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Arsenic carcinogenicity: Relevance of c-Src activation

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Abstract

Environmental and occupational exposure to arsenic is associated with increased risk of skin, urinary bladder and respiratory tract cancers. Increasing evidence indicates that arsenic acts at the level of tumor promotion by modulating the signaling pathways responsible for cell growth. One of this pathways might include c-Src dependent EGFR and MAPK activation. (Mol Cell Biochem **234/235**: 277–282, 2002)

Key words: gene expression, reactive oxygen species, skin cancer, bladder cancer

Epidemiological data of arsenic toxicity

Trivalent and pentavalent forms of inorganic arsenic are ubiquitous elements found in nature that result in significant human exposure. Oral exposure to arsenic occurs primarily from contamination of drinking water and food constituents and is particularly high in certain regions of the world including areas of the Southwestern United States, Eastern Europe, India, China, Taiwan and Mexico [1, 2]. Humans can also be exposed to arsenic through inhalation. This occurs primarily in occupations involved in mining/smelting operations, agriculture or microelectronics [3, 4]. Epidemiologic studies from Finland, Taiwan, China, Bangladesh, Mexico, Southwestern United States and Central and South America have demonstrated that exposure to inorganic arsenic is associated with increased risk of cancers of the skin, and internal organs including the urinary bladder, respiratory tract, liver and kidney in populations [3–8]. Arsenic-induced skin cancers usually develop 20–30 years after exposure, and occur in sun-exposed as well as non-exposed areas. The types of skin tumors found include either Bowen's disease, squamous cell carcinomas, basal cell carcinomas or combined lesions [9–11]. The key to identifying patients with arsenic-induced skin tumors is that they normally occur at multiple sites and unusual locations. Internal tumors are also common and are most frequently associated with the bladder. The association between arsenic exposure and urinary bladder cancers, typically transitional cell carcinomas, has been observed in the same endemic areas of the world where skin cancer populations have been identified [3, 6, 12, 13]. Lung tumors from arsenic are often associated with occupational exposure, such as smelters or agriculture workers, and occur from inhalation [14]. On the basis of numerous epidemiological studies, arsenic has been classified as a potent human carcinogen; and population cancer risk due to arsenic has been suggested to be comparable to environmental tobacco smoke and radon in homes with risk estimates of around 1 per 1000 [11]. In addition to neoplasia, other pathological manifestations of chronic arsenic exposure include skin hyperpigmentation and hyperkeratosis [9, 15], as well as vascular disease [16, 17]. In contrast to carcinogenicity, little research has been conducted regarding the vascular effects of arsenic exposure. Circulatory manifestations of arseniasis include increased prevalence of ischemic heart disease and peripheral vascular disease. The latter is commonly known as blackfoot disease in southwestern Taiwan.

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Epigenetic mechanisms of arsenic carcinogenicity

Understanding the mechanism of action for arsenic carcinogenicity is an important factor in assessing cancer risk, particularly at low levels of exposure. The mechanism by which arsenic causes cancer has been under intense investigation (reviewed in [18, 19]). Although arsenic itself is not mutagenic, some deleterious effects on DNA have been observed including inhibition of DNA repair, potentiation of DNA damage by other agents, sister chromatid exchange and gene amplification [20–22]. However, these effects do not adequately explain arsenic carcinogenic properties, and epigenetic mechanisms have been proposed (reviewed in [18]). Central to the epigenetic hypothesis is the evidence indicating that arsenic stimulates cellular stress responses and proliferation by affecting specific cell signal transduction pathways. Increasing evidence supports the hypothesis that arsenic shares many properties of tumor promoters. Similarly to classic tumor promoters, such as phorbol esters, okadaic acid and UV radiation, arsenic activates transcription factors, such as AP-1 and AP-2, and induces immediate early genes including *c-fos*, *c-jun* and *c-myc* [23–25] whose products stimulate cell proliferation. Consistent with these observations, arsenic induces a moderate, albeit persistent increase in keratinocyte proliferation *in vitro* as evidenced by increases in thymidine incorporation [26], cell cycling [27], labeling of Ki-67, a proliferating cell marker [27] and ornithine decarboxylase activity [28]. Recently, it has been demonstrated that fibroblasts [29] and human urinary bladder epithelial cells [30] also respond to arsenic *in vitro* by moderate enhanced cell growth. Electromobility shift assays (EMSAs) and proliferating cell nuclear antigen (PCNA) immunostaining has helped establish that activation of the AP-1 transcription factors and hyperplasia can occur concurrently in urinary bladder epithelial cells and epidermis of mice and rats within 8 weeks following exposure to arsenite [26, 30, 31]. The ability of arsenic to activate AP-1 *in vivo* has recently been confirmed in transgenic mice which contain an AP-1 luciferase reporter construct [32]. Characterization of arsenic-induced AP-1 DNA binding complex has demonstrated that the complex consists of fos/jun heterodimers [23, 30], a common heterodimer responsible for regulating cell mitogenesis [33]. Of particular relevance to these studies is evidence that *c-jun* expression occurs simultaneously with urinary bladder transitional carcinoma [34, 35]. Additionally, cDNA microarray analysis of human uroepithelial cell line, UROtsa, identified genes activated by arsenic whose products are involved in cell cycle regulation and malignancies, such as early-growth response gene (EGR)-1, growth arrest and DNA damage (GADD)153, GADD45, and repair associated protein (RAD) [30].

Taken together, these data suggest that arsenic can act as a co-carcinogen by providing a microenvironment of a ready-to-go cell growth machinery. Consistent with this hypothesis are *in vivo* studies demonstrating increased numbers of phorbol ester-induced papillomas in arsenic-treated Tg:AC transgenic mice [26], a tumor initiated mice which over-expresses H-ras, and hairless Skh1 mice initiated with ultraviolet radiation [36]. This is also consistent with studies demonstrating that urinary bladder cancers can develop in arsenic treated rats following exposure to N-butyl-N-(4-hydroxybutyl) nitrosamine [37], a potent chemical tumor initiator.

Arsenic-induced mitogen activated protein kinase (MAPK) and EGF receptor (EGFR) activation

The expression of genes involved in the regulation of cellular growth has been related historically to the action of growth factors, such as epidermal growth factor (EGF), and their ability to induce a cascade of events following receptor binding, and this includes activation of receptor tyrosine kinases and phosphorylation of members of the MAPK family. The MAPKs are a family of serine/threonine kinases that control cellular responses to growth, apoptosis, and stress signals. There are four main MAPKs, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNKs), p38 MAPKs, and big MAPK-1. All members of MAPK family are activated through complex phosphorylation cascades and their activation leads to the phosphorylation of a variety of proteins with diverse functions including downstream kinases, transcription factors and components controlling protein synthesis [38, 39]. Although there is a similarity between MAPKs' characteristics, distinct regulatory molecules and specific targets of their action have been identified. For example, ERKs are highly activated in response to mitogenic stimuli and may be required for cell growth because they phosphorylate the ternary complex factor (TCF), which promotes transcription of the immediate-early gene *c-fos* or *elk-1* [38]. In contrast, JNK, as well as p-38, predominate in response to cellular stresses and are primary associated with activation of *c-jun* and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), respectively [40]. Additionally, important cell-specific differences appear to influence the participation of particular cellular factors involved in these signal transduction pathways.

Several studies have been suggested that arsenic activates gene expression by modulation of intracellular phosphorylation events and MAPK. Cavigelli *et al*. [41] demonstrated that arsenite, in contrast to arsenate, induces JNK and p38 activation in HeLa cells and this occurs in parallel with AP-1 activation and *c-jun* /*c-fos* gene expression. They suggested

that arsenite interacts with the sulfhydryl groups on cysteine at the catalytic site of JNK phosphatase to inhibit its activity, resulting in prolonged JNK and p38 activation. A study conducted with PC12 cells has demonstrated that arsenite treatment potently activates both JNK and p38, but only moderately activates ERK [42]. The activation of all three kinases by arsenic was prevented by addition of NAC, suggesting a role of glutathione and/or oxidative stress in the initiation of this response. It has been suggested that arsenite may induce ERK activation in PC-12 cells by binding to the cysteine-rich domains of the epidermal growth factor receptor (EGFR) and subsequent activation of the Ras-dependent pathway [43]. MAPK activation by arsenite has been shown to occur in the JB6 mouse epidermal cell line, as evidenced by ERK phosphorylation and increased ERK activity at doses ranging from 0.8–200 μM while higher doses (> 50 μM) were required for JNK activation [44]. Furthermore, arsenite-induced cell transformation of this cell line was blocked by overexpression of dominant negative ERK, indicating a direct role of ERK in arsenic related cell transformation. Recently, we demonstrated that, in the UROtsa, human uroepithelial cell line as well as urinary bladder, arsenic-activated AP-1 and cell cycle progression, are accompanied by EGFR and ERK activation [45]. This is important as arsenic exposure is associated with urinary bladder cancer and EGFR activation is an integral part of human urinary bladder carcinogenesis [46].

Arsenic-induced EGFR phosphorylation is associated with triggering of a cascade of events including recruitment of adaptor proteins such as Shc and GRB2 to the cytoplasmic domain of the EGFR and consequent ERK activation [42, 45]. Although this similarity, there are some differences between EGF and arsenic-induced EGFR phosphorylation, at least in UROtsa cells [45]. First, monoclonal antibody, specific for one of the major autophosphorylation sites of EGFR (Tyr^{1173}) discriminates between EGF- and arsenic-induced EGF receptor phosphorylation. Secondly, the ability of arsenic to phosphorylate EGFR and activate ERK is slightly, but consistently, delayed compared to the endogenous ligand. EGF stimulates tyrosine phosphorylation of its receptor by homodimerization of EGFR and activation of receptor tyrosine kinases [47]. The stressor-induced tyrosine phosphorylation of EGFR might be caused by activation of receptor tyrosine kinases, as a result of direct effects on the receptor and its kinases or dephosphorylation events through inactivation of protein tyrosine phosphatases, or alternatively, by non-receptor tyrosine kinases including c-Src. Phosphotyrosine phosphatases have highly conserved sulfhydryl groups in their catalytic site and they can be potential targets for oxidation by UV or sulfhydryl reagents [48]. Arsenite has been shown to activate JNK through sulfhydryl dependent inactivation of JNK phosphatase [41]. However, c-Src is strongly applicable in arsenic-induced EGFR in UROtsa cells, since arsenic-induced EGFR or ERK phosphorylation, in contrast to the EGF response, was prevented by PP-1, a selective inhibitor of Src activity or by transfection with a dominant-negative c-Src construct [45]. Arsenic activates c-Src in these cells and the activation precedes EGFR and ERK phosphorylation. In addition, a similar interaction between c-Src and EGFR occurs *in vivo* in urinary bladder of mice exposed to arsenic through drinking water.

Non-receptor tyrosine kinase c-Src

The cytoplasmic tyrosine kinase Src has been studied for many years, either in the form of v-Src, an activated form encoded by the *v-src* transforming gene of Rous Sarcoma virus (RSV), or in the form of c-Src, encoded by the *c-src* gene of multicellular organisms. Several lines of evidence have demonstrated that c-Src is associated with the inner cell membrane, particularly in the vicinity of growth factor or integrin clusters [49]. c-Src activation involves phosphorylation and de-phosphorylation events which can be triggered by diverse stimulants including, growth factors, integrins or conformational changes from disulfide bond interactions which result in aggregation of c-Src molecules [49]. Recently, the latter paradigm has been shown to occur by nitric oxide [50]. Arsenic may act through some of these mechanisms via its reactivity to vicinal sulfhydryl groups. Macromolecules, such as EGFR, integrins, c-Src or protein phosphatases, contain high numbers of vicinal sulfhydryls and are capable of reacting with arsenic. Previous studies established that arsenic serves as a ligand for receptors which have vicinal thiols in their binding sites, such as glucocorticoid receptors [51]. Alternatively, inorganic arsenic may accumulate in the extracellular matrix (ECM), bound to keratin or other sulfhydryl-containing molecules in skin or urinary bladder tissue, resulting in cellular integrin rearrangements and c-Src activation. In this respect, we recently demonstrated that inorganic arsenic accumulates in the bladder epithelium following oral exposure [18, 45]. ROS or modulation of GSH intracellular levels may also play a role in arsenic-induced c-Src activation. Recent studies on c-Src activation demonstrated its involvement in redox-sensitive signaling such as that induced by UV radiation and ROS including H_2O_2 [52–54].

c-Src has been demonstrated to be involved in multiple pathways and interactions that have significant roles in carcinogenicity and also in vascular pathophysiology [49, 52]. c-Src can physically associate with EGFR resulting in two unique tyrosine phosphorylations of the receptor (Tyr^{845}, Tyr^{1101}), distinct from the autophosphorylation sites, and potentiate the receptor's mitogenic/tumorigenic activity [55, 56]. The synergistic role of c-Src and EGFR in tumorigenesis was confirmed by increased DNA synthesis in cells co-overexpressing both c-Src and EGFR compared to cells

overexpressing either of these molecules alone [57]. Parallel activation of c-Src and EGFR has been identified in many human cancers [58]. Arsenic-induced c-Src-dependent activation of EGFR may serve to augment the effects of growth factors and carcinogens which is consistent with the hypothesis of a co-promoting role of arsenic in carcinogenesis. Alternatively, arsenic through c-Src activation can assure EGFR independent ERK activation. It has been demonstrated that Src can directly activate Shc or FAK, creating binding sites for Grb2 and activation of Ras dependent ERK phosphorylation [55, 59]. ERK phosphorylation by arsenic in EGFR deficient B82 cells, has been demonstrated [45]. In addition to ERK activation, c-Src can be responsible for the tyrosine phosphorylation of numerous actin-binding proteins, such as cortactin, and can impact the cortical actin assembly [60]. The c-Src-dependent mechanisms of cytoskeleton reorganization might also contribute to arsenic-induced pathophysiological processes. Other important functions of c-Src, that can be related to the molecular mechanisms of arsenic toxicity include its ability to phosphorylate Cγ [61] and Janus kinase (JAK)-2 [62] as well as activate JNK [53] and big MAPK-1 [63]. In addition to the role of c-Src in carcinogenesis, recent evidence has demonstrated that c-Src is an important signaling molecule in vascular pathophysiology [52] and this respect, may be involved in arsenic related vascular effects.

In conclusion, c-Src is a candidate molecule in arsenic initiation of expression of genes related to cell cycle regulation (Fig. 1). c-Src might facilitate gene expression cascades through multiple mechanisms, including EGFR- or FAK-dependent ERK activation. A role of c-Src in JNK related arsenic-induced gene expression may also be considered. Understanding the molecular mechanisms of arsenic action will help determine the safe exposure levels and may help provide targets for specific therapeutic or prevention interventions.

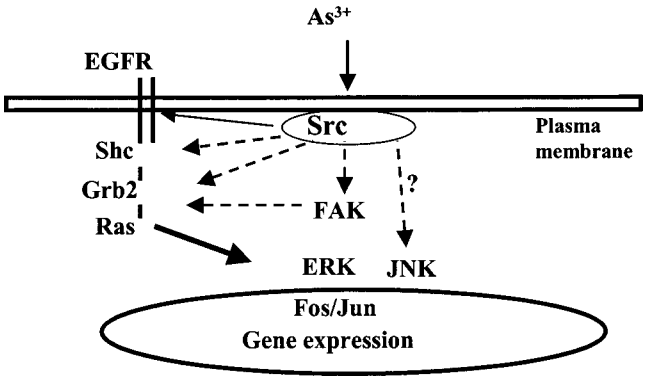

Fig. 1. Schematic representation showing the role of c-Src in arsenic-induced signaling pathways of growth-related gene expression.

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