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Arsenic inhibition of the JAK-STAT pathway

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The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is an essential cascade for mediating normal functions of different cytokines in the development of the hematopoietic and immune systems. Chronic exposure to arsenic has been found to cause immunotoxicity and has been associated with the suppression of hematopoiesis (anemia and leukopenia). Here, we report the novel finding of arsenic-mediated inactivation of the JAK-STAT signaling pathway by its direct interaction with JAK tyrosine kinase. Pretreatment with sodium arsenite strongly inhibited IL-6-inducible STAT3 tyrosine phosphorylation in HepG2 cells and did not affect its serine phosphorylation. As a result, sodium arsenite completely abolished STAT activity-dependent expression of suppressors of cytokine signaling (SOCS). Both cellular and subcelluar experiments showed that the inhibition of JAK-STAT signaling resulted from JAK tyrosine kinase's direct interaction with arsenite, and that arsenic's suppression of JAK tyrosine kinase activity also occurred in the interferon γ (IFNγ) pathway. The ligand-independent inhibition by arsenic indicates that JAK was the direct target of arsenic action. Other inflammatory stimulants, stress agents, and metal cadmium failed to induce similar effects on the tyrosine phosphorylation of STAT3 as arsenic does. Our experiments also revealed that arsenic inactivation of the JAK-STAT pathway occurred independent of arsenic activation of MAP kinases. Taken together, our findings indicate that arsenic directly inhibits JAK tyrosine kinase activity and suggest that this direct interference in the JAK-STAT pathway may play a role in arsenic-associated pathogenesis.
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Introduction

The Janus kinases (JAKs) are cytoplasmic tyrosine kinases that relay signals from a variety of cell-surface receptors, particularly cytokine receptors. There are four mammalian JAK family members: JAK1, JAK2, JAK3, and Tyk2. JAK1 is required by gp130 cytokine receptors (IL-6, LIF, OSM, CNTF, and IL-11); JAK2 is essential for EPO signaling; and JAK3 is used only by cytokine (IL-2, IL-4, IL-7, IL-9, IL-15) receptors having the common γ chain (γc) subunit. The other family member Tyk2 appears to be activated by a wide range of cytokines in different tissues (Aringer *et al*., 1999; O'Shea *et al*., 2002). Upon receptor activation, JAKs are responsible for receptor phosphorylation and create docking sites for the association of the latent cytosolic transcription factors called signal transducers and activators of transcription (STATs). STATs are in turn phosphorylated by JAKs, dimerized via reciprocal SH2-phosphotyrosine interaction, and translocated into the cell nucleus for the regulation of gene transcription (Darnell, 1997; Bromberg and Darnell, 2000). Conditionally targeted inactivation of *Jak1* or *Stat3* genes results in defective lymphoid development and severely impairs IL-6/gp130-induced biological responses in hematopoietic cells (Guschin *et al*., 1995; Kunisada *et al*., 1998).

Worldwide, millions of individuals are exposed to levels of arsenic that are believed to suppress the hematopoietic and immune systems and are associated with an increased risk of cancer (National research Council, 2001). Acute, subchronic, or chronic exposure of the hematopoietic system to arsenic is commonly associated with anemia and leukopenia (Woods, 1996; National research Council, 2001). The immunotoxicity caused by exposure to arsenicals includes the damages of humoral- and cell-mediated immunity (Burns and Munson, 1993a, b; Bustamante *et al*., 1997; Yu *et al*., 1998; Vega *et al*., 1999; Ishitsuka *et al*., 2002; Galicia *et al*., 2003) and appears to be associated with suppression of T- and B-lymphocyte maturation. It has been shown that arsenic induces apoptosis in B lymphocytes, macrophages, and primary thymic T lymphocytes. The antibody-forming ability was significantly reduced with mice exposed to semiconductor gallium arsenide. In addition to B-cell toxicity, the suppression of T-cell-specific cytokine or cytokine receptor expression by arsenic has also been reported (Yu *et al*., 1998; Vega *et al*., 1999). In human peripheral blood mononuclear cells, arsenic inhibited cell proliferation and the IL-2 secretion, a result believed to be impaired protein synthesis. Other study also showed

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that, compared with health controls, patients with arsenic-induced Bowen's disease have exhibited sharp decreases in IL-2 receptor expression on lymphocytes and impaired PHA-inducible interferon γ (IFN-γ) and TNF-α production (Yu *et al*., 1998).

This ability of arsenic both to suppress hematopoiesis and to induce immunotoxicity suggests that arsenic may interfere with cytokine receptors signaling at many levels or that arsenicals may act on a target shared by different cytokine signaling pathways, or perhaps both. In any case, the underlying molecular mechanism by which arsenic affects cytokine intracellular signaling is unknown. Considering the essential role that the JAK-STAT cascade plays in mediating a variety of cytokine receptor signaling pathways and the regulation of cell growth, it is important to determine if the JAK-STAT cascade is affected by arsenic.

Therefore, using IL-6 as a model system, we studied the perturbational effects of arsenic on the activation of the JAK-STAT signaling cascade. The resulting evidence suggests that arsenite can inhibit the JAK-STAT signaling pathway, and that the interaction of arsenic with JAK tyrosine kinase may play a role in arsenic-associated pathogenesis.

Results

Arsenite inhibits STAT3 activity

The perturbational role of arsenite in the inhibition of STAT3 activity was investigated by electrophoretic mobility shift assay (EMSA) and immunoblotting analysis. Two types of arsenite exposure were performed to determine the acute and cumulative toxicity of arsenite to STAT3 activity: (i) transient exposure, in which we pretreated cells with sodium arsenite for 30 min, and (ii) long-term exposure, in which we pretreated cells with sodium arsenite for 48 h. As expected, addition of IL-6 for 30 min induces STAT3 activation (Figure 1a, top panel, lane 7, left to right), and this IL-6-induced STAT3 activity decreased along with increasing arsenite concentration (compare lane 7 with lanes 8, 9, 10, 11, and 12). Arsenite itself had no direct effect on STAT3 activity (Figure 1a, left six lanes).

To investigate the cumulative effects of arsenite on STAT3 activity, STAT3 activity was assessed in cells exposed to arsenite for 48 h. STAT3 was activated by IL-6 stimulation (Figure 1b, lane 7, left to right). The induction of STAT3 activity significantly decreased after 48 h of sodium arsenite exposure (Figure 1b, left five lanes). Prolonged exposure to low concentrations of arsenite (4–40 μM) achieved the same effect as transient exposure to high concentrations of arsenite (compare Figure 1a with Figure 1b), indicating that the inhibitory effect of arsenite on STAT3 activity was cumulative and time dependent.

To differentiate arsenic inhibition of STAT3 activity from heavy metal toxicity, we exposed cells to different levels of cadmium for 48 h and found that cadmium itself did not inactivate STAT3 (Figure 1b, right five lanes). We also failed to find cadmium effects on JAK-STAT activity with short-term exposure (data not shown). Thus, arsenic inhibition of STAT3 activity is not an issue of nonspecific heavy metal toxicity. In addition, arsenite exposure has been linked to the induction of stress responses (Ahmad, 1995; Barchowsky *et al*., 1999); therefore, we evaluated the effects of arsenic and other stress stimulants (ansiomycin 100 ng/ml; sorbitol 500 μM; IL-1α 10 ng/ml; and IL-1β 10 ng/ml) on STAT3 activity on a comparison with arsenic by EMSA (data not shown). None of these agents was able to block IL-6-inducible STAT3 activation, thus ruling out the stress response as a cause of arsenic's inhibition of STAT3.

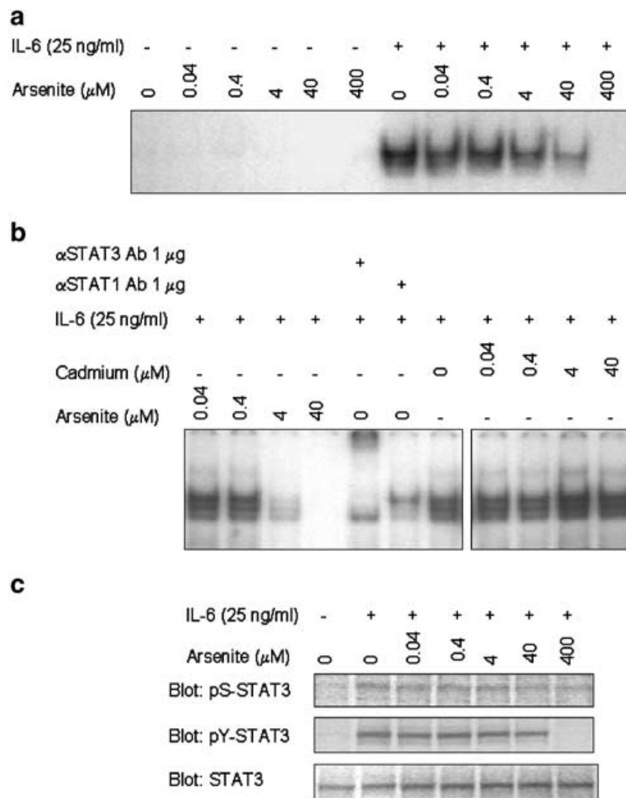

Figure 1 Arsenic inhibition of IL-6 inducible STAT3 activity. (**a**) EMSA analysis of arsenic inhibition of STAT3 activity. Clarified whole-cell extracts from treated HepG2 cells were incubated with end-labeled STAT3 consensus binding sequence (hSIE) and assessed for STAT3 activity by DNA binding ability with EMSA. Cell extracts without IL-6 induction were examined as a control. (**b**) Evaluation of cumulative inhibition of STAT3 activity. The activity of STAT3 in HepG2 cells subjected to prolonged exposure to arsenite or cadmium chloride (48 h) was analysed by EMSA. For supershift EMSA, cell extracts were preincubated with specific antibody (1 μg of αSTAT3 polyclonal or αSTAT1 polyclonal antibody; Santa Cruz Biotechnology). (**c**) Suppression of IL-6 inducible STAT3 tyrosine phosphorylation by arsenite. Whole-cell lysates from treated HepG2 cells were analysed for STAT3 tyrosine phosphorylation with antibody recognizing STAT3 pY705 (Cell Signaling) or STAT3 serine phosphorylation with antibody recognizing STAT3 pS727 (Cell Signaling). STAT3 protein expression was measured as control

Tyrosine phosphorylation of STAT3 (Y705) is required for STAT3 activation and DNA binding. Serine

phosphorylation of STAT3 on conserved residue (S727), on the other hand, is reportedly involved in the regulation of STAT3 activity in gene transcription and tyrosine phosphorylation (Lim and Cao, 1999; Haq *et al*., 2002). We then examined the effect of arsenite on the phosphorylation of STAT3 tyrosine (Y705) and serine (S727) by immunoblotting. Consistent with the EMSA results, IL-6 treatment induced tyrosine phosphorylation of STAT3 on tyrosine Y705 (Figure 1c, middle panel, lane 2, left to right), while arsenite exposure inhibited it at high concentration (Figure 1c, middle panel, compare lane 2 with lanes 3–7, left to right). However, increased exposure to arsenic did not significantly affect serine phosphorylation of STAT3 (Figure 1c, top panel, compare lane 1 with lanes 2–7). The fact that STAT3 expression (protein level) was the same under all conditions indicated that the difference in tyrosine phosphorylation was not the result of a decrease in expression (Figure 1c, bottom). Taken together, the data shown in Figures 1 indicate that arsenite blocked IL-6-inducible activation of STAT3, and that the inhibition of STAT3 by arsenic was limited to its inhibition of STAT3 tyrosine phosphorylation.

Transient transfection experiments of reporter gene activity assay were performed to confirm arsenic inhibition of STAT3 activity *in vivo*. The construct that contains STAT3 binding sites upstream of the *junB* promoter and luciferase reporter gene (see Materials and methods) has been previously shown to be STAT3 activity dependent for transcription (Fujitani *et al*., 1994). Here, we show that luciferase activity, as expected, was strongly induced upon IL-6 stimulation (Figure 2a). IL-6-induced luciferase activity was inhibited by arsenite in a dose-dependent fashion, and the luciferase activity was completely abolished in cells pretreated with 400 μM of arsenite.

To determine whether arsenite could disrupt the DNA binding ability of active STAT3, we used whole-cell extracts from cells transfected with a constitutively active STAT3 (STAT3C) (Bromberg *et al*., 1999). By EMSA, we found that STAT3C comigrated with a labeled probe in the absence of cytokine stimulation (Figure 2b), and this comigration was unaffected by increasing concentrations of arsenite (compare left five lanes). Thus, our data suggested that arsenite disruption of STAT3 activity is likely to occur only upstream in this signaling pathway.

To further investigate the effects of arsenic on the integrity of the JAK-STAT signal pathway and the regulatory feedback loop, we analysed the suppressor of cytokine signaling (SOCS) mRNA level by ribonuclease protection assay (RPA). Transcription of SOCS genes induced by cytokines is strictly dependent on the activity of STATs and the resultant proteins negatively regulate the JAK-STAT signaling (Heinrich *et al*., 1998; Iwamoto *et al*., 2000). The results showed that mRNA levels of several different SOCS were rapidly induced by IL-6 stimulation for 1 h (Figure 3a, lane 5). In particular, CIS, SOCS-3, and SOCS-1 strongly responded to this stimulation. The induction of SOCS mRNA was inhibited by sodium arsenite, confirming that JAK-

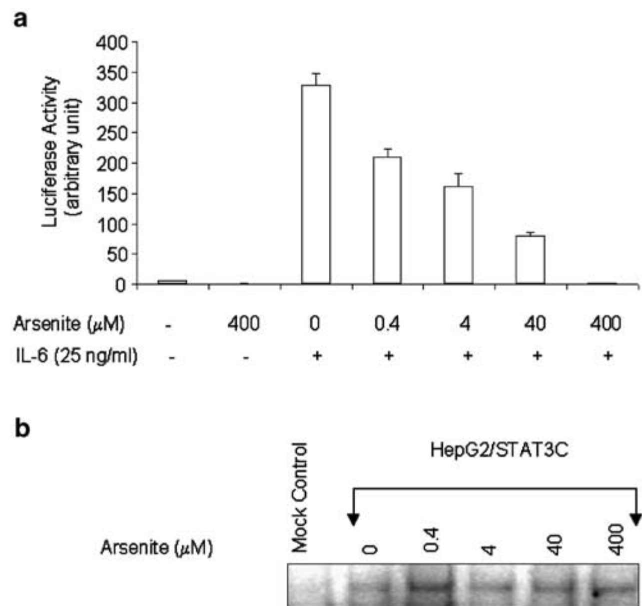

Figure 2 (**a**) Inhibition of STAT3-dependent reporter gene activity by arsenic. HepG2 cells transfected with the reporter construct 4 × APRE-luciferase were exposed to sodium arsenite for 12 h and stimulated with IL-6 for another 4 h. Each experiment was performed in triplicate, and the results from five independent experiments were averaged. (**b**) Evaluation of arsenite interference with active STAT3 DNA binding activity. Stably transfected cells (HepG2/STAT3C) were evaluated by EMSA for the effect of arsenite on active STAT3 DNA binding ability. Cytokine-independent STAT3 DNA binding ability in the presence of sodium arsenite was measured. Data represent the results of six different experiments

STAT signaling had been turned off. Since the suppression of SOCS transcription by arsenite correlated with arsenic inhibition of STAT3 activity, these results suggested that the inhibition of STAT3 by arsenite was not associated with negative feedback from SOCS expression. The mRNA levels of all STAT family members was analysed and not shown to be affected by exposure to sodium arsenite (Figure 3b), consistent with our immunoblotting data.

Arsenite inhibits JAK1 tyrosine kinase in vitro and in vivo

It has been known that the JAK1 tyrosine kinase, an enzyme essential for the receptor signaling of the IL-6 family (IL-6, LIF, OSM, CNTF, and IL-11), is located upstream of STAT3 (Guschin *et al*., 1995; Rodig *et al*., 1998). To determine whether JAK1 is the target for arsenic action, we evaluated the response of JAK1 tyrosine kinase activity to IL-6 stimulation in cells treated with sodium arsenite. Autophosphorylation of tyrosine (Y1022/1023) of JAK1 was induced in response to IL-6 stimulation (Figure 4a, lane 2). However, JAK1 tyrosine phosphorylation was inhibited with costimulation of arsenite (Figure 4a, lane 4). No change JAK1 tyrosine phosphorylation was found within arsenic-only treatment (Figure 4a, lane 3). Upon ligand stimulation, activated JAK1 phosphorylates tyrosine residues on

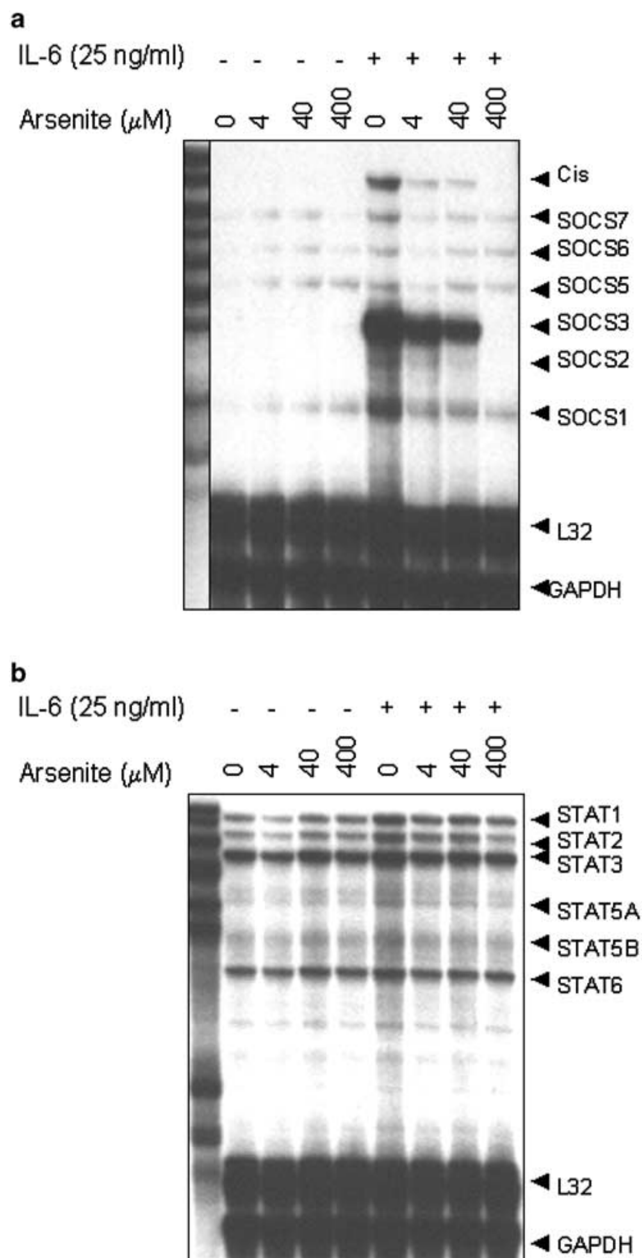

Figure 3 Effect of arsenite on SOCS and STAT transcription. Total RNA from treated HepG2 cells was examined for SOCS mRNA (**a**) and STATs (**b**). (**a**) HepG2 cells were starved in serum-free medium for 16 h before being exposed to sodium arsenite (30 min). In the presence of sodium arsenite, the cells were exposed to 25 ng/ml IL-6 or PBS (control) for an additional hour. Levels of SOCS or STAT mRNAs were determined by RPA. Data represent the results of three independent experiments

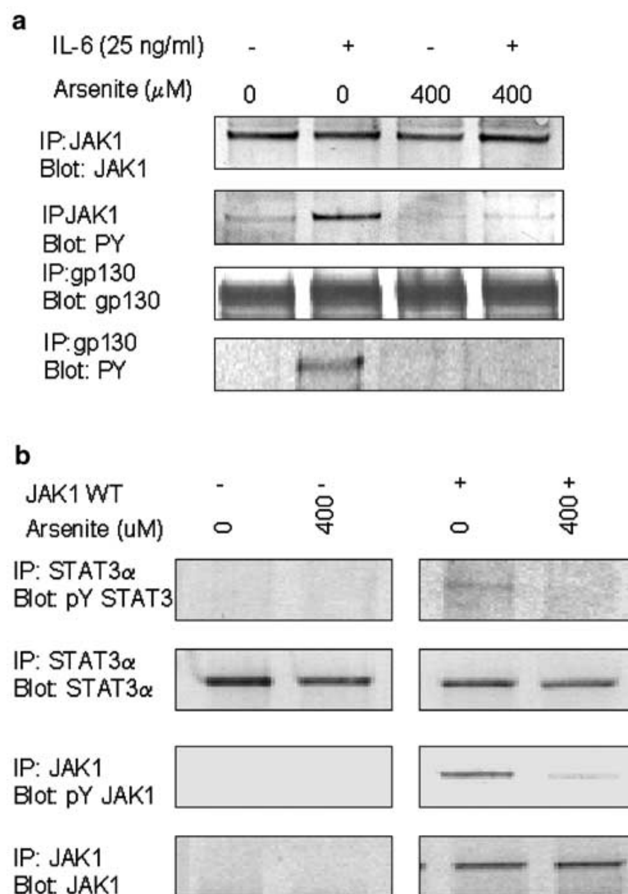

Figure 4 Arsenic suppression of JAK1 and gp130 tyrosine phosphorylation is independent of the receptor action. (**a**) Arsenic inhibition of IL-6-inducible tyrosine phosphorylation of JAK1 and gp130. Endogenous JAK1 kinase from treated HepG2 cells was immunoprecipitated with monoclonal antibody (Transductional Laboratories) and analysed for tyrosine phosphorylation with antibody recognizing JAK1 pYpY1022/1023 (Biosource International) (top two panels). Resolved proteins were immunoblotted for JAK1 protein (top panel) or phosphorylated JAK1 (second panel). Arsenite inhibition of gp130 tyrosine phosphorylation was also examined (bottom two panels). The receptor subunit gp130 from treated cells was immunoprecipitated and immunoblotted for generic tyrosine phosphorylation (bottom panel) with general tyrosine phosphorylation antibody (PY99; Santa Cruz Biotechnology) or gp130 protein level was probed with anti-gp130 antibody (Santa Cruz Biotechnology). (**b**) Receptor-free activation of JAK1 is suppressed by arsenite. Overexpression of JAK1 in JAK1$^{-/-}$ Hela cells was introduced to induce receptor-independent activation of JAK1. JAK1 or STAT3 was immunoprecipitated from lysates of transfected cells and analysed for JAK1 or STAT3 tyrosine phosphorylation (anti-JAK1 pYpY 1022/1023 or anti-STAT3 pY705) in the presence or absence of arsenite. Untransfected JAK1$^{-/-}$ Hela cells were examined as a control

gp130, which in turn provide docking sites for STAT3 receptor recruitment and activation. To determine whether this cascade of events was affected by arsenic exposure, immunoprecipitated gp130 from treated HepG2 cells was also analysed for tyrosine phosphorylation (PY). IL-6-stimulation induced gp130 tyrosine phosphorylation (Figure 4a, lane 2, bottom panel). Exposure to sodium arsenite abolished JAK1 and gp130 tyrosine phosphorylation (compare lane 4 with lane 2), supporting the idea that arsenic-induced JAK-STAT inactivation is a result of JAK tyrosine kinase inhibition.

To rule out the possibility that arsenic inhibition of JAK activation was the result of the disruption of ligand–receptor interaction and to determine that arsenite directly inhibited JAK tyrosine kinase activity, we examined the inhibitory effect of arsenite on JAK1 kinase activity in a receptor-free system (Figure 4b). In this experiment, wild-type JAK1 was introduced into

JAK$^{-/-}$ Hela cells. Heterologous overexpression of JAK1 released JAK1 tyrosine kinase activity and induced kinase autophosphorylation as well as the STAT3 tyrosine phosphorylation (Figure 4b, right column, panel 3, top to bottom). Arsenite suppressed the activity of JAK1 and STAT3 tyrosine phosphorylation. This result demonstrates that the effect of arsenite on JAK-STAT inactivation is independent of ligand–receptor action and is a result of the direct action of arsenic on JAK1 protein.

To determine if arsenic inhibition of JAK1 tyrosine kinase activity results from direct interaction with JAK1 kinase, we used an *in vitro* immunocomplex kinase assay to examine the interaction of JAK1 with arsenite. In this experiment, immunoprecipitated JAK1 protein from untreated HepG2 cells was labeled with γ-[^{32}P]ATP in the presence of arsenic *in vitro*. Direct incubation of JAK1 protein with sodium arsenite *in vitro* resulted in a dose-dependent inhibition of JAK1 autophosphorylation (Figure 5), suggesting that arsenic exerts direct interference with protein kinase activity.

Activation of MAP kinases is not required for arsenic inhibition of JAK1

Others have found that cytokine-activated MAP kinase (Erks or p38) may contribute to JAK-STAT down-regulation (Sengupta *et al*., 1996; Sengupta *et al*., 1998; Ahmed and Ivashkiv, 2000). In the experiments described below, we show that although arsenite also activates the MAP kinase pathway, inhibition of this pathway does not interfere with the ability of arsenite to inactivate JAK-STAT signaling (Figures 6 and 7). Arsenite induced the activation of all three subfamilies of MAP kinases and it clearly did so independently of cytokine stimulation (compare Figure 6a with Figure 6b). When we used specific inhibitors of Erks, p38 and JNKs, we found that the inhibition did not prevent arsenic's inhibition of STAT3 activity (Figure 7). For control purposes, we have conducted a control experiment to determine that specific inhibitor PD98059 (25 uM) and SB203580 (10 uM) indeed disrupted Erk and p38 MAP kinase. The inhibition of Erk phosphorylation after pretreatment of PD98059 and blocking of p38 substrate HSP27 phosphorylation after pretreatment of SB203580 were confirmed (data not shown). Taken together, the findings indicate that arsenite selectively targeted JAK tyrosine kinase and that the inhibition of JAK-STAT activity by arsenic did not require the participation of any MAP kinases.

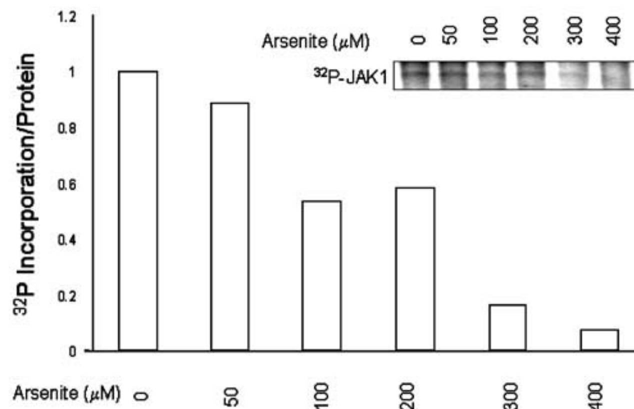

Figure 5 Direct inhibition of JAK kinase activity by arsenic. *In vitro* kinase assay of JAK1 immunocomplex was used to assess the direct effect of arsenic on JAK1 tyrosine kinase autophosphorylation. Purified JAK1 proteins from untreated HepG2 cells were incubated with [γ-^{32}P]ATP in the presence of different amounts of sodium arsenite at room temperature for 30 min. The ^{32}P incorporation was analysed by BioRad densitometry using QuantityOne software. Results were replotted on the ordinate as percentage of ^{32}P incorporation. Data represent the results of five separate kinase assay experiments

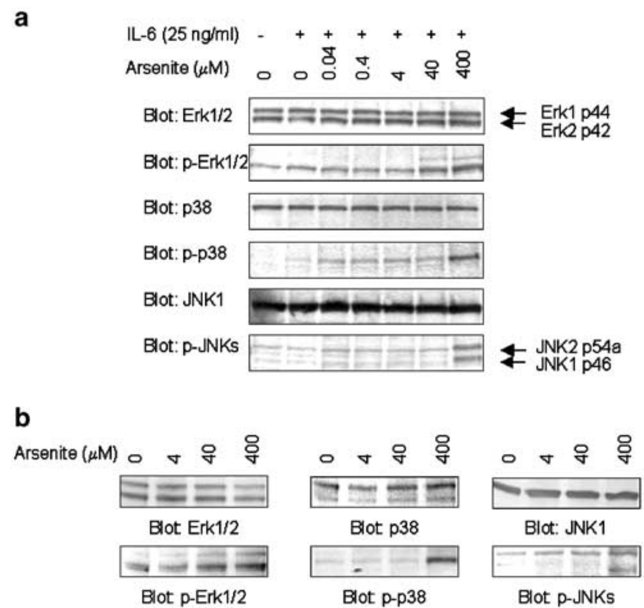

Figure 6 Activation of MAP kinases by arsenite. (**a**) Activity of the three individual MAP kinases in HepG2 cells costimulated with arsenite and IL-6 was determined by immunoblotting with antibodies recognizing phosphorylation of ERK1/2 pTpY185/187, or p38 pTpY180/182, or JNK1/2 pTpY183/185 (Biosource International). Individual MAP kinase protein levels were measured as a control. (**b**) In parallel, arsenite-treated cell lysates were assayed for IL-6-independent activation of MAP kinases

Inhibition of JAK family members by arsenic

To determine if arsenic can inhibit JAK1 tyrosine kinase activity in different cytokine receptor pathways, HepG2 cells were stimulated with different cytokines including IFNα, IFNβ, IFNγ, and IL-6, and their lysates were examined by immunoblotting for effects of arsenite on JAK1 activity. We found that IFNγ and IL-6 induced JAK1 activation (Figure 8a, compare lane 1 with lanes 4 and 5). The induced JAK1 activity was inhibited by arsenite in both type I and type II cytokine receptor groups (Figure 8a, compare lanes 4 and 5 with lanes 9 and 10, left to right). These data support that arsenic's ability to inhibit JAK tyrosine kinase activity is a result of direct action and is cytokine receptor specificity independent. We also examined JAK2 activity after stimulation by these cytokines (Figure 8b). In HepG2

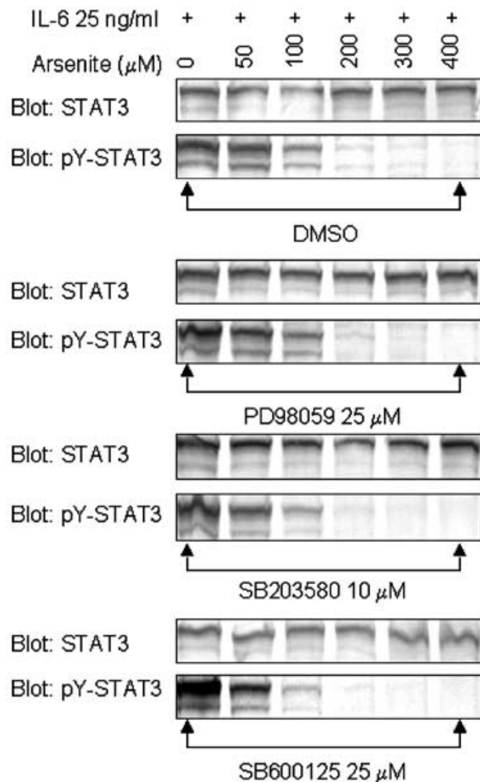

Figure 7 Activation of MAP kinase is not required for the inhibition of the JAK-STAT pathway by arsenic. Cells were pretreated with different specific inhibitors of the individual MAP kinases (PD98059 for ERKs, SB203580 for p38, and SB600125 for JNKs) or with DMSO (1 : 100 dilution, 30 min) before costimulation with arsenite and IL-6. In the presence of the individual inhibitor or DMSO, cells were then treated with sodium arsenite for 30 min and then stimulated with IL-6 for another 30 min. Clarified whole-cell lysates were analysed for STAT3 activity using antibody against STAT3 Y705 phosphorylation

cells, IFN-γ stimulation induced clear activation of JAK2 (Figure 8b, lane 4). Similarly, the induction of JAK2 kinase activity was inhibited by arsenic. Under the conditions of these experiments, the activation of JAK1 or 2 by other ligands was not observed. The lack of induction of JAK1 or 2 activation by other cytokines in HepG2 cells may suggest either JAK specificity for the signaling or relatively low receptor density.

We used the Tel-JAK fusion proteins, a constitutive construct for JAK tyrosine kinase (see Methods and materials), to further test whether or not arsenic affects other JAK family members. As shown in Figure 9, arsenic inhibited tyrosine phosphorylation of Tel-JAK1, Tel-JAK2, and Tel-JAK3 (Figure 9, compare lane 1 to lanes 2–5 in right four panels). We made several attempts to analyse Tel-Tyk2 but were unable to get Tel-Tyk2 protein expression with these stable cell lines.

Active STAT3 can rescue arsenic suppression of cell growth

STAT3 activity is a key factor for cell proliferation and plays a critical role in promoting cell cycle progression

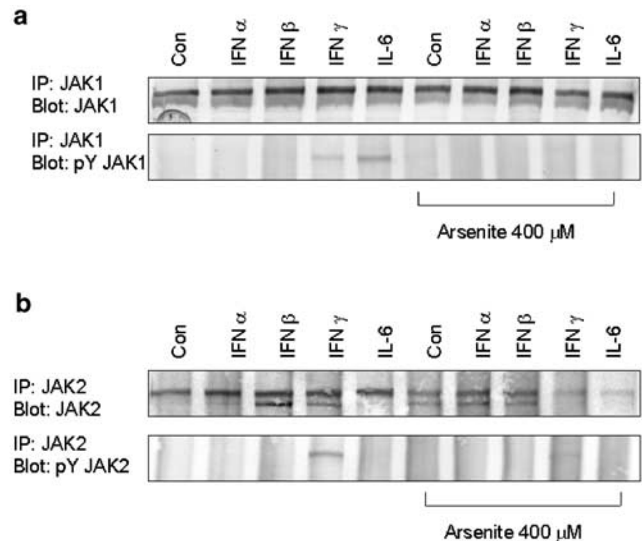

Figure 8 Arsenic suppression of cytokine-induced JAK kinase activation. (**a**) Arsenite inhibition of JAK1 activity. HepG2 cells were treated with PBS, IFNα, IFNβ, IFNγ, and IL-6, respectively, to induce JAK1 activation as described in Materials and methods. Treated cells were subjected to JAK1 immunoprecipitation and analysed for JAK1 tyrosine phosphorylation (anti-JAK1 pYpY 1022/1023; Biosource International), in the presence or absence of sodium arsenite exposure. JAK1 protein levels were probed for control. (**b**) Arsenite inhibition of JAK2 activity. Similarly treated cells were immunoprecipitated with anti-JAK2 antibody. After electrophoresis, resolved proteins were subjected for immunoblotting analysis of JAK2 tyrosine phosphorylation (anti-JAK2 pYpY1007/1008, Biosource International) and total JAK2 protein

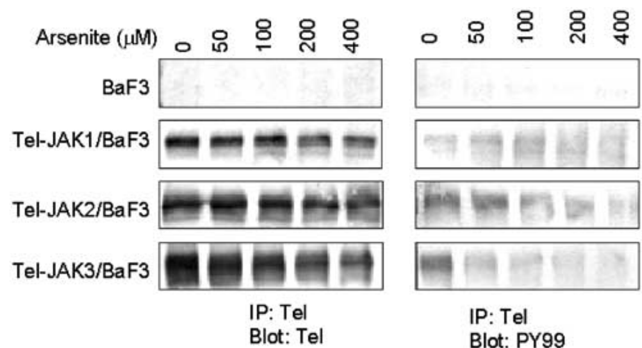

Figure 9 Arsenic inhibition of activity of the Tel-JAK fusion proteins in hematopoietic BaF3 cells. (**a**) Cytokine-independent BaF3 cells with stable expression of Tel-JAK1, or Tel-JAK2 or Tel-JAK3 were treated with arsenite as indicated for 1 h. Treated cells were immunoprecipitated for Tel-tagged proteins (Tel-fusion JAKs) with anti-Tel antibody. Immunocomplex was resolved and analysed for tyrosine phosphorylation (PY99, right column), or probed for Tel-JAKs with anti-Tel antibody. Lysate from cytokine-dependent BaF3 lysate was used as a control

(Bromberg *et al*., 1999). Aberrantly elevated STAT3 activity is associated with cell oncogenic transformation *in vitro* and tumorgenesis *in vivo*. To further define whether arsenic-induced cell growth suppression is a result of inhibition of STAT3 activity, we evaluated growth curves of HepG2 with stable expression of STAT3C (constitutively activated STAT3) using a

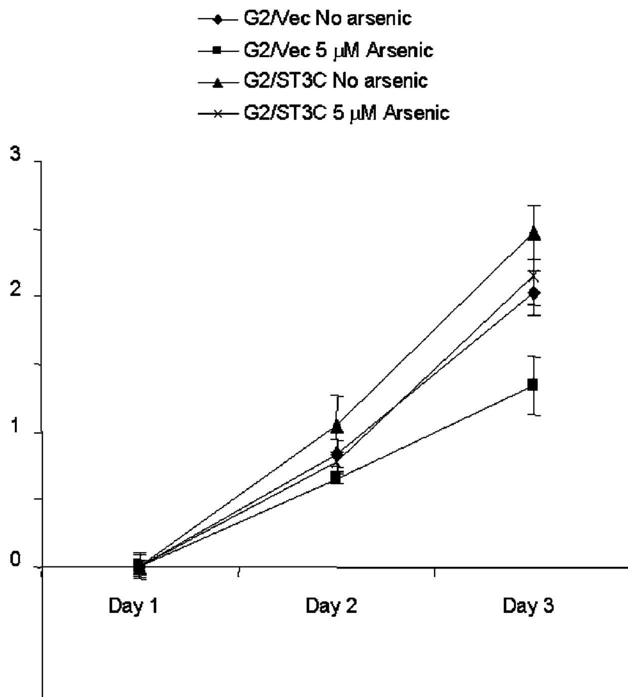

Figure 10 Active STAT3 (STAT3C) protects cells against arsenic-induced suppression of cell growth. Constitutively activated STAT3-transfected HepG2 cells were selected with G418 for the establishment of stable cell line (G2/ST3C). Empty vector (pCDNA3)-transfected HepG2 cells were selected and used as controls (G2/Vec). In all, 1×10^4 cells/well (G2/Vec or G2/ST3C) were plated in 96-well plates and evaluated for cell growth (see Materialsand methods) on days 1, 2, and 3. All OD readings at 570 nM were normalized to day 1 value (–◆–: G2/Vec, no arsenic; –■–: G2/Vec, 5 μM sodium arsenite; –▲–: G2/ST3C, no arsenic; –×–: G2/STAT3C, 5 μM sodium arsenite)

colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Exposed cells were grown continuously with arsenite for up to 72 h. Under the conditions, arsenite suppressed HepG2 growth while active STAT3 significantly reversed arsenic suppression of cell growth (Figure 10). The data suggest that arsenic inhibition of STAT3 activity in the JAK-STAT pathway contributes to arsenic toxicity.

Discussion

JAK-STAT signaling plays a critical role in the regulation of cell proliferation, differentiation, and function of hematopoietic cells. Here, we provide evidence that sodium arsenite plays a previously unrecognized role in the inhibition of JAK tyrosine kinase activity (Figures 4, 5, 8, 9). Inactivation of JAK tyrosine kinase activity results in the inhibition of STAT3 activation and STAT3-dependent gene transcription (Figures 1–3). Sodium arsenite inhibits STAT3 through JAK inactivation, and this inhibition is not mimicked by a variety of other agents that induce stress. Even though arsenite is a potent activator of ERKs, p38, and JNKs, our data demonstrate that the arsenic inhibition of JAK kinase activity is independent of MAP kinase activation (Figures 6 and 7). Further investigation demonstrates that arsenic exerts direct interaction with JAK kinase protein and thus may affect different cytokine-related biological functions ranging from erythropoiesis to maturation of myeloid lineages (Figures 8, 9). In addition to hematopoiesis, the JAK-STAT pathway is also essential for the normal maturation and function of lymphoid cells (Rodig *et al*., 1998; Yoo *et al*., 2002). Hence, it is possible that the inhibition of JAK by arsenite may contribute to some of the environmental or clinical effects of arsenicals.

We present evidence that arsenite can inhibit the activity of other JAK family members (JAK1, JAK2, and JAK3); however, the molecular mechanism of this inhibition is still unclear. Structurally, all JAK family members contain JH (JAK homology) domains (JH1-7, from C-terminal to N-terminal) (Zhou *et al*., 2001). The Tel-fusion JAKs in this study retain only the carboxyl-JH1 domain of JAK kinase protein and these three chimeric JAKs were all inhibited by arsenite. Since the JH1 domain is highly conserved among the isoforms of JAK family members, it is possible that this is the site of action for arsenic inhibition of JAK kinase activity. Arsenite may exert direct action on the ATP-binding site within JH1 domain. Nevertheless, based on other data from our laboratory, arsenite is not a general tyrosine kinase inhibitor. We have found that arsenite activates, rather than inhibits, the EGF receptor, another tyrosine kinase (manuscript in preparation). As the ATP binding motifs among the tyrosine kinases are very similar, it is unlikely that arsenite blocks ATP binding using same mechanism of recruitment. It is likely that some other aspect of the JH1 domain is involved in JAK inhibition by arsenic.

In this study, we have found that arsenite is a potent activator for all the family members of MAP kinases. The carboxyl-terminal region of STAT contains the MAP kinase consensus phosphorylation sequence and is a putative substrate for MAP kinases (Chung *et al*., 1997; Decker and Kovarik, 2000). STAT3 serine phosphorylation has been proposed to participate in the regulation of tyrosine phosphorylation. Nevertheless, we found that sodium arsenite selectively inhibits JAK-STAT3 tyrosine phosphorylation but does not affect STAT3 Ser727 phosphorylation. This finding indicates that arsenite inhibition of JAK-STAT signaling is independent of serine phosphorylation. Although it has been reported that activated MAP kinase pathways could negatively regulate JAK-STAT signaling, our data suggest that the molecular mechanism of action for arsenic inhibition of JAK kinase activity is the direct interaction of arsenic with JAK protein kinase. The activation of MAP kinase by arsenic is not necessary for the inhibition of JAK-STAT signaling.

Normal biological responses to cytokines such as IL-6 are the combined results of downstream signaling such as JAK-STAT and MAP kinase pathways. Sodium arsenite differentially interferes in the IL-6 pathway by downregulating JAK-STAT activity and upregulating MAP kinase activity. Since the JAK-STAT and MAP

kinase pathways are involved in many different biological processes, the biological responses to sodium arsenite exposure may vary from tissue to tissue. Further studies are warranted to determine if the differential biological effects from arsenic exposure can be explained by the multitargeting effects of arsenic on the different signal pathways.

Materials and methods

Cell culture and cDNA constructs

HepG2 cells were originally purchased from the American Type Cell Collection (ATCC, VA, USA). All experiments described below were performed on HepG2 cells except the experiments of ligand-independent activation of JAK1 in Hela $JAK^{-/-}$ cells. The Myc-tagged active form of STAT3 cDNA construct (STAT3C) was generated as described previously by Bromberg *et al.* (1999). In brief, STAT3C cDNA was constructed by inserting cysteines into the carboxyl-terminal loop of the SH2 domain. This insertion allowed STAT3 to form a dimer without undergoing tyrosine phosphorylation. To establish a cell line that would stably express STAT3C, HepG2 cells were transfected with STAT3C and then selected in 400 μg/ml G418 (Life Technologies, Inc., Rockville, MD, USA) for 14–20 days. The expression of STAT3C was confirmed by immunoblotting with anti-Myc antibody. The generation of $JAK1^{-/-}$ Hela cells has been described previously (Loh *et al.*, 1994). To induce ligand-independent activation of JAK1, wild-type JAK1 was introduced into $JAK1^{-/-}$ Hela cells by transient transfection. All cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO/Invitrogen Corporation, New York, USA).

The original BaF3 and cytokine-independent BaF3 cell lines with stable expression of Tel-JAKs were provided by Dr Virginie Penard as described elsewhere (Lacronique *et al.*, 2000). BaF3 was cultured with RPMI1640 supplemented with 10% FBS and 10% WEHI medium for the supplement of IL-3. Cytokine-independent growth of the stable cell lines of BaF3 with Tel-JAKs were cultured with RPMI1640 supplemented with 10% FBS only. Cells were subjected to 2 h serum-free starvation prior to arsenite treatment. Starved cells of BaF3 and Tel-JAK transformed BaF3 cell lines were exposed to arsenite for 1 h in serum-free RPMI1640 medium to determine the arsenic action on the activity of Tel-JAK fusion proteins.

Cell treatment

Sodium arsenite (Sigma-Aldrich, St Louis, MO, USA) was prepared in phosphate-buffered saline (PBS), pH 7.4, as a 100 × stock solution and used at a final concentration ranging from 0 to 400 μM. MAP kinase inhibitors PD98059, SB203580, and SB600125 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). For short-term exposure, serum-free starved HepG2 cells were exposed to different concentrations of sodium arsenite ranging from 0 to 400 μM for 30 min. To induce MAP kinase inhibition, starved HepG2 cells were pretreated with individual inhibitor PD98059 (25 μM), p38 inhibitor SB203580 (10 μM), and JNK inhibitor SB600125 (25 μM) for 30 min before an additional 30 min of exposure to sodium arsenite. Treated HepG2 cells were stimulated with IL-6 (25 ng/ml), IFNα (10 ng/ml), IFNβ (10 ng/ml), and IFNγ (10 ng/ml) (R&D Systems Inc., Minneapolis, MN, USA) for an additional 30 min. For the assessment of mRNAs, cells were treated for 1 h with IL-6, and treated cells were subjected to the determination of level of mRNA for STAT and the upstream STAT inhibitor SOCS by ribonuclease protection assay (RPA). For long-term exposure to sodium arsenite, HepG2 cells were cultured in decreased serum (3% FBS) of DMEM for up to 48 h in the presence of either sodium arsenite or cadmium chloride. Cells were then starved in serum-free medium for 2 h and finally treated with either PBS or IL-6 for 30 min.

To induce ligand-independent activation of JAK1 in JAK1 null cells, $JAK1^{-/-}$ Hela cells were transfected with wild-type JAK1 and placed as duplicates into a 100-mm dish 24 h after transient transfection. Cells were incubated for another 24 h. Then, the cells were starved in serum-free medium for 2 h and finally treated with either PBS or sodium arsenite.

EMSA

Whole-cell extracts were prepared for EMSA. In brief, treated cells grown in six-well plates were washed once with ice-cold PBS and collected. Cell pellets were resuspended in 50 μl 2 × high-salt buffer (840 mM NaCl; 40 mM HEPES, pH 7.9; 2 mM EDTA; 2 mM EGTA; 40% glycerol). Clarified cell extracts were analysed for STAT activity by electrophoresis in 5% nondenaturing polyacrylamide gels after incubation with [γ-^{32}P] end-labeled double-stranded oligonucleotides (*hSIE*, 5′-GTGCATTTCCCGTAAATCTTGTCTACA-3′). The identified STAT3 binding activity was confirmed by coincubation with unlabeled hSIE oligonucleotides (cold probe competition) or with antibodies of αSTAT1 or αSTAT3 (supershift assay) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Immunoprecipitation and immunoblotting analysis

Treated cells were collected by centrifugation and resuspended in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton X-100, 0.1%. SDS, 1 mM EDTA, 1% sodium deoxycholate), supplemented with 25 μg of aprotinin/ml, 50 μg of leupeptin/ml, 1 mM phenylmethylsufonyl fluoride, 20 mM NaF, 1 mM Na_3VO_4, and 50 μM Na_2MoO_4. The protein of JAK1, gp130, or STAT3 from clarified lysates was immunoprecipitated with 1 μg specific polyclonal antibody against JAK1 or STAT3 (BD Transductional Laboratories; PharMingen, San Diego, CA, USA) or gp130 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 20 μl protein G agarose (GIBCO BRL/Invitrogen, Grand Island, NY, USA) for 2 h at 4°C. Immunoprecipitated proteins were washed, resolved by electrophoresis in 7.5% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes, and then analysed by immunoblotting. Tyrosine phosphorylation of gp130 and JAK1 was analysed using general tyrosine phosphorylation antibody (PY99; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with specific phosphotyrosine antibody to JAK1 (pYpY1022/1023) or JAK2 (pYpY1007/1008) (Biosource International). Tyrosine phosphorylation of STAT3 was analysed using antibodies against STAT3 (pY705; Cell Signaling Technology, Inc., Beverly, MA, USA) or a serine phosphorylation-specific antibody to STAT3 (pS727; Cell Signaling). For the immunoblotting analysis of whole-cell lysates, clarified cell lysate was resolved by electrophoresis on 7.5% SDS–polyacrylamide gels or 4–12% Nupage® gels (GIBCO BRL/Invitrogen). Resolved proteins were transferred to PVDF membranes and analysed by immunoblotting accordingly.

For arsenic action on hematopoietic BaF3 cells, Tel-fusion proteins were immunoprecipitated with anti-Tel antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immu-

nocomplex was washed three times with RIPA buffer and resolved with 7.5% SDS–PAGE. The transferred PVDF with resolved proteins were probed with anti-tyrosine phosphorylation (PY99) and anti-Tel antibodies, respectively. Individual antibodies recognizing carboxyl termini of JAK1, JAK2, and JAK3 (Santa Cruz Biotechnology) were also used for the confirmation (data not shown).

In vitro kinase assay

The endogenous JAK1 proteins from untreated HepG2 cells were immunoprecipitated as described above and resuspended in kinase assay buffer (50 mM HEPES, pH 7.4; 10 mM $MgCl_2$). The final pellets were aliquoted and incubated with [γ-^{32}P]ATP (10 μCi; Pharmacia Corp., Peapack, NJ, USA) and 2 μl of sodium arsenite/kinase buffer in a total incubation volume of 20 μl. After incubation for 30 min at room temperature, the reaction was stopped by heating in 2 $\times$ SDS sample loading buffer. Phosphorylated proteins were resolved by electrophoresis in SDS polyacrylamide gels and visualized by exposure to X-ray film. Incorporation of ^{32}P in JAK1 autophosphorylation was analysed by densitometry (QuantityOne, BioRad Laboratories, Inc., Hercules, CA, USA).

Luciferase reporter gene assay

A cDNA construct, containing four repeats of the STAT3 binding sequence known as the acute phase response element (APRE) in front of the minimal *jun*B promoter-luciferase gene (Fujitani *et al*., 1994), was kindly provided by Dr S Dong (Baylor College of Medicine). At 24 h after transfection, HepG2 cells were split into six-well plates and incubated at 37°C for an additional 24 h. Cells were exposed to different concentrations of sodium arsenite ranging from 0 to 400 μM in a serum-free medium for 12 h and then with 25 ng/ml IL-6 for an additional 4 h. Clarified cell lysates were analysed for luciferase activity using a commercially available substrate kit (Sigma-Aldrich, St Louis, MO, USA).

RPA

The total RNA was isolated from treated HepG2 cells using TriZol reagent (GIBCO BRL/Invitrogen), according to the manufacturer's instructions. Aliquots (2 μg) of total RNA were analysed for SOCS and STAT mRNA levels by RPA using two preassembled, commercially available multiprobe kits (PharMingen, San Diego, CA, USA). One of these kits contained the probes for CIS, SOCS1, SOCS2, SOCS3, SOCS5, SOCS6, and SOCS7. The other kit contained the probes for STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. All of the probes were labeled with [α-^{32}P]UTP with *in vitro* transcription (Promega, Madison, WI, USA). Sample RNAs were hybridized with labeled probes, and unprotected RNAs were digested with RNase A/T (Torrey Pines Biolabs, Inc., Houston, TX, USA). The protected mRNAs were then resolved on 6% polyacrylamide gels containing 8 M urea and visualized by exposure to X-ray film.

Cell growth MTT assay

The thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), MTT) was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and dissolved in PBS (5 mg/ml). 1×10^4 cells were plated per well for the MTT proliferation assay. For arsenic treatment, cells were continuously exposed to different concentrations of sodium arsenite. On days 1–3, cells were evaluated for cell growth by measuring absorbance at 570 nm. Growth curves were plotted and normalized to day 1.

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