Estrogen Signaling in Livers of Male Mice With Hepatocellular Carcinoma Induced by Exposure to Arsenic In Utero

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Background: Exposure of pregnant mice to inorganic arsenic induces a spectrum of tumors, including hepatocellular carcinoma (HCC), in their adult offspring similar to that induced by exposing adult mice to estrus compounds. To investigate whether arsenic exposure in utero causes altered estrogen signaling, we examined expression of estrogen receptor-\(\alpha\) (ER-\(\alpha\)), cyclin D1 (an estrogen-responsive hepatic oncogene), and several cytochrome P450 genes (with sexually dimorphic liver expression patterns) in livers from adult male mice with in utero arsenic-induced HCC. Methods: Quantitative real-time reverse transcription–polymerase chain reaction was used to evaluate gene expression in livers of adult male mice that had (i.e., exposed mice; \(n = 8\)) or had not (i.e., control mice; \(n = 5\)) been exposed to arsenic in utero. DNA methylation status of portions of the ER-\(\alpha\) and cyclin D1 gene promoters in liver tissue was measured using methylation-specific polymerase chain reaction. Statistical tests were two-sided. Results: ER-\(\alpha\) mRNA levels were 3.1-fold (95% confidence interval [CI] = 2.0-fold to 4.3-fold) higher in livers of exposed mice than in those of control mice, and cyclin D1 levels were 3.0-fold (95% CI = 1.7-fold to 4.3-fold) higher. Exposed mice showed a feminized expression pattern of several cytochrome P450 genes, expressing the female-dominant CYP2A4 \((P = .017\) versus control) and CYP2B9 \((P < .001\) at 8.7 and 10.5 times, respectively, the level in control mice and expressing the male-dominant CYP7B1 at approximately one-fourth the level in control mice \((P = .0012\)). Exposed mice exhibited reduced (by approximately 90%) methylation of the ER-\(\alpha\) gene promoter in liver DNA as compared with control mice; the cyclin D1 gene promoter was not methylated in either exposed or control mice. Conclusion: Altered estrogen signaling may play a role in induction of HCC by arsenic exposure in utero. Specifically, overexpression of ER-\(\alpha\), potentially through promoter region hypomethylation, in livers of such mice may be linked to the hepatocarcinogenicity of arsenic. [J Natl Cancer Inst 2004;96:466–74]

Inorganic arsenic is considered to be one of the most hazardous substances in the United States, largely based on the carcinogenic potential of this metalloid and the chance of exposure in U.S. populations (1,2). Inorganic arsenic in the human environment has been etiologically linked to tumor development in a variety of tissues, including skin, bladder, lung, liver, and prostate (1–5). The main source of environmental arsenic exposure in most populations is drinking water, in which inorganic forms of arsenic (trivalent arsenite and pentavalent arsenate) predominate (2). High levels of arsenic in drinking water and the associated adverse health effects are found in many developing areas around the world, including Taiwan, China, Chile, India, Mexico, and Bangladesh (2). However, recent evidence suggests that lower levels of arsenic, such as those found in drinking water in many areas of the United States, may also pose a substantial carcinogenic risk to humans for various tumors (6,7). The risks associated with these lower levels are underscored by the recent decision by the U.S. Environmental Protection Agency to reduce the limit for the acceptable level of arsenic in drinking water by 80%, from 50 \(\mu\)g/L to 10 \(\mu\)g/L.

Gestation in mammals is a period of high sensitivity to chemical carcinogenesis (8). For example, maternal exposure of rodents to a variety of inorganic agents, including lead (9), cisplatin (10), and nickel (11) can result in cancers in the offspring after they reach adulthood. Several studies (2,12,13) have shown that arsenic can readily cross the placenta and enter the fetal blood system in mammals, including humans. A study of people living in an area of endemic arsenic exposure in Argentina (14) showed that the concentration of arsenic in fetal cord blood approaches that in maternal blood, suggesting that arsenic is easily transferred to the human fetus.

The importance of fetal exposure in arsenic-induced carcinogenesis is demonstrated by our recent finding (15) that adult mice that had been briefly exposed to arsenic during gestation form malignant and benign tumors at multiple sites. Specifically, the offspring of mice whose drinking water contained inorganic arsenic from day 8 through 18 of gestation developed tumors of the liver, ovary, adrenal gland, and lung and preneoplastic lesions of the uterus and oviduct after they reached adulthood and long after arsenic exposure had ceased (15). Among the tumors induced in male mice exposed to arsenic in utero were hepatocellular carcinoma (HCC) (15), a tumor site and type that has been associated with arsenic exposure in humans (5,16,17). In addition, multiple primary tumors (adenomas and carcinomas) of the liver were often observed in these mice (15).

A similar spectrum of tumors and proliferative lesions (i.e., of the liver, ovary, uterus, oviduct, and adrenal gland) is interestingly, associated with treatment with estrogenic compounds in

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See “Notes” following “References.”

DOI: 10.1093/jnci/djh070

Journal of the National Cancer Institute, Vol. 96, No. 6, © Oxford University Press 2004, all rights reserved.
humans and animals (18–21). Further evidence that arsenic exposure may influence estrogen response pathways comes from the finding that adult mice chronically exposed to inorganic arsenic (22) overexpress both estrogen receptor-α (ER-α) and cyclin D1 in the uterus. Strong evidence links overexpression of ER-α with estrogen-induced tumors in mice (20). In addition, estrogens can activate, through ER-α, genes that are important in proliferation and carcinogenesis, such as cyclin D1 (23). Indeed, overexpression of both ER-α and cyclin D1 has been implicated in the development of various malignancies, including liver tumors (24–26), and cyclin D1 is considered to be a hepatic oncogene (25).

To further investigate the connection between in utero arsenic exposure and estrogen response pathways, we have examined the effect of transplacental arsenic exposure on expression of ER-α and several estrogen-responsive genes, including cyclin D1, in the liver of adult male mice. In preliminary exploratory analyses, we also compared ER-α and cyclin D1 gene expression in human liver biopsy samples from an arsenic-exposed population in Guizhou Province, China (27), with that in men not known to have such exposure.

### Materials and Methods

#### Animals and Liver Samples

For studies of the effects of in utero arsenic exposure, we analyzed liver samples from adult male mice collected as part of a tumor endpoint study on the transplacental carcinogenic potential of inorganic arsenic in C3H mice (15). For the present work, we used liver samples from five of the adult male mice born to mothers supplied drinking water containing 0 ppm arsenic and eight of the adult male mice born to mothers supplied drinking water containing 85 ppm arsenic from gestation day 8 through day 18. The samples from arsenic-treated animals were selected from normal-appearing areas of the livers of mice that had grossly visible liver tumors (all of which were later diagnosed as HCC). Samples were snap-frozen in liquid nitrogen for later processing.

For studies of sexually dimorphic gene expression patterns in untreated adult mice, male and female C3H mice (five of each sex) were obtained from The Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and were housed there in a standard barrier facility at temperatures of 20–22 °C with a relative humidity of 50% ± 5% and a 12-hour light/dark cycle. Food (NIH-31 Open Formula, 6% Modified; Harlan Teklad Standard Diets, Madison, WI) and drinking water were provided ad libitum. Liver samples were obtained when the animals were 3 months old and were snap-frozen in liquid nitrogen for later processing. Animal care was provided in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] Publication 86-23).

#### Human Samples

Samples from liver biopsies were obtained from male patients (n = 3) selected at random from a larger group of eligible patients living in an area of endemic arsenic exposure in Guizhou Province, China (27). The patients had originally been selected on the basis of a history of high arsenic exposure, and all had skin lesions (e.g., keratosis or hyperpigmentation) that are indicative of chronic arsenicalism. The subjects did not have a history of hepatitis, and were negative for hepatitis B and C viruses by serology. These patients had been selected to receive a traditional Chinese medicine therapy for arsenic-induced liver disease under a human subjects protocol approved by the Chinese Ministry of Public Health. To evaluate the efficacy of the therapy, liver biopsies were performed before and after therapy in local hospitals by certified physicians. The results of the present report concern only samples taken prior to therapeutic intervention. As controls, normal human liver samples (n = 5) from rejected transplants or surgical resections (all from men) were obtained from the University of North Carolina Hospital (27). All samples were snap-frozen in liquid nitrogen for later processing. No unique identifiers exist for any of the human samples used in this study.

#### Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis

Human liver samples, samples of histologically normal liver from the same mice used for immunohistochemical analysis after in utero arsenic exposure that had been snap-frozen at necropsy, and liver samples from untreated adult male and female C3H mice were used for quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis of gene expression. Total RNA was isolated from all frozen samples using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by purification on RNeasy columns (Qiagen, Palo Alto, CA). The mouse and unexposed human samples were processed in North Carolina, and the arsenic-exposed human samples were processed in China; however, the same reagents (purchased separately) were used under the oversight of a single individual (J. Liu) to reduce any variation that could have been caused by different treatment of samples. For quantitative real-time RT–PCR analyses, which were all carried out in a single laboratory at the National Institute of Environmental Health Sciences (NIEHS), total RNA from each sample was reverse transcribed with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamer primers. The SYBR Green PCR Kit (Applied Biosystems) was used for quantitative real-time RT–PCR analyses. The primers for mouse and human ER-α and cyclin D1 and mouse CYP2A4, CYP2B9, CYP7B1, and β-actin (available at http://jnci.cancerspectrum.oupjournals.org/jnci/content/vol96/issue6) were designed using Primer Express software (Applied Biosystems). Relative differences in gene expression between groups were expressed using cycle time values; these values were first normalized with that of β-actin in the same sample, and the expression in the experimental group was expressed as a percentage of expression in controls. A standard curve for expression of each gene was generated according to the method of Liu and Saint (28).

#### DNA Methylation Analyses

Global DNA methylation was determined by the methyl acceptance assay of Balaghi and Wagner (29) in liver samples from five control mice and five of the eight mice exposed to arsenic in utero. These samples were obtained from the same mice whose samples were used for protein and gene expression analysis. In addition, the methylation status of CpG islands in portions of the promoters of the ER-α and cyclin D1 genes were assessed in the same samples using a modification of the technique of Clark et al. (30). In this
method, sodium bisulfite treatment of single-strand DNA converts unmethylated cytosines to uracils but leaves methylated cytosines unchanged. Briefly, separate samples of 10 μg of genomic DNA each isolated from livers of the five arsenic-treated and five control mice were used. The 10 DNA samples were separately digested with EcoRI for 2 hours at 37 °C and then denatured with 0.3 M NaOH at 37 °C for 10 minutes. The digested single-strand DNA was sulfonated and deaminated by incubation with 3.1 M sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone at 50 °C for 16 hours. The modified DNA was purified using the Wizard DNA Clean-Up System (Promega, Madison, WI), incubated with 0.3 M NaOH at room temperature for 10 minutes, and re-purified with the QIAquick DNA Cleanup System (Qiagen). Bisulfite-modified DNA was PCR amplified with primers for the promoter region of ER-α (forward: 5'-AAATTTTAGGAATGTTGATTATTAG-3'; reverse: 5'-CCGATCTCTCTCTCTTACTACATAATTCAA-3') and cyclin D1 (forward: 5'-GTGCATCTACGTACACACCTCTATCCG-3'; reverse: 5'-GTGGTTGGAAATGAAACTTCACATCG-3'). The use of these primers allowed us to assess the methylation status of a 341-base-pair (bp) DNA fragment (~2402 to ~2601) within the ER-α promoter region that includes 13 CpG sites (31) and of a 215-bp DNA fragment (~5 to ~220) within the cyclin D1 promoter that includes 16 CpG sites. The PCR products were subjected to electrophoresis on agarose gels, purified using a QIAquick Gel Extraction Kit (Qiagen), and cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen). Plasmid DNA from each clone was purified using a QIAprep Spin Miniprep Kit (Qiagen), and the inserts were sequenced using a BigDye Terminator Cycle Sequence Kit (Applied Biosystems) and an M13 reverse primer, according to the manufacturer's instructions. Sequencing products were purified using DyeEx Spin Kits (Qiagen) to remove the unincorporated dye and analyzed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). For each treatment group (i.e., control and arsenic exposed), the five individual DNA samples were used to generate at least 16 individual clones for assessment of promoter region methylation at which point the clonal data were pooled. For each individual CpG site, the data are given as the proportion of clones in which that site is methylated and are expressed as a percentage of the total number of assessments at that site. Total promoter region methylation is defined as the percentage of methylated CpG sites of the total number of all sites assessed.

Immunohistochemical Analysis

Formalin-fixed, paraffin-embedded histologically normal sections of liver from in utero arsenic–exposed (n = 8 [all of which had grossly visible HCC tumors], from six different mothers) and control mice (n = 5) obtained in the original study (15) (all mice were at least 36 weeks old) were used for immunohistochemical analysis of ER-α and cyclin D1 protein expression. We used polyclonal antibodies against ER-α (Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal antibodies against cyclin D1 (Upstate Biotechnology, Lake Placid, NY) as primary antibodies (at dilutions of 1:1000 and 1:1500, respectively) and a streptavidin-conjugated secondary antibody (Santa Cruz Biotechnology). Antibody binding was visualized with an avidin–biotin–peroxidase kit (VECTORSTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromagen and hematoxylin as a nuclear counterstain. For controls, we omitted the primary antibodies.

Statistical Analysis

For DNA methylation analysis, the clonal incidence of methylation at individual CpG sites in the promoter region was analyzed by using Fisher's exact test. Unpaired Student's t tests were used for all other data, which are expressed as means with 95% confidence intervals (CIs). All statistical tests were two-sided.

RESULTS

Because the spectrum of transplacentally arsenic-induced tumors in mice (15) is similar to that induced by estrogenic compounds, and because estrogens are known hepatic carcinogens, we first examined the effect of gestational arsenic exposure on hepatic ER-α expression. We assessed ER-α protein levels by immunohistochemistry in pathologically normal liver samples from eight adult male mice (from six different mothers) bearing HCC after gestational exposure to inorganic arsenic and in samples of normal liver from five unexposed male mice. Livers from adult mice exposed to arsenic in utero showed marked increases in ER-α protein compared with livers from unexposed mice (Fig. 1, A and B). ER-α was uniformly overexpressed in the livers from mice exposed to arsenic in utero, with almost every cell showing higher staining intensity (Fig. 1, B) than cells in livers from unexposed mice (Fig. 1, A). In addition, the immunostaining revealed that, in livers from mice exposed to arsenic in utero, ER-α was localized primarily within the nucleus, the cellular site of action for this transcription factor/receptor. The finding that the overexpressed ER-α is often localized to the nucleus indicates that it is probably active. To determine whether the increased level of ER-α protein in livers of mice exposed to arsenic in utero was due to increased gene expression, ER-α mRNA levels were examined by quantitative real-time RT–PCR. As shown in Fig. 1, C, ER-α mRNA levels were 3.1-fold higher (95% CI = 2.0-fold to 4.3-fold) in the livers of adult male mice exposed to arsenic in utero than in livers of unexposed mice.

Next, we addressed the functional significance of ER-α overexpression in livers from mice exposed to arsenic in utero and its possible relationship with hepatocarcinogenesis. Immunohistochemical staining revealed that cyclin D1 was expressed at higher levels in the livers of mice exposed to arsenic in utero than in the livers of control mice (Fig. 2, A and B). Like ER-α, cyclin D1 was uniformly overexpressed in the livers of mice exposed to arsenic in utero and exhibited nuclear localization. Quantitative real-time RT–PCR revealed a 3.0-fold increase (95% CI = 1.7-fold to 4.3-fold) in cyclin D1 mRNA in livers of exposed mice relative to control mice (Fig. 2, C).

Cyclin D1 can be activated by factors other than estrogens and is therefore not a definitive indicator of ER-α activity. However, several cytochrome P450 genes have been shown to have sexually dimorphic expression patterns in the liver, making them useful tools for examining potential functional effects of arsenic-induced ER-α overexpression. CYP2A4 is a female-dominant gene (i.e., it is expressed at much higher levels in females than males), the expression of which is imprinted by estrogens through ER-α during development; after CYP2A4 is activated, its expression persists into adulthood (32). Quantitative real-time RT–PCR analysis revealed an 8.7-fold increase (95% CI = 3.7-fold to 13.7-fold; P = .017) in CYP2A4 expres-
Expression of CYP2B9, a female-dominant P450 that is strongly estrogen-inducible in males (33), was elevated 10.5-fold (95% CI 6.7-fold to 14.4-fold; P < .001) in male mice gestationally exposed to arsenic as compared with control male mice (Fig. 3). Expression of CYP7B1, which is expressed more strongly in males than females (i.e. male-dominant), was reduced to only 25% of control values (95% CI 13.7% to 36.4%; P < .001) in adult male mice exposed to arsenic in utero relative to control mice (Fig. 3). These results are consistent with the feminized gene expression pattern that would be expected as a result of ER-α overexpression, and they suggest that the ER-α overexpression resulting from in utero arsenic exposure has functional consequences.

To compare the magnitude of the differences in expression of sex-dominant genes in the livers of male mice born to arsenic-exposed mothers and those of control male mice with the magnitude of the differences between female and male mice, we determined female-to-male expression ratios for ER-α, CYP2A4, CYP2B9, and CYP7B1 in liver samples from untreated adult female and male C3H mice (five of each). The female-to-male expression ratios in these mice were 2.9 for ER-α (95% CI 1.7 to 4.2), 55 for CYP2A4 (95% CI = 50 to 59), 5.2 for CYP2B9 (95% CI = 4.6 to 5.7) and 0.05 for CYP7B1 (95% CI = 0.03 to 0.11). Thus, for ER-α the expression ratio in untreated adult
females relative to untreated adult males is similar to the ratio in males exposed to arsenic in utero relative to control males. In addition, in utero arsenic exposure of males induced a sexually dimorphic pattern of CYP gene expression that was qualitatively similar to that observed in untreated adult males and females.

The effect of arsenic on gene expression in vitro may be due to decreased methylation within the promoter region (34). There is also increasing evidence that expression of the ER-α gene is inversely related to the level of promoter methylation (35,36).

Therefore, we used a PCR-based technique that distinguishes methylated and unmethylated cytosines to examine ER-α promoter methylation at 13 CpG sites within a segment of the ER-α promoter. Frequency of methylation was reduced at 12 of these 13 sites in DNA from the livers of adult male mice transplacentally exposed to arsenic (n = 22 clones derived from five different mice) compared with DNA from livers of unexposed mice (n = 20 clones from five different mice). The reduction was statistically significant at nine of the 13 sites (Fig. 4). The alterations observed in ER-α promoter methylation did not appear to be due to overall DNA methylation changes, because genomic DNA methylation in the livers of mice exposed to arsenic in utero by the methyl acceptance assay was 101.4% (95% CI = 72.9% to 129.8%; n = 5) of the value in control mice.

We also examined the methylation status of a portion of the promoter region of the cyclin D1 gene. The 16 CpG sites in this segment of the cyclin D1 promoter were all unmethylated in samples from mice exposed to arsenic in utero (n = 16 clones derived from five different mice) and control mice (n = 18 clones derived from five different mice). None of these sites was methylated in either treated or control mice. Therefore, overexpression of cyclin D1 after arsenic exposure does not appear to be due to altered methylation of the promoter region (data not shown).

To investigate the relevance to human arsenic exposure of the gene expression changes observed in our mouse model, we carried out preliminary exploratory analyses of the expression of the human ER-α and cyclin D1 genes in liver RNA samples from three individuals residing in Guizhou Province, China, who were known to be exposed to high levels of environmental inorganic arsenic. The three patients consistently exhibited dermal lesions typical of chronic arsenicalism. As controls, ER-α and cyclin D1 gene expression in liver samples from persons without known exposure to elevated environmental arsenic were analyzed. Quantitative real-time RT–PCR analysis revealed that, consistent with the results in mice, both ER-α and cyclin D1 were overexpressed in the livers of humans exposed to high levels of environmental arsenic compared with controls (Fig. 5). The expression of ER-α was increased by 4.2-fold (95% CI = 3.2-fold to 5.3-fold) and that of cyclin D1 was increased by 2.4-fold (95% CI = 1.6-fold to 3.2-fold).

**DISCUSSION**

Our results suggest that in utero exposure to inorganic arsenic results in persistent and widespread overexpression of ER-α mRNA and protein in the livers of adult male mice bearing arsenic-induced HCC. The overexpressed ER-α was localized in the nucleus after arsenic exposure, the site where the transcription factor is normally active, indicating that it could potentially...
Fig. 5. Analysis of estrogen receptor-α (ER-α) and cyclin D1 mRNA expression in liver samples from men chronically exposed to arsenic and from control (unexposed) men. mRNA levels were measured using quantitative real-time reverse transcription-polymerase chain reaction. Data were first normalized to β-actin mRNA levels and are expressed as a percentage of unexposed control. Graph shows means (control, n = 5; arsenic-exposed, n = 3) with 95% confidence intervals. * = statistically significant differences from control (ER-α, P <.001; cyclin D1, P <.001; unpaired Student's t tests).

be active in regulating gene expression (37). This conclusion is supported by our findings that the ER-α-regulated hepatic oncogene cyclin D1 was expressed at higher levels in the livers of mice exposed to arsenic in utero than in the livers of control mice and that livers of mice exposed to arsenic in utero exhibited a feminized pattern of expression of cyclochrome P450 genes with sexually dimorphic expression patterns. In addition, preliminary analyses showed that liver biopsy samples from a population heavily exposed to environmental arsenic in Guizhou Province, China, exhibited much higher expression of ER-α and cyclin D1 than samples from an unexposed population. Thus, the accumulated results from the present study suggest that overexpression of ER-α is an important molecular event in arsenic carcinogenesis, at least in the liver.

The underlying basis of arsenic-induced ER-α overexpression appears to be pronounced demethylation of the ER-α promoter, a phenomenon that has been associated with ER-α activation in other studies (35,36,38). Hypomethylation of the ER-α promoter is also consistent with previous work showing that a loss of DNA methylation can occur after chronic arsenic exposure, resulting in malignant transformation of rodent hepatocytes (34). In addition, hypomethylation of the H-ras gene promoter has been observed in arsenic-exposed mice fed methyl-deficient diets (39). Thus, changes in DNA methylation may underlie the persistence of arsenic-induced ER-α overexpression that, in turn, plays a role in oncogenesis. Such a mechanism for the effects of gestational arsenic exposure would explain our observation that a brief period of exposure to inorganic arsenic during gestation caused aberrant gene and protein expression that could be detected long after exposure to the metalloid because mice were assessed at 9 months old or older. Initiation of carcinogenesis is thought to involve the irreversible establishment of phenotypic changes, which is consistent with the persistence of the arsenic-induced altered gene expression in the present study.

The overexpression of hepatic ER-α observed in the present work confirms and extends our earlier observation of ER-α activation in the uterus after chronic exposure of adult mice to inorganic arsenate (22). In addition, the combined results indicate that both inorganic arsenate (22) and arsenite (present study) exposure can result in ER-α overexpression. The present work also shows that increased ER-α expression is associated with arsenic-induced hepatocarcinogenesis. These results indicate that a persistent increase in ER-α expression and activity occurred in adult male mice exposed to arsenic in utero. An association between increased expression of the ER gene and estrogen-induced tumor formation has been demonstrated by MT-mER transgenic mice, which overexpress the ER gene and are highly sensitive to the carcinogenic effects of synthetic estrogens (20). The spectrum of tumors and/or preneoplastic lesions induced by inorganic arsenic in mice (in liver, ovary, uterus, oviduct, testes, adrenal gland) in prior studies (15,22) and the enhanced expression of the ER-α in the liver (present study) and uterus (22) after arsenic exposure raise the possibility that arsenic carcinogenesis may, in some cases, involve enhanced cellular responsiveness to endogenous or exogenous estrogenic compounds.

Arsenic-induced overexpression of ER-α in this mouse model may have profound implications for arsenic-induced carcinogenesis in humans. Estrogens are widely used as pharmaceuticals, often in an elective fashion, such as in hormone replacement therapy. In addition, a large number of natural and synthetic chemicals present in the environment have been shown to act as xenoestrogens (40). The results of the present study raise the possibility that people exposed to arsenic may be more sensitive to the well-established oncogenic effects of estrogenic compounds (18–21,41–43) than those who have not been exposed to arsenic. Conversely, pharmacologic or environmental exposure to estrogens may exacerbate the carcinogenic effects of arsenic. In this context, it is interesting to note that endometrial carcinoma cells that have been transfected with the ER-α gene and overexpress ER-α are more invasive than untransfected cells (44), indicating the possibility that arsenic, through its promotion of ER-α expression, may enhance the progression of estrogen-related tumors. Further research in this area is warranted; we are presently using our transplacental model to examine carcinogenic interactions between arsenic and synthetic estrogens. The persistent overexpression of cyclin D1 in the livers of adult mice exposed to arsenic during gestation may also have implications for carcinogenesis. The D-type cyclins appear to be essential for the progression of the cell cycle through the G1 phase (45). Overexpression of the cyclin D1 gene has been associated with aggressive forms of human HCC (25), and organ-specific targeted overexpression of cyclin D1 in transgenic mice is sufficient to initiate hepatocellular carcinogenesis (25). In addition, expression of a cyclin D1 antisense construct that prevents cyclin D1 expression in rodent hepatoma cells inhibits tumor cell proliferation in vitro and reduces tumor growth after inoculation of cells into nude mice (46). These results indicate that cyclin D1 overexpression is a cause, and not a consequence, of hepatic carcinogenesis (25,46). Thus, overexpression of cyclin D1 in normal liver tissue from mice that developed HCC after exposure to arsenic in utero may be potentially important as a contributing factor in liver carcinogenesis. Cyclin D1 overexpression may be involved in the development of other arsenic-induced cancers in addition to
HCC. Indeed, overexpression of this cell-cycle regulator has been reported in chronic arsenic exposed and/or transformed cells (47,48), in arsenic-induced co-mutagenicity in vitro (49), co-carcinogenicity in mice (50), and in dimethylarsinic acid–induced urinary bladder proliferative lesions in rats (51).

Given the poor mutagenicity reported for arsenic (52) and the low basal methylation of the cyclin D1 promoter, the cyclin D1 overexpression we observed in the livers of adult mice gestationally exposed to arsenic may be a result of the increased ER-α activity. There is strong support for ER-α regulation of cyclin D1; two studies (53,54) have shown an association between estrogen-induced expression of cyclin D1 and steroid-dependent cell proliferation in human breast cancer cells. Moreover, ER-α and cyclin D1 are concurrently overexpressed in several malignant cells and tumors (55,56). We have also seen concurrent overexpression of ER-α and cyclin D1 in arsenic-induced proliferative lesions of the uterus in mice (22).

Our analysis of the expression of several cytochrome P450 genes with sexually dimorphic expression patterns demonstrates that the elevated ER-α levels observed in the livers of adult male mice transplacentally exposed to arsenic are of potential functional significance in that they affect hepatic gene expression. One of the genes analyzed, CYP7B1, encodes an oxysterol 7α-hydroxylase that is normally expressed at higher levels in males than in females (57). CYP7B1 expression has not been definitively shown to be regulated by ERs, but our finding that this gene is repressed in the livers of male mice gestationally exposed to arsenic supports the conclusion that the feminization of hepatic tissue is associated with the elevated ER-α levels we observed. The CYP7B1 product functions to hydroxylate, and thereby inactivate, 5α-androstane-3-β, 17 β-diol, an estrogenic metabolite of dihydrotestosterone (58). Thus, one consequence of the reduced CYP7B1 expression in livers of male mice exposed to arsenic in utero would be elevated levels of an estrogenic compound.

The observed expression levels of two other cytochrome P450 genes, CYP2B9 and CYP2A4, in male mice exposed to arsenic in utero was also consistent with metabolic feminization. CYP2B9 is normally expressed at much higher levels in females but can be induced in males by treatment with estrogen (33). The overexpression of CYP2B9 in adult males exposed to arsenic in utero indicates that the elevated levels of the ER-α observed in these same livers may be affecting hepatic gene expression. CYP2A4 is also a female-dominant gene, the expression of which was increased in the livers of male mice exposed to arsenic in utero. Not only is CYP2A4 female-dominant, there is also evidence that the adult expression pattern is imprinted during development in an ER-dependent fashion (32). The ER-α overexpression associated with gestational arsenic exposure may therefore have altered CYP2A4 expression to a female mode as a fairly early event, although our results do not allow us to define the specific timing of ER-α activation of CYP2A4 during development.

In this regard, estrogen treatment during development can clearly alter genetic imprinting patterns [reviewed in (59)], and it is possible that arsenic-induced ER-α overexpression may affect adult gene expression patterns by altering sensitivity to estrogen-regulated events during development. Genes are often imprinted through DNA methylation during development (60), and arsenic-induced malignant transformation in vitro and subsequent aberrant gene expression have been associated with altered DNA methylation patterns (34). However, because the gene expression studies in the present work were performed in adult mice, we do not know when the observed expression changes occurred. Studies are currently underway to examine the time course of ER-α expression and related events in male mice after in utero exposure to arsenic. In any event, the present data clearly demonstrate that relatively brief in utero arsenic exposure can profoundly influence gene expression patterns in adult mice.

To investigate the human relevance of the overexpression of the ER-α and cyclin D1 genes observed in male mice exposed to arsenic in utero, we examined the expression of these genes in liver samples from men residing in Guizhou Province, China, where environmental exposure to arsenic is high (27). This human population has been shown to have an elevation in HCC incidence related to arsenic exposure (16). Although this study was preliminary and involved a very small number of samples, we found that ER-α and cyclin D1 were expressed at much higher levels in the livers of men from this population than in livers from men not known to have been exposed to arsenic. Thus, it appears plausible that hepatic ER-α and cyclin D1 could play a role in the molecular response to environmental arsenic exposure in humans. In agreement with these observations, a marked overexpression of cyclin D1 protein was detected in human skin samples from this arsenic-exposed population from Guizhou Province (61) relative to an unexposed population, although there is no evidence yet that oncogenic mechanisms in the skin and liver are similar. Indeed, it seems likely that multiple mechanisms would be involved in arsenic-induced carcinogenesis.

Our study has several limitations. One is, as already mentioned, the inability to define the time point at which in utero arsenic exposure in mice caused the observed changes in DNA methylation and aberrant gene expression, because assessments were carried out in adults 36 weeks old or older. In addition, only a few sexually dimorphic genes were investigated, and other genes may not show arsenic-induced female-type patterns of expression. The dose of inorganic arsenic used for in utero exposure of mice (85 ppm) is approximately 50 to 100 times higher than the thought to pose a risk to humans for development of liver cancers (7,15), raising the possibility that overt acute toxicity of arsenic may have played a role in the observed responses. However, this level of in utero arsenic exposure did not alter litter size at birth (8.7 pups per litter, 95% CI = 7.7 to 9.7 pups per litter; n = 10 litters) compared with no arsenic exposure (9.5 pups per litter, 95% CI = 8.7 to 10.3 pups per litter; n = 10 litters), and data published in the original carcinogenesis study (15) show that this dose did not affect maternal weight during pregnancy, maternal water consumption during pregnancy, or the body weight of the offspring at any time during the study. Finally, the human samples assessed in the present study came from a population that has a complex mixture of chronic exposure to inorganic arsenic, including inhalation, ingestion, and dermal exposure (27,61). In contrast, the mice used in this study came from mothers who were exposed only orally to arsenic and for 10 days only.

In summary, we have shown that overexpression of ER-α occurred in the livers of male mice in association with hepatocarcinogenesis induced by in utero exposure to inorganic arsenic. Overexpression of ER-α may result from the hypomethylation of the promoter region of the ER-α gene, which in turn
may cause aberrant expression of other genes, including those encoding cyclin D1 and several cytochrome P450 enzymes. Indeed, the three cytochrome P450 genes analyzed showed feminized expression patterns in male mice exposed to arsenic in utero. Overexpression of ER-α and cyclin D1 was also observed in liver samples from arsenic-exposed men as compared with those from unexposed men. Taken together, these data indicate that aberrant expression of the ER-α gene, as a result of changes in methylation, could be an important molecular event in carcinogenesis induced by inorganic arsenic, at least in the liver.

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NOTES
Supported in part by contract N01-CO-12400 (to SAIC Frederick) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.
We thank Dr. Jerrold M. Ward for assistance with the pathological analyses. We also thank Dan Logsdon, Keith Rodgers, Barbara Kasprzak, and Drs. Shuan Fang Li and Jeannie Herring for technical assistance and Drs. Jingbo Pi, Elaine Leslie, and Larry Keefer for critically reviewing the manuscript.
Manuscript received July 16, 2003; revised December 23, 2003; accepted January 15, 2004.