Requirement of Erk, but Not JNK, for Arsenite-induced Cell Transformation*

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Trivalent arsenic (arsenic, As3+) is a human carcinogen, which is associated with cancers of skin, lung, liver, and bladder. However, the mechanism by which arsenic causes cancer is not well understood. In this study, we found that exposure of Cl 41 cells, a well characterized mouse epidermal cell model for tumor promotion, to a low concentration of arsenite (<25 μM) induces cell transformation. Interestingly, arsenite induces Erk phosphorylation and increased Erk activity at doses ranging from 0.8 to 200 μM, while higher doses (more than 50 μM) are required for activation of JNK. Arsenite-induced Erk activation was markedly inhibited by introduction of dominant negative Erk2 into cells, while expression of dominant negative Erk2 did not show inhibition of JNK and MEK1/2. Furthermore, arsenite-induced cell transformation was blocked in cells expressing the dominant negative Erk2. In contrast, overexpression of dominant negative JNK1 was shown to increase cell transformation even though it inhibits arsenite-induced JNK activation. Our results not only show that arsenite induces Erk activation, but also for the first time demonstrates that activation of Erk, but not JNK, by arsenite is required for its effects on cell transformation.

Arsenite is introduced into the environment during energy production based on coal, oil shale, and geothermal sources. Once in the environment, arsenite represents a potential health hazard of unknown magnitude. Arsenite is associated with increased risks of human cancer of the skin, respiratory tract, hematopoietic system, and urinary bladder (1–4). Epidemiological investigations indicated that long-term arsenic exposure results in promotion of carcinogenesis, especially in lung and skin via inhalation and ingestion (5). Many cases of skin cancer have been documented in people exposed to arsenite through medical or other occupational exposures. It has been reported that high arsenic levels in drinking water (0.35–25 μg/L) increased risks of cancer of skin, bladder, kidney, lung, and colon (1, 2, 5, 6). Hence, arsenite is a well documented human carcinogen (5, 7).

Previously, several hypotheses have been proposed to describe the mechanism of arsenic-induced carcinogenesis (8–14). It has been suggested that arsenic induces chromosome aberration and sister chromatid exchange which may be involved in arsenite-induced carcinogenesis (11, 12). Recently, Zhao et al. (13) reported that arsenic may act as a carcinogen by inducing DNA hypomethylation, which in turn facilitates aberrant gene expression. Additionally, it was found that arsenite is a potent stimulator of extracellular signal-regulated protein kinase (Erk)1 and AP-1 transactivation activity and an efficient inducer of c-fos and c-jun gene expression (10, 14). Induction of c-jun and c-fos by arsenite is associated with activation of JNK (10). However, the role of JNK activation by arsenite in cell transformation or tumor promotion is unclear. We have established cell culture conditions for studying arsenite-induced cell transformation in this report. Furthermore, our data have shown that activation of Erk, but not JNK, is required for cell transformation induced by arsenite.

MATERIALS AND METHODS

Plasmids and Reagents—CMV-neo vector plasmid was constructed as previously reported (15, 16); dominant negative JNK1 (pcDNA-flag-JNK1 (APF)) was from Dr. Roger J. Davis, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School (17, 18); fetal bovine serum (FBS) and Eagle's minimal essential medium (MEM) were from BioWhittaker; LipofectAMINE was from Life Technologies, Inc.; TPA was from Sigma; rabbit polyclonal IgG against PKCα was from Santa Cruz Biotechnology; EGF was from Collaborative Research; luciferase assay substrate was from Promega; and PhosphoPlus MAPK antibody kit, phospho-MEK1α antibody, and p44/42 MAP kinase assay kit were from New England Biolabs.

Cell Culture—JB6 P1 mouse epidermal cell line, Cl 41, and its dominant negative Erk2-K52R transfectants, Cl 41 DN MAPK-DN B3 mass1 (19), as well as dominant negative JNK1 (pcDNA-flag-JNK1 (APF)) transfectant, C141 DN JNK1 mass2, were cultured in monolayers at 37 °C, 5% CO2 using Eagle's minimal essential medium containing 5% fetal calf serum, 2 mM L-glutamine, and 25 μg of gentamicin/ml (20, 21).

Generation of Stable Co-transfectants—JB6 Cl 41 cells were cultured in a 6-well plate until they reached 85–90% confluence. We used 1 μg of CMV-neo vector with or without 12 μg of dominant negative JNK1 (pcDNA-flag-JNK1 (APF)) plasmid DNA and 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced by 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection the cells were digested with 0.033% trypsin and cell suspensions were plated into 75-ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfectants were identified by using phospho-specific antibodies against phosphorylated JNK. Stable transfected Cl 41 mass1, and Cl 41 DN JNK1 mass2, were established and cultured in G418-free MEM for at least two passages before each experiment.

Phosphorylation Analysis for Erk and JNK—Immunoblot analysis for phosphorylated proteins of Erk and JNK was carried out using phospho-specific MAPK antibodies against phosphorylated sites of Erk and JNK as described previously (22, 23). Antibodies were from New England Biolabs and used according to the manufacturer's recommendations. PKCα was used as an internal control for sample protein loaded. Antibody bound proteins were detected by chemiluminescence (ECL, New England Biolabs).

JNK Activity Assay—JNK assay was carried out as described previ-

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1 The abbreviations used are: Erk, extracellular signal-regulated protein kinase; AP-1, activated protein-1; BME, basal medium Eagle; CMV, cytomegalovirus; EGF, epidermal growth factor; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; TPA, 12-O-tetradecanoylphorbol-13-acetate.
were starved for 48 h in 0.1% FBS MEM at 37 °C, 5% CO2 atmosphere in each well of 6-well plates. The cultures were maintained in 37 °C, 5% CO2 incubator for 4 weeks. Then, another 3 ml of 0.33% BME agar containing 15% FBS was added to each well and the cultures continued in the 5% CO2 incubator for another 4 weeks. The TPA- and arsenite-induced cell colonies were scored at the end of the second and eighth week after cells were exposed to TPA or arsenite, respectively.

Fig. 1. Cell transformation induced by low concentrations of arsenite. C141 cells (10³ cells) were or were not exposed to TPA (10 ng/ml) or arsenite at the concentration and times indicated in the figure legends. Then, the cells were washed once with ice-cold phosphate-buffered saline and exposed to UVC (60 J/m²) or arsenite at the concentration and times indicated in the figure legends. Then, the cells were washed once with ice-cold phosphate-buffered saline and lysed in 300 µl of lysis buffer per sample (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₃, 1 mM leupeptin). The lysates were sonicated and centrifuged, and the supernatant was incubated with 2 µl of N-terminal c-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 (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂). The kinase reactions were carried out in the presence of 100 µM ATP at 30 °C for 30 min. c-Jun phosphorylation was selectively measured by Western immunoblotting using a chemiluminescent detection system and specific c-Jun antibodies against phosphorylation of c-Jun at serine 63.

Erk Activity Assay—Erk activity was carried out as described using the protocol of New England Biolabs. In brief, JB6 C141 transfectants were starved for 48 h in 0.1% FBS MEM at 37 °C, 5% CO₂ atmosphere incubator. The cells were exposed to arsenite at the concentration and times indicated. The cells were washed once with ice-cold phosphate-buffered saline and lysed in 300 µl of lysis buffer per sample (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM NaVO₃, 1 mM leupeptin). The lysates were sonicated and centrifuged, and the supernatant was incubated with phospho-specific p44/p42 MAP kinase (Thr202/Tyr204) monoclonal antibody for 4 h at 4 °C, then, incubated with protein A-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 (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂). For determination of Erk-induced phosphorylation of Elk-1 measured by quantitative immunoblotting with phospho-Elk-1 antibody, the kinase reactions were carried out in the presence of 2 µl of Elk-1 fusion protein and 100 µM ATP at 30 °C for 30 min. Elk-1 phosphorylation is selectively measured by Western immunoblotting using a chemiluminescent detection system and specific antibodies against phosphorylation of Elk-1 at serine 383. For measurement of Erk-induced phosphorylation of Elk-1 by direct assessment of phosphate incorporation from [γ-32P]ATP, the kinase reactions were carried out in the presence of 2 µg of Elk-1 fusion protein and 100 µM cold ATP. The results were presented as SDS-polyacrylamide gel electrophoresis autoradiography and the phospho-Elk-1 bands were cut off and counted.

Anchorage-independent Transformation Assay—JB6 C141 cells or their transfectants (1 × 10⁵ cells) were exposed to arsenite and TPA in 1 ml of 0.33% BME agar containing 15% FBS over 3.5 ml of 0.5% BME agar containing 15% FBS in each well of 6-well plate. The cultures were maintained in 37 °C, 5% CO₂ incubator for 4 weeks, then another 3 ml of 0.33% BME agar containing 15% FBS was added to each well and the cultures continued in the 5% CO₂ incubator for another 4 weeks. The TPA- and arsenite-induced cell colonies were scored at the end of the second and eighth week after cells were exposed to TPA or arsenite, respectively.

Fig. 2. Time course of activation of Erk and JNK by arsenite. A, for assay of phosphorylated Erk and JNK, 8 × 10⁵ of JB6 C141 cells were seeded into each well of the 6-well plates. After culturing at 37 °C for 24 h, the cells were starved for 48 h by replacing medium with 0.1% FBS MEM. Four h before cells were exposed to TPA or arsenite, the medium was changed to serum-free MEM. Then, the cells were or were not exposed to TPA (10 ng/ml) or arsenite (200 µM). The cells were harvested and JNK activity was measured as described previously (22, 23). B, assays of JNK activity. JB6 C141 cells were cultured in monolayers in 100-mm diameter dishes to 90% confluency. The cells were starved by changing the medium with 0.1% FBS MEM medium for 48 h. The cells were or were not exposed to 200 µM arsenite for the times indicated. The cells were harvested and JNK activity was measured as described previously (24).
were not exposed to UVC (60 J/m²), TPA (10 ng/ml) for 30 min or medium with 0.1% FBS MEM medium for 48 h. Then, the cells were on dishes to 90% confluency. The cells were starved by changing the medium to serum-free MEM. Then, the cells were or were not exposed to TPA or arsenite, the medium was changed to serum-free MEM. Four h before cells were exposed to arsenite, the medium was changed to serum-free medium. Then the cells were treated with different concentrations of arsenite for 15 min. The cells were extracted at the time points as indicated. The phosphorylated proteins of ERK (A) and JNK (B) as well as internal control protein kinase C (PKC) were determined as described in the phospho-specific antibody kit (New England Biolabs). C, assay of JNK activity. JB6 C1 41 cells were cultured in monolayers in 100-mm diameter dishes to 90% confluence. The cells were starved by changing the medium with 0.1% FBS MEM medium for 48 h. Then, the cells were or were not exposed to UVC (60 J/m²), TPA (10 ng/ml) for 30 min or different concentrations of arsenite for 120 min. The cells were harvested and JNK activity was measured as described previously (24).

\[ ^{[\text{H}]} \text{thymidine incorporation assay. For the study of the influence of expression of dominant negative mutants of JNK, or Erk, on cell proliferation, } 5 \times 10^5 \text{ of CI 41 AP-1 mass } \alpha, \text{ CI 41 MAPK-DN } B_2, \text{ mass } \alpha, \text{ or CI 41 DN-JNK, mass } \alpha \text{ cells were seeded into each well of a 96-well plate. After } 12 \text{ h of culture, the cells were or were not treated with TPA (10 ng/ml) or EGF (10 ng/ml) for } 24 \text{ h. Then } 0.5 \text{ M Ci of } ^{[\text{H}]} \text{thymidine was added to each well. The cells were harvested } 12 \text{ h later, and the incorporation of } ^{[\text{H}]} \text{thymidine was detected with a liquid scintillation counter. The results were presented as counts per minute.}

RESULTS

Induction of Cell Transformation by Low Concentration of Arsenite—Arsenite is a known carcinogen (1–4). Some previous studies have suggested that arsenite acts as a tumor promoter rather than an initiator (25). However, there is no convenient anchorage-independent cell transformation model for studying the molecular mechanism of the tumor promotion effect of arsenite. The mouse epidermal JB6 cell system is a model to study tumor promotion in vitro. To study whether arsenite induces JB6 cell transformation, we exposed JB6 C1 41 cells to arsenite in soft agar. Anchorage-independent colonies were observed in the eighth week after arsenite exposure. However, the transformation rate was lower and the colonies were smaller than those induced by TPA, which were observed after 2 weeks of exposure (Fig. 1). Also, cell transformation can only be observed in cells exposed to low concentration (25 μM) of arsenite, while no cell transformation colonies were observed at high concentrations of arsenite (50–100 μM) (Fig. 1A).

Differential Activation of Erk and JNK by Arsenite—Previously, we and others have reported that signal transduction pathways leading to AP-1 activation are required for cell transformation induced by tumor promoters, such as TPA and EGF to occur (23, 26, 27). It was also reported that arsenite is a potent stimulator of AP-1 activity and JNK activity (10). To study the molecular basis for neoplastic transformation activity of arsenite, we examined its effects on MAP kinase signal transduction pathways in our system. We found that arsenite could induce activation of both JNK and Erk. However, the activation of JNK and Erk by arsenite is different. During the time course and dose-response studies, marked Erk activation could be observed at 15 min after exposure and at all dosages studied (Figs. 2 and 3). There was no significant induction of Erk by arsenite after a 30-min exposure (Fig. 2). In contrast, activation of JNK was only observed at high dosage (>50 μM).
and after 60 min of exposure (Figs. 2 and 3). These results indicated that dosages of arsenite for activation of Erk, but not JNK, are consistent with those for cell transformation. Therefore, Erk activation may be involved in arsenite-induced cell transformation. Interestingly, we also observed phosphorylated Erk-like bands in cells treated with arsenite for 15 min when we used antiphospho-JNK antibody (Fig. 2A). These bands were very consistent with the Erk activation when we used the anti-phosphorylated Erk antibody (Fig. 2A). The reason for this may be due to cross-reaction of antiphospho-JNK antibody with phosphorylation of Erk (Fig. 2A). To directly measure the Erk activity induced by arsenite, we assessed the Erk activity by measuring phosphate incorporation from $\gamma^{-32}$PATP. The results showed that exposure of cells to arsenite caused markedly an increase of phosphate incorporation to the Erk substrate Elk-1 (Fig. 4A). These increases appear to be in a dose-dependent manner (Fig. 4A). The maximus induction of Erk activity by arsenite is similar to these induced by 10 ng/ml of TPA or EGF (Fig. 4A). We have compared dose-responses between Erk phosphorylation and Erk-induced phosphorylation of Elk-1 by direct measurement of phosphate incorporation from $\gamma^{-32}$PATP. Results from both methods are generally correlated. However, the method by using $\gamma^{-32}$PATP is more sensitive than that by directly measuring Erk phosphorylation (Fig. 4, A and B).

No Inhibition of Arsenite-induced Cell Transformation by Expression of Dominant Negative JNK1—To rule out a role of JNK activation in arsenite-induced cell transformation, we established a dominant negative JNK1 stable transfectant, Cl41 DN-JNK1 mass2. The dominant negative JNK1 mutant (APF) is the double point mutation that changes the phosphorylation sites Thr183 and Tyr185 to Ala and Phe, respectively (18, 19). This mutation blocks JNK activation. The stable transfectant was generated by “mass culture selection” of pooled clones as described previously (23). To determine whether dominant negative JNK1 have blocking effects on JNK activation, we compared the JNK phosphorylation induced by arsenite between dominant negative transfectant Cl41 DN-JNK1 mass2 and the control transfectant Cl41 CMV-neo mass1. The results show that arsenite-induced JNK phos-
phorylation was impaired by introduction of dominant negative JNK1, while there were no significant effects on arsenite-induced Erk phosphorylation (Fig. 5). Expression of dominant negative JNK1 was shown to increase the cell transformation rate by arsenite (Fig. 6). Taken together with the results regarding the difference between the dose-response curves for JNK activation (Fig. 3, B and C) and the transformation (Fig. 1) at different arsenite concentrations, we ruled out the possible role of arsenite-induced JNK activation in cell transformation.

**Inhibition of Erk Activation Blocks Arsenite-induced Cell Transformation**—The results described above revealed that Erk activation by arsenite may be involved in its cell transformation. To test this possibility, we used dominant negative Erk2-K52R stable transfecant, CI 41 MAPK-DN B3 mass1 (19). We found that overexpression of dominant negative Erk2 blocks arsenite-induced Erk activation and cell transformation (Figs. 6 and 7A), while there is no marked influence on arsenite-induced phosphorylations of JNK or MEK1/2 (Fig. 7, B and C). However, the cell proliferation of the C1 41 MAPK DN B3 mass1 cells are not significantly different from those of C1 41 AP-1 mass1 cells and CI 41 DN-JNK1 mass1, Table I). This data is consistent with previous findings that the cell transformation is dissociated from mitogenesis in JB6 cells (15, 50). These data also indicate that lack of cell transformation of C1 41 MAPK DN B3 mass1 cells in response to arsenite is not due to inhibition of cell growth by transfection of dominant negative Erk2. Our results demonstrate that Erk activation, but not JNK activation, is required for arsenite-induced cell transformation.

**DISCUSSION**

This study investigated the arsenite-induced signal transduction pathway and its role in arsenite-induced cell transformation. Exposure of JB6 Cl 41 cells to low concentrations (<25 μM) of arsenite lead to cell anchorage-independent growth, while there are no cell transformation colonies at a high concentration (100 μM) of arsenite. In contrast, Erk activation could be seen at all dosages studied, whereas JNK activation could only be observed at high doses of arsenite. Furthermore, introduction of dominant negative Erk2-K52R into cells blocks Erk activation as well as cell transformation induced by arsenite, while it does not block JNK activation and MEK1/2 activation. In addition, overexpression of dominant negative JNK1 increases arsenite-induced cell transformation even though it blocks arsenite-induced JNK activation. These results demonstrate that arsenite induces Erk activation and for the first time provides strong evidence that Erk activation, but not JNK activation, is required for arsenite-induced cell transformation.

Arsenic is the first metal to be identified as a human carcinogen (28). Arsenic can exist in trivalent and pentavalent forms and in organic or inorganic compounds (7, 28). Both inorganic and organic forms are absorbed by human and animal skin (7). Autoradiographic animal studies show that following chronic exposure, arsenic accumulates in the skin and hair (7). It is known that long-term arsenic exposure can result in carcinogenesis (5). Many cases of skin cancer have been reported among people exposed to arsenic through medical or occupational exposures to trivalent arsenic compounds (7). Epidemiological studies in areas of high arsenic in drinking water are associated with increased risk of cancer of skin, bladder, kidney, lung, and colon (6). Recently, Zhao et al. (13) reported that chronic exposure of culture cells to low concentrations of arsenic lead to 70% of cells exhibiting morphological changes indicative of transformation at 18 weeks of exposure (13). However, in animal experiments, arsenic exposure shows no reliable evidence of its carcinogenicity (29). One possible explanation for the positive carcinogenicity of arsenic compounds in humans and the negative carcinogenicity in experimental animals is that arsenite may be a tumor promoter, but not an initiator of carcinogenesis (10, 25). This explanation is supported by the observation that arsenic acts with other agents to alter or enhance other biological effects, which are potentially involved in progression of carcinogenesis (30–33). For example, arsenic accumulation in skin increases the sensitivity of skin to ultraviolet (UV) light and sequentially increased its carcinogenic effect (34, 35). Smoking may synergistically interact with arsenic (30, 31).

The JB6 C1 41 cell is a post-initiated mouse epidermal cell line and represents an excellent in vitro model for studying
Role of MAPKs in Arsenite-induced Cell Transformation

**TABLE I**

Influence of expression of dominant negative mutants of Erk or JNK, on cell proliferation

| Cell line                  | Medium | TPA | EGF |
|----------------------------|--------|-----|-----|
| Cl 41 AP-1 mass<sub>1</sub>| 179,716 ± 11,629 | 196,598 ± 6,781 | 191,523 ± 3,032 |
| Cl 41 MAPK-DN B<sub>1</sub> mass<sub>1</sub>| 164,923 ± 6,038 | 215,939 ± 7,290 | 231,675 ± 18,763 |
| Cl 41 DN-JNK<sub>1</sub> mass<sub>1</sub>| 187,016 ± 2,284 | 257,178 ± 14,931 | 257,285 ± 3,829 |

* Cell proliferation was done as described under "Materials and Methods." The results were presented as the average and standard deviation of counts per minute for assays of triplicate wells.

During the preparation of this paper, Ludwig et al. (14) reported that arsenite induces Erk activation in the human embryonic kidney cell line HEK292 and this Erk activation appears to be late phase and dependent on activation of p38 kinase. However, in JB6 cells, we found that Erk activation occurs very early, while p38 kinase activation was observed later than 60 min after cell exposure to arsenite. Our recent studies indicated that expression of dominant negative p38 kinase in Cl 41 cells could not block arsenite-induced Erk activation, while it blocks p38 kinase activation (data not shown). Therefore, Erk activation is not dependent on p38 kinase activation in JB6 cells. The reason for this may be due to the difference of cell lines used. Taken together, our results strongly suggest that arsenite induces Erk activation and Erk activation induced by arsenite is required for its cell transformation to occur. The biological role of JNK activation induced by arsenite is under current study.

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