is an up-stream, rather than a down-stream event in GADD45α induction by arsenite.

Arsenite is a non-genotoxic carcinogen ubiquitously existed in a number of environmental and occupational settings (Simeonova and Luster, 2000; Simeonova *et al*., 2000). A better understanding of arsenite effects on MAP kinase activation, NF-κB activity and cell cycle regulation may provide better insights into elucidating the oncogenic properties of arsenite and establishing novel preventive and therapeutic strategies of arsenite-induced human diseases.

Acknowledgments

We are grateful to Dr Hiroyasu Nakano (Juntendo University, Japan) for providing pCR-Flag-IKKβ and pCR-Flag-IKKβ-KM (K44A) expressing vectors; to Dr Roger Davis (University of Massachusetts, Boston, MA, USA) for his gift of pcDNA-SEK1-KM vector; and to Drs Murali Rao, Val Vallyathan and Vince Castranova (NIOSH) for their critical reading of this manuscript. Fei Chen is supported by a Career Development Award under a cooperative agreement from the Centers for Disease Control and Prevention through the Association of Teachers of Preventive Medicine.

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