Sodium Arsenite Inhibits and Reverses Expression of Adipogenic and Fat Cell-Specific Genes during in Vitro Adipogenesis

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Arsenic causes cancer in humans, but its mechanism of action is unique among known carcinogenic agents. As a naturally occurring component of sediments and ground water, human exposure to arsenic is inevitable, necessitating the establishment of exposure limits. Because cancer is characterized as an imbalance between cell growth and differentiation, it has been hypothesized that arsenic exerts its carcinogenic effect, in part, by perturbing the balance between these antagonistic processes. Previous work in this laboratory has demonstrated that sodium arsenite prevents adipocytic differentiation of C3H 10T1/2 cells, leading to the hypothesis that the underlying mechanism involves downregulation of genes associated with adipogenesis. In support of this hypothesis, it was found that mRNA levels of peroxisome proliferative-activated receptor γ (PPARγ), CCAAT-enhancer binding protein α (C/EBPα), and adipocyte-selective, fatty acid-binding protein (aP2) are decreased in arsenic-treated cells; arsenic-induced phenotypic reversion of differentiated adipocytes correlates with reduced aP2 expression. Arsenic also blocks upregulation of p21<sup>Cip1/Waf1</sup>, a factor whose expression is tightly regulated during adipogenesis. The differentiating effect of pioglitazone, which in the presence of C/EBP<sup>d</sup>, C/EBP<sup>α</sup>, and C/EBP<sup>β</sup>, is inhibited by arsenic, suggesting that arsenic interferes with adipogenic signaling at or below the level of PPARγ. Because C/EBPα is important in the expression of certain keratinocyte-specific genes, the negative effect of arsenic on C/EBPα might also contribute to the development of skin cancer. PPARγ, C/EBPα, and p21<sup>Cip1/Waf1</sup> are important in numerous normal and pathological processes, including carcinogenesis, leading us to postulate that perturbation of these factors by arsenic might contribute to the carcinogenic effect of this metalloid.

Key Words: arsenic; differentiation; proliferation; cancer; adipocytes; adipogenesis; aP-2; PPARγ, C/EBPα, p21<sup>Cip1/Waf1</sup>.

Chronic ingestion of inorganic arsenic causes basal- and squamous-cell skin carcinomas and also is associated with cancers of the bladder, lung, liver, and kidney (Callen and Headington 1980; Hopenhayn-Rich et al., 1998; Mazumder et al., 1998; Smith et al., 1998). In addition to its carcinogenic effects, arsenic exposure has been linked to type II diabetes mellitus (Tseng et al., 2000) and cardiovascular diseases (Engel et al., 1994), including atherosclerosis, hypertension (Rahman et al., 1999), and blackfoot disease (Tseng et al., 1996). Since arsenic is a natural constituent of soil and water, some degree of human exposure is inevitable. Therefore, the limits set for the amount of arsenic to which people are exposed must balance safety with the cost incurred by decreasing exposure. Establishing the acceptable concentration of arsenic in the drinking water is an ongoing challenge; this task would be greatly facilitated by discovery of the mechanisms involved in arsenic-induced neoplasia.

Although arsenic exhibits characteristics of both initiators and promoters, its unique spectrum of effects precludes its classification as either (for review, see Kitchin 2001). Data from several studies indicate that arsenic alters cell proliferation, and this effect is thought to contribute to its carcinogenicity (Germolec et al., 1997, 1998; Shimizu et al., 1986; Trouba et al., 2000a,b). Arsenic also inhibits differentiation of cultured cells, including keratinocytes (Kachinskas et al., 1994, 1997) and adipocytes (Trouba et al., 2000b). Since proliferation and differentiation are antagonistic toward one another (Freytag and Geddes, 1992), determining the effects of arsenic on the molecular mechanisms underlying each process will contribute to our understanding of the carcinogenicity of arsenic.

Adipogenesis is a well-characterized cellular process, and cultured preadipocyte cell lines have been an invaluable tool in delineating the induction pathway. C/EBPβ and C/EBPδ are immediate-early targets of adipogenic hormones, and each of these transcription factors is upregulated transiently early in the adipogenic process (Cao et al., 1991). In turn, C/EBPβ and C/EBPδ induce the expression of PPARγ, a transcription factor absolutely required for adipogenesis (Rosen and Spiegelman 2000). A member of the nuclear hormone superfamily, PPARγ forms a heterodimeric complex with the retinoid-X receptor α (RXRα) and induces transcription of a number of fat cell-specific genes, including aP2 and adipin (Tontonoz et al., 1994a).

In addition to physiological stimulation by C/EBPβ and C/EBPδ, pharmacological induction of PPARγ can be achieved with dexamethasone (Wu et al., 1996). Although
endogenous, high affinity PPARγ ligands have not been identified, a class of antidiabetic agents called thiazolidinediones (TZDs) function as potent PPARγ ligands (Lehmann et al., 1995). C/EBPα, which is induced by C/EBPβ and C/EBPδ, is an important transcription factor that works in conjunction with PPARγ to induce expression of most fat cell-specific genes. In the current model of adipogenesis, PPARγ induces C/EBPα; however, C/EBPα appears to enhance the levels of PPARγ, thereby setting up a positive feedback loop (reviewed in Rosen et al., 2000). C/EBPα is sufficient for differentiation of the preadipocyte cell line 3T3-L1 (Freytag and Geddes 1992) and appears to be important in maintaining the post-mitotic growth arrest of differentiated adipocytes (Tao and Umek 2000).

Expression of the negative growth regulator p21<sup>Cip1/Waf1</sup> is regulated tightly during adipogenesis. During the confluent stage prior to induction of differentiation, p21<sup>Cip1/Waf1</sup> levels are high, a state consistent with its negative influence on cell proliferation. As cells undergo subsequent clonal expansion, p21<sup>Cip1/Waf1</sup> levels decrease, but expression again increases as cells enter post-mitotic growth arrest. The inability of cells to upregulate p21<sup>Cip1/Waf1</sup> is postulated to contribute to their continued potential for proliferation and lack of ability to differentiate.

Based on the hypothesis that arsenic inhibits and reverses morphological differentiation of adipocytes by disrupting expression of the panel of genes involved in adipogenesis, including downregulation of fat cell-specific genes, we examined the transcript level of a number of genes associated with adipogenesis and/or growth arrest. Our data support a model in which arsenic perturbs cell programming in a manner consistent with a shift away from differentiation and toward the proliferation pathway.

**MATERIALS AND METHODS**

**Cells and culture conditions.** C3H 10T1/2 cells represent a pluripotent cell line that can be induced to differentiate into adipocytes with the appropriate stimuli. C3H 10T1/2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FBS, penicillin, and streptomycin (complete media; Life Technologies, Grand Island, NY). The long-term effects of arsenic were assessed by growing C3H 10T1/2 cells in DMEM plus 10% FBS, 25 μg/ml insulin, and 10 μg/ml streptomycin (complete media; Life Technologies). The C3H 10T1/2 cells were subjected to the differentiation protocol as previously described (Trouba et al., 2000b). Briefly, cells were seeded in DMEM plus 10% FBS. After reaching confluence, the medium was replaced with IDM (DMEM with 10% FBS containing the appropriate concentrations of pioglitazone). To determine if arsenic blocks differentiation upstream or downstream of PPARγ, control C3H 10T1/2 cells or cells treated long term with arsenic were treated with pioglitazone, in either the presence or absence of 1 μM arsenite (NaAsO<sub>2</sub>). These cells fail to undergo morphological differentiation (not shown). Similarly, cells treated long term with 6 μM of sodium arsenite and subjected to the differentiation protocol exhibit diminished levels of aP2, PPARγ and C/EBPα, regardless of whether arsenic is removed from the media (lane 3 for aP2 and PPARγ; lane 2 for C/EBPα); these cells fail to undergo morphological differentiation (not shown). We reported previously that arsenic inhibits morphological differentiation of C3H 10T1/2 cells, but the effect of arsenic on expression of fat-specific genes was not examined (Trouba et al., 2000b). To determine if arsenic inhibits adipogenesis at the biochemical level, the mRNA levels of 3 fat cell-specific genes were examined following the dexamethasone/insulin differentiation protocol. As can be seen in Figure 1, control C3H 10T1/2 cells induced to differentiate exhibit a high level of aP2, PPARγ and C/EBPα (lane 1). In contrast, the mRNA levels of these 3 fat cell-specific genes is greatly diminished in control cells to which 6 μM arsenite has been added (lane 2 for aP2, PPARγ; lane 3 for C/EBPα); these cells fail to undergo morphological differentiation (not shown).

**Insulin/dexamethasone-induced adipogenesis.** The C3H 10T1/2 cells were subjected to the differentiation protocol as previously described (Trouba et al., 2000b), essentially following protocols established by others (Brownell et al., 1996; Lu et al., 1992). Cells were seeded in DMEM plus 10% FBS. After reaching confluence, the medium was replaced with IDM (DMEM containing 10% FBS, 25 μM dexamethasone (Sigma), and 10 μg/ml insulin (Life Technologies)). After 2 days in IDM, cells were then changed to IM (DMEM without dexamethasone), and morphological differentiation became evident after another 7–10 days in culture. Cells were photographed using a Nikon Diaphot inverted microscope system (× 25). Unless otherwise stated, analysis was performed at day 12 of the differentiation protocol. In some experiments, mitogenic signaling was inhibited by the addition of the MEK-1 and -2-specific inhibitor U0126 (Favata et al., 1998).

**RESULTS**

We reported previously that arsenic inhibits morphological differentiation of C3H 10T1/2 cells, but the effect of arsenic on expression of fat-specific genes was not examined (Trouba et al., 2000b). To determine if arsenic inhibits adipogenesis at the biochemical level, the mRNA levels of 3 fat cell-specific genes were examined following the dexamethasone/insulin differentiation protocol. As can be seen in Figure 1, control C3H 10T1/2 cells induced to differentiate exhibit a high level of aP2, PPARγ and C/EBPα (lane 1). In contrast, the mRNA levels of these 3 fat cell-specific genes is greatly diminished in control cells to which 6 μM arsenite has been added (lane 2 for aP2, PPARγ; lane 3 for C/EBPα); these cells fail to undergo morphological differentiation (not shown). Similarly, cells treated long term with 6 μM of sodium arsenite and subjected to the differentiation protocol exhibit diminished levels of aP2, PPARγ and C/EBPα, regardless of whether arsenic is removed from the media (lane 3 for aP2 and PPARγ; lane 2 for C/EBPα) or retained (lane 4). These results indicate that repression of adipogenesis by arsenic involves not only inhibition of lipid accumulation, but also decreased expression of adipocyte marker genes.

Pioglitazone initiates differentiation by acting directly on PPARγ, thereby bypassing the requirement for insulin and dexamethasone-activation of C/EBPβ and C/EBPδ. To determine if arsenic blocks differentiation upstream or downstream of PPARγ, control C3H 10T1/2 cells or cells treated long term with arsenic were treated with pioglitazone, in either the pres-
FIG. 1. Inhibitory effect of arsenic on the expression of fat cell-specific genes in C3H 10T1/2 cells induced to differentiate. RNA was isolated from cells subjected to the insulin/dexamethasone differentiation protocol and hybridized to radiolabeled probes for aP2, PPARγ, or C/EBPα. The data shown represent 1 of 3 experiments performed using either insulin/dexamethasone or pioglitazone to induce differentiation. (A) Results of Northern-blot analysis with each probe. For aP2 and PPARγ (lane 1) control cells (morphologically differentiated); (lane 2) cells to which 6 μM As3+ was added at the start of differentiation; (lane 3) cells treated long-term with arsenic but switched to arsenic-free media at the start of differentiation; (lane 4) cells cultured long-term in arsenic and maintained in arsenic-containing media during the differentiation protocol. For C/EBPα (lanes 1 and 4), identical to aP2 and PPARγ; (lane 2) cells treated long-term with arsenic but switched to arsenic-free media at the start of differentiation; (lane 3) cells to which 6 μM As3+ was added at the start of differentiation. (B) Results of the Northern blot quantified by densitometry and normalized to the housekeeping genes GAPDH (aP2 and PPARγ) or tubulin (C/EBPα).

ence or absence of 6 μM arsenite. As can be seen in Figure 2A, pioglitazone-treated control cells assume an adipocyte morphology and accumulate lipid droplets. In contrast, addition of 6 μM arsenite to control cultures completely eliminates the response to pioglitazone. Regardless of whether cells treated long term with arsenic are exposed to pioglitazone in the presence or absence of arsenic, no morphological differentiation is detected. These results are corroborated by Oil Red-O staining of cells treated with 1 to 10 μM pioglitazone (Fig. 2B). It can be concluded from these data that signaling events occurring upstream of PPARγ are not relevant targets of arsenic for its negative effect on adipogenesis.

Previous data reported by this laboratory showed that long-term treatment of C3H 10T1/2 cells with arsenic causes an upregulation in MKP-1 (Trouba et al., 2000a), which is postulated to increase MAPK activity over time. Since activated MAPK is antagonistic to the process of adipocyte differentiation (Font de Mora et al., 1997), we hypothesized that arsenic prevents adipogenesis, in part, by maintaining MAPK in the activated state. To determine if activation of MAPK is required for arsenic to inhibit adipogenesis, the potent and specific MEK-1 and –2 inhibitor U0126 was utilized. As can be seen in Figure 3, the inhibition of MEK, and therefore MAPK, does not block the ability of arsenic to inhibit adipogenesis induced by insulin/dexamethasone or pioglitazone, regardless of the concentration of U0126 added. Only control cells respond to the differentiation protocol by accumulating lipids. These results indicate that MAPK activity is not required for inhibition of adipogenesis by arsenic. Interestingly, it appears that U0126 enhances differentiation of control cells, an observation in agreement with the finding that MAPK activation opposes adipogenesis (Font de Mora et al., 1997) and with the observation that mitotic clonal expansion is not an essential step in adipogenesis (Qiu et al., 2001).

Since the addition of sodium arsenite to terminally differentiated adipocytes causes phenotypic reversion characterized by a reduction in accumulated lipids (Trouba et al., 2000b), it was predicted that a corresponding decrease in the adipocyte genes aP2, PPARγ and C/EBPα would be observed. As shown in Figure 4, the mRNAs for all 3 genes are undetectable in control cells and in cells grown long term in 6 μM arsenite (lanes 1 and 2, respectively). Control cells induced to differentiate display an increase in all 3 transcripts at t = 12 days (lane 3), with aP2 levels elevated most noticeably. Further culture of differentiated cells results in a further increase in aP2 and C/EBPα at day 17 (lane 4). In contrast, aP2 mRNA levels drop dramatically 5 days after addition of arsenic to the differentiated cells (lane 5); levels of PPARγ and C/EBPα also decrease, but to a lesser extent. Thus, arsenic-induced phenotypic reversion is accompanied by a decrease in the levels of mRNA corresponding to adipocyte marker genes.

In addition to adipocyte-specific genes, the expression of the negative growth regulator p21Cip1/Waf1 was examined. As can be seen in Figure 5, mRNA levels of p21Cip1/Waf1 are decreased significantly in cells grown long-term in arsenic as compared to control cells under conditions of confluence. The inability of arsenic-treated cells to increase p21Cip1/Waf1 expression during density-dependent growth arrest is consistent with a decreased capacity to undergo terminal differentiation. The effects of arsenic on adipogenic and fat cell-specific gene expression are summarized in Figure 6.

DISCUSSION

We have shown previously that arsenic inhibits and reverses morphological differentiation of C3H 10T1/2 pre-adipocytes as assessed by lipid accumulation (Trouba et al., 2000b). However, these studies did not distinguish between an effect of arsenic to decrease lipids secondary to effects on adipocyte biochemistry (e.g., inhibition of lipogenesis or glucose uptake)
versus an arsenic-dependent alteration in adipogenic cellular programming. The results described herein indicate clearly that arsenic decreases expression of fat cell-specific and adipogenic genes, thereby supporting the hypothesis that arsenic perturbs regulatory processes fundamental to maintaining the appropriate balance between proliferation and differentiation.

FIG. 2. Effect of arsenic on C3H 10T1/2 cells induced to differentiate with pioglitazone. Control cells or cells treated long term with 6 μM arsenite were treated with pioglitazone to induce differentiation. (A) Lipid accumulation in cells treated with pioglitazone in the presence or absence of arsenic. Cells were treated as described in the legend to Figure 1, except that pioglitazone was used to induce adipocytic differentiation. Lipids were stained with Oil Red-O. (B) Quantification of Oil Red-O accumulation: Oil Red-O was extracted from the cells, quantified by spectrophotometry as described in the text, and depicted as a percentage of the control value (no pioglitazone). Asterisks (*) denote a statistically significant increase in absorbance in control cells compared to arsenic-treated cells.
The finding that arsenic inhibits PPARγ expression could be an important key in determining the mechanism by which this metalloid perturbs normal control of growth and differentiation. PPARγ can be considered a “master switch” in adipogenesis, acting to coordinate expression of numerous genes involved in the differentiation process. In addition, PPARγ initiates withdrawal from the cell cycle, an event necessary in the induction of adipogenesis (Altiok et al., 1997). In smooth
muscle cells, PPARγ activation results in accumulation of cells in G0/G1, an effect accompanied by upregulation of the negative growth regulator p27Kip1 (Wakino et al., 2000). It follows that arsenic-treated cells exhibiting decreased PPARγ expression are predicted to be less likely to enter G0/G1 and less likely to exhibit a normal increase in p27Kip1 expression when induced to differentiate. Therefore, inhibited PPARγ expression is a reasonable explanation for our previous data showing that arsenic treated cells are primed to enter the cell cycle and display decreased p27Kip1 levels (Trouba et al., 2000a,b).

Our finding that arsenic causes decreased expression of PPARγ also is of potential importance in understanding the mechanisms underlying diverse pathologies induced by arsenic. In exposed human populations, arsenic exposure has been linked to type II diabetes mellitus (Rahman et al., 1998; Tseng et al., 2000) and hypertension (Rahman et al., 1999), and downregulation of PPARγ has been correlated with both of these disease states (Barroso et al., 1999). Conversely, PPARγ agonists inhibit the growth and/or initiate differentiation of a variety of cultured tumor cell lines, including colorectal, breast, and prostate cancer cells (Brockman et al., 1998; Elstner et al., 1998; Kubota et al., 1998; Mueller et al., 1998). Therefore, it is intriguing to speculate that many of the carcinogenic and noncarcinogenic pathologies resulting from arsenic exposure might result from downregulation of PPARγ.

Another piece of evidence indicating that PPARγ is an important target of arsenic is provided by the results of experiments using pioglitazone to induce adipogenesis. Direct activation of PPARγ circumvents the requirement for hormonally...
induced expression of C/EBPβ and C/EBPα, two factors that contribute to PPARγ induction. Because arsenic efficaciously inhibits adipogenesis induced by this PPARγ agonist, it can be concluded that upstream signaling events are not required for the inhibitory effects of arsenic on differentiation.

In cells induced to undergo adipogenesis, treatment with arsenic tips the balance away from differentiation and toward proliferation. At the molecular level, adipogenesis is inhibited by phosphorylation of PPARγ by MAP kinase (Hu et al., 1996), and arsenic potentially increases MAPK activity secondary to down regulated MKP-1 expression (Trouba et al., 2000a). Therefore, it is conceivable that arsenic blocks adipogenesis simply by maintaining cells in a state of proliferative activity. This leads to a major question of whether arsenic inhibits adipogenesis indirectly by its ability to maintain cells in a mitogenically competent state, or by a more direct mechanism involving adipogenic processes. We found that UO126-mediated inhibition of mitogenic signaling does not abrogate the differentiation inhibition effect of arsenic, thereby supporting the hypothesis that arsenic interferes with the molecular events leading to differentiation.

For full phenotypic differentiation of adipocytes, expression of both PPARγ and C/EBPα is required (El-Jack et al., 1999). In addition to playing a pivotal role in establishment of insulin-sensitive glucose transport, C/EBPα is necessary for maintenance of post-mitotic growth arrest in adipocytes (Tao and Umek 2000; Timchenko et al., 1996). Downregulation of C/EBPα, accomplished by expression of antisense RNA, enables quiescent cells to reenter the cell cycle (Tao and Umek 2000), thereby illustrating the importance of C/EBPα in maintaining proliferative inactivity. Because C/EBPα positively regulates protein levels of p21^Waf1^Cip1 (Timchenko et al., 1996), the abnormally low level of p21^Waf1^Cip1 seen in arsenic-treated cells (Fig 5) might be a consequence of arsenic-induced down regulation of C/EBPα. Using animals genetically engineered to lack C/EBPα, a correlation has been made between loss of C/EBPα, decreased p21^Waf1^Cip1 levels, and unregulated cell proliferation in vivo (Timchenko et al., 1997).

There is increasing evidence that C/EBPs, including C/EBPα, are important in regulating epidermal differentiation (Maytin and Habener 1998; Maytin et al., 1999; Oh and Smart 1998; Swart et al., 1997; Zhu et al., 1999). C/EBPα and C/EBPβ are greatly reduced in squamous cell carcinomas when compared to control cells (Oh and Smart 1998), establishing a link between changes in C/EBP expression and malignant transformation. In light of the fact that arsenic causes primarily skin cancer, arsenic-induced alterations in C/EBPα could contribute to inhibition of differentiation.

Like other members of the family of cyclin-dependent kinase inhibitors, p21^Cip1^Waf1 plays an important role in regulating cell cycle progression by inhibiting the cyclin-dependent kinases (CDKs). A putative target gene of C/EBPα, p21^Cip1^Waf1 is upregulated following induction of C/EBPα (Heath et al., 2000; Timchenko et al., 1996). Furthermore, the proliferative inhibition exerted by C/EBPα is mediated through p21^Cip1^Waf1 (Timchenko et al., 1996). Therefore, decreased p21^Cip1^Waf1 expression in arsenic-treated cells might be a consequence of downregulated C/EBPα expression. Regardless of the mechanism, the changes observed in cellular p21^Cip1^Waf1 expression following arsenic exposure are consistent with maintenance of proliferative competence during conditions favorable for differentiation. The loss of p21^Cip1^Waf1-dependent negative regulation has been implicated in the cocarcinogenic property of arsenic (Vogt and Rossman 2001).

The results of the present study corroborate our earlier report that arsenic inhibits morphological differentiation of C3H 10T1/2 cells into adipocytes. The data extend our understanding of this effect by demonstrating that arsenic disrupts adipogenesis via perturbations of the cellular programming underlying this phenomenon. It is anticipated that this information will contribute to a definitive identification of the molecular target(s) of arsenic that lead to its adverse effects.

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