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Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis

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Inorganic arsenic is a human carcinogen that can target the liver, but its carcinogenic mechanisms are still unknown. Global DNA hypomethylation occurs during arsenic-induced malignant transformation in rodent liver cells. DNA hypomethylation can increase gene expression, particularly when occurring in the promoter region CpG sites, and may be a non-genotoxic mechanism of carcinogenesis. Thus, in the present study liver samples of male mice exposed to 0 (control) or 45 p.p.m. arsenic (as $NaAsO_2$) in the drinking water for 48 weeks were analyzed for gene expression and DNA methylation. Chronic arsenic exposure caused hepatic steatosis, a lesion also linked to consumption of methyl-deficient diets. Microarray analysis of liver samples showed arsenic induced aberrant gene expression including steroid-related genes, cytokines, apoptosis-related genes and cell cycle-related genes. In particular, the expression of the estrogen receptor-α (ER-α), and cyclin D1 genes were markedly increased. RT–PCR and immunohistochemistry confirmed arsenic-induced increases in hepatic ER-α and cyclin D1 transcription and translation products, respectively. Arsenic induced hepatic global DNA hypomethylation, as evidenced by 5-methylcytosine content of DNA and by the methyl acceptance assay. Arsenic also markedly reduced the methylation within the *ER*-α gene promoter region, as assessed by methylation-specific PCR, and this reduction was statistically significant in 8 of 13 CpG sites within the promoter region. Overall, in controls 28.3% of the *ER*-α promoter region CpG sites were methylated, but only 2.9% were methylated after chronic arsenic exposure. Thus, long-term exposure of mice to arsenic in the drinking water can induce aberrant gene expression, global DNA hypomethylation, and the hypomethylation of the *ER*-α gene promoter, all of which could potentially contribute to arsenic hepatocarcinogenesis.

Introduction

Inorganic arsenic is a common environmental pollutant and a high priority hazardous substance for human exposure both in the US and around the world (1,2). The general population is exposed to inorganic arsenicals primarily through contaminated drinking water, food and soil while occupational exposure is usually from arsenic inhalation in a number of industrial settings (1,2). Epidemiological studies provide compelling evidence that inorganic arsenic exposure induces tumors at multiple sites including the skin, liver, lung, urinary bladder and prostate (1–4). The single most important route of human exposure is through inorganic arsenic contaminated drinking water. Indeed, elevated levels of drinking water arsenic have been associated with an increased risk of cancer in US populations (4).

The liver is clearly a target of arsenic in humans and arsenic exposure is associated with development of hepatocellular carcinomas as well as other toxic lesions (1–3,5). Evidence indicates that inorganic arsenic can target the liver in animal model systems. After long-term exposure to inorganic arsenic in drinking water, the mouse liver shows toxic lesions including widespread fatty infiltration (5). In a 6-month bioassay in rainbow trout, which is thought to be particularly sensitive to carcinogenesis, arsenic induced liver hyperplasia (6). In addition, repeated injections of arsenate can induce hepatocellular proliferative lesions, including neoplasia, in mice (7). Recently, it was shown that inorganic arsenic exposure in the drinking water of pregnant mice results in a high incidence of hepatocellular carcinomas in the offspring when they reach adulthood (8). Thus, it appears the liver can be a potential target of arsenic carcinogenesis in both humans and non-human species.

Our prior work has shown that chronic exposure of a rat liver epithelial cell line (TRL 1215) to a low concentration of arsenite ($\leq$500 nM) produces malignant transformation concurrently with global DNA hypomethylation and aberrant gene expression (9,10). The extent of DNA hypomethylation in these transformed cells was positively correlated with tumorigenicity of the cells upon inoculation into Nude mice (11), strongly implicating arsenic-induced DNA hypomethylation as a causative factor.

Inorganic arsenic is enzymatically methylated to mono- and di-methylated species in most mammals (12,13). Arsenic methylation occurs at a high level in the liver (12). The methylation process requires *S*-adenosyl-methionine (SAM), as the methyl donor and is accomplished by incompletely characterized methyltransferases (12,14). SAM is also required for most other cellular methylation reactions, including for instance, DNA methylation (15). Chronic, *in vitro* exposure to arsenite induces SAM depletion in rodent liver cells, and this chronic depletion of SAM appears to contribute to a global loss of DNA methylation during malignant transformation (9). The role of DNA methylation in cancer causation is complex and diverse. However, hypomethylation of DNA is thought to be a non-genotoxic mechanism of carcinogenesis that acts by facilitating aberrant gene expression, and can be a causative factor in hepatocarcinogenesis (16–18). The DNA hypomethylation resulting from chronic *in vitro* arsenite exposure in rat liver

Abbreviations: ER-α, estrogen receptor-α; SAM, *S*-adenosyl-methionine.

cells is clearly associated with aberrant expression of genes of potential importance in tumor development including *c-myc*, *c-met* and cyclin D1 (10,11).

Because the liver is a target of arsenic carcinogenesis in humans and rodents (2,8), and altered DNA methylation has been associated with arsenic-induced malignant transformation and aberrant gene expression in rodent liver cells (9–11), the present study was designed to investigate genome-wide and site-specific DNA methylation status in mouse liver after chronic oral inorganic arsenic exposure. In addition, cDNA microarray techniques were used to profile gene expression changes. Critical expression changes were confirmed by RT–PCR at the transcriptional level and by immunohistochemical analysis at the translation level. The results indicate that arsenic induces global DNA hypomethylation and aberrant gene expression *in vivo*, as well as the specific hypomethylation of the *ER*-α promoter region. All these factors could play a potential role in arsenic hepatocarcinogenesis.

Methods and materials

Chemicals

Sodium arsenite ($NaAsO_2$) was purchased from Sigma Chemical Company (St Louis, MO). The Atlas mouse cDNA expression microarray (588 genes) and the primer of mouse ER-α, and cyclin D1 were purchased from the Clontech (Palo Alto, CA). All other chemicals are of reagent grade.

Animals and sample collection

The current study was performed using samples collected during a previous chronic arsenic exposure study concerning the liver and kidney pathology of arsenicals (5). Briefly, 129/SvJ mice were given unaltered water (controls) or 45 p.p.m. arsenic as sodium arsenite in the drinking water for 48 weeks. Liver samples were frozen at −80°C until analysis, or fixed in 10% formalin for histology or immunohistochemistry.

Global DNA methylation determination

Global DNA methylation was determined by methyl acceptance assay and by HPLC assessment of deoxyribonucleosides. The methyl acceptance assay was performed by the method of Balaghi and Wagner (18). This assay measures unmethylated cytosines in DNA by the use of bacterial DNA methyltransferase that indiscriminately methylates all unmethylated cytosine residues using tritiated SAM. Thus, the extent of ^{3}H-methyl acceptance is positively related to the number of endogenously unmethylated cytosines. As such, an increase in incorporation represents a loss of DNA methylation in the original samples. Briefly, 1 μg of DNA was incubated with 3 U of *Sss*I Methylase (New England Biolabs, Beverly, MA) and 1 μM ^{3}H labeled SAM (79 Ci/mmol), 10 mM EDTA, 5 mM DTT and 100 mM Tris–HCl (pH 8.2) in 30 μl mixture and incubated for 1 h at 37°C. The reaction was stopped by chilling on ice, and 15 μl of the reaction mixture was transferred onto Whatman DE 81 filter paper. The filters were washed with suction by 0.5 M sodium phosphate buffer (pH 7.0) twice, followed by 70 and 100% ethanol. After drying, the levels of methyl-^{3}H incorporated into DNA were determined by a Beckman LS 9800 Liquid Scintillation System. An increase in methyl-^{3}H incorporation indicates hypomethylation of endogenous DNA.

HPLC assessment of deoxyribonucleosides was performed according to the method of Gehrke *et al.* (19) with slight modifications. Briefly, 50 μg of purified genomic DNA was denatured by boiling for 2 min, and immediately put on ice. DNA samples were incubated in a mixture containing 10 mM sodium acetate (pH 5.3), 0.1 mM zinc sulfate, and 5 U of nuclease P1 (Sigma, St Louis, MO) for 16 h at 37°C. Then, 10 μl of bacterial alkaline phosphatase (Sigma, 150 U/ml) and 10 μl of 0.5 M Tris buffer (pH 8.5) were added, and the samples were incubated for an additional 4 h at 37°C. The samples were then centrifuged and the supernatants were stored frozen at −20°C until analysis. A HPLC system equipped with a C-18 ODS ion paring reverse-phase column (Advantage-60, 60 Å, 250 × 4.6 mm) and a UV detector was used for separation and quantification of deoxycytidine (dC) and 5-methyl-deoxycytidine (5 mdC). Elution was carried out at 1 ml/min in a mixture of buffer A (50 mM NaPO4, pH 4.0) and buffer B (100% methanol) 97.5:2.5 for 10 min, followed by a mixture of buffer A:B (92:8) for 10 min, and eluted with buffer A:B mix 85:15 for 15 min. The nucleosides were detected at 280 nm. The 5 mdC content of DNA, as percent of total dC (5 mdC + dC), was calculated using area under the peaks for 5 mdC and dC.

Gene-specific methylation analysis

Gene-specific methylation was performed by the bisulfite-modification method (20). Briefly, 10 μg of genomic DNA was digested with *Eco*RI for 2 h at 37°C, and then denatured with 0.3 M NaOH at 37°C for 10 min. The digested single-stranded DNA was sulfonated and deaminated by incubation with 3.1 M sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone, under mineral oil at 50°C for 16 h. Eluted DNA was used immediately or stored at −20°C until analysis. For sequencing, bisulfite modified DNA was amplified using the following oligonuclotide primers for the ER-α gene: 5′-AAATTTTAG-GAATGT TGATTTTAG-3′ (sense) and 5′-CCGATCCTCCTACCCTAC-TAATTCAA-3′ (anti-sense), overlapping the mouse ER-α promoter region (from −2402 to – 2061, a 341 bp DNA fragment) and untranslated Exon C (21); and cyclin D1: 5′-GGTTTTGATT TTTGTTTAATAATAG-3′ (sense) and 5′-GACCATCTAAAAAAAACTA TAATCT-3′ (anti-sense), a 216 bp DNA fragment from −5 to −220. PCR amplification was performed and the PCR products were visualized by ultraviolet illumination after electrophoresis on a 1.5% agarose gel with 0.5 μg/ml ethidium bromide. DNA fragments from agarose gel were extracted using QIAquick Gel Extraction Kits (Qiagen, Valencia, CA). The PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) with pCR® 2.1-TOPO as the plasmid vector and chemically competent One Shot TOP10 cells as the host *Escherichia coli*. Each DNA sample was screened by culture with LAX plate. Ten positive clones were picked from each sample and cultured overnight in Luria–Bertain medium. Plasmid DNA from each clone was purified using QIAprep Spin miniprep kits (Qiagen). Recombinant plasmid DNA were amplified using DNA sequencing Kit (Applied Biosystem, Foster, CA) and M13 reverse primer for 30 cycles after 5 min at 96°C, 30 s at 96°C, 15 s at 50°C and 4 min at 60°C and last 7 min at 4°C. PCR products were purified by using DyeEx Kits (Qiagen) to remove the unincorporated dye, and then sequenced by ABI PRISM 377 DNA Sequencers (Applied Biosystem). In this method, sodium bisulfite first converts all unmethylated cytosines to uracil residues, while 5-methyl-cytosine remains unchanged. The sequence is then amplified with primers converting uracil to thymidines. Hence, unmethylated cytosines are detected as thymidines allowing for determination of site-specific methylation.

Microarray analysis

Total RNA was isolated using Tri-zol (Gibco BRL, Grand Island, NY), followed by DNase I digestion and purification. Microarray was performed as described previously (10). Briefly, 5 μg of total RNA (which was a pooled sample of equal amounts of RNA from 4 mice/group) was converted to ^{32}P-labeled cDNA probes using MuLV reverse transcriptase and [α-^{32}P]dATP with the Clontech Atlas mouse cDNA synthesis primer mix (Clontech). The ^{32}P-labeled cDNA probe was purified using chroma spin-200 columns, denatured in 0.1 M NaOH, 10 mM EDTA at 68°C for 20 min, followed by neutralization with equal volume of 1 M NaH_2PO_4 for another 10 min. The microarray membranes were pre-hybridized with Expresshyb (Clontech) for 1 h at 68°C, followed by hybridization overnight at 68°C with the cDNA probes. The array membranes were washed four times in 2 × standard saline citrate SSC/1% SDS, 30 min each, and two times in 0.1 × SSC/0.5% SDS for 30 min. The array membranes were then wrapped and exposed to a phosphoimage screen. The image was analyzed densitometrically using AtlasImage software.

RT–PCR analysis

For reverse transcription (RT–PCR) analysis, the Advantage one-step RT–PCR kit (Clontech) was used. One milligram of purified total RNA was used for each RT–PCR reaction. Primers for ER-α gene (5′-GGAGACTCGC-TACTGTGCCGTGTG-3′, 5′-CTGACGCTTGTGCTTCAAC ATTCTCC-3′), and cyclin D1 (5′-GTGCATCTACACTGACAACTCTATC CG-3′, 5′-GTGGGTTGGAAATGAACTTCACATCTG-3′) were synthesized by OPERON (Alameda, CA). The target genes were amplified simultaneously using the β-actin as control. The RT–PCR products were visualized at the end of a series of amplification cycles (25, 30 and 35 cycles) by ultraviolet illumination after electrophoresis through a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide at 50 V for 2 h. Gels were photographed with a digital camera and analyzed with Kodak gel analysis 2.0 software (Kodak, Rochester, NY).

Immunohistochemical and histopathological analysis

Immunohistochemical analysis for ER-α was performed as reported previously (7). The formalin-fixed, paraffin-embedded sections of liver from arsenic exposed and control mice were microwaved after deparaffinization for 10 min in citrate buffer. The specimens were incubated with the polyclonal rabbit anti-ER-α antibody or polyclonal rabbit anti-cyclin D1 antibody (Santa Cruz, Santa Cruz, CA), followed by incubation with the secondary antibody. Reactions were visualized with an avidin–biotin–peroxidase kit (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromagen. To demonstrate the specificity of immunostaining, primary

antibodies were omitted from each staining series (7). Hemotoxylin and eosin stained slides were additionally used for general pathological analysis.

Statistical analysis

For RT–PCR, results represent the mean ± SEM ($n = 3$–4) and a Student's *t*-test was used for comparison. For gene-specific methylation analysis, 19 (control) or 21 (arsenite exposed) clones for ER-α, and 22 (control) or 23 (arsenite) clones for cyclin D1 derived from three separately processed samples of DNA were assessed, and are expressed as incidence of methylated CpG sites out of total sites assessed. The Fisher's exact test was used for comparing clonal incidence to control. The significance level was set at $P \leq 0.05$ in all cases.

Results

Histological analysis

In the present work, mice were chronically (48 weeks) exposed to arsenite (45 p.p.m.) in the drinking water in order to determine correlations between liver pathology, aberrant gene expression, and potential genome-wide and gene-specific errors in methylation. Although arsenic exposure did not affect body weight or liver weight during the exposure period (5), pathological analysis of livers showed overt hepatocyte hypertrophy and fatty infiltration as widespread vacuoles consistent with fatty droplets after arsenic exposure (Figure 1). Thus, chronic oral inorganic arsenic exposure induced hepatocyte hypertrophy and steatosis. Fatty liver often is associated with dietary manipulations that produce methyl deficiency and results in formation of hepatocellular tumors.

Aberrant gene expression associated with chronic arsenic exposure

In order to profile aberrant gene expression after chronic arsenic exposure and select specific genes for assessment of aberrant methylation, microarray analysis was performed. Microarray analysis showed that among the 588 genes tested, ~5% (30 genes) were aberrantly expressed after chronic arsenic exposure. Altered gene expression induced by arsenic exposure is listed in Table I. Aberrantly expressed genes included: genes encoding for cell cycle regulators, such as cyclin D1; genes encoding for growth factors and hormone receptors, such as ER-α and EGF. Genes for which expression was significantly altered also included apoptosis- and stress-related genes. The expression of ER-α was increased 2.3-fold over control, while cyclin D1 increased 3.6-fold. Both ER-α and cyclin D1 have been associated with hepatocarcinogenesis (22,23). Thus, these two genes were selected for in-depth analysis of promoter region methylation (see below).

Over-expression of ER-α and cyclin D1 observed by microarray after arsenic exposure was confirmed at both the transcriptional and translational levels. RT–PCR analysis showed hepatic ER-α and cyclin D1 mRNA levels were dramatically increased with chronic exposure to arsenic (Figure 2). Quantification revealed ER-α transcript increased 2.1-fold while cyclin D1 transcript increased 2.3-fold with arsenic treatment. In addition, immunohistochemical analysis revealed arsenic induced a marked increase in ER-α (Figure 3) and cyclin D1 (Figure 4) protein in arsenic-treated mouse liver. Staining for both ER-α and cyclin D1 protein was intense and widespread in livers from arsenic exposed mice, and often most intense within nuclei.

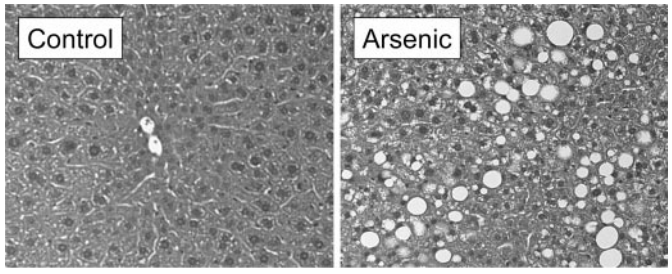

Fig. 1. Pathological analysis of liver lesions after arsenic chronic exposure in mice. Representative section of control liver showing normal histology. Representative arsenic-treated liver showing cellular hypertrophy and fatty infiltration seen as widespread vacuoles.

Table I. Gene expression changes in livers of mice exposed to drinking water containing arsenite (As 45 p.p.m.) or unaltered water (control, As 0 p.p.m.) for 48 weeks

Gene/protein	GenBank	Cont	As	As/Con
Cell cycle regulators				
Cyclin D1	S78355	1330 ± 360	4720 ± 670	3.57*
Cyclin D2	M83479	480 ± 210	1460 ± 310	3.04*
Cyclin D3	U43844	1870 ± 730	4000 ± 1320	2.14
Cyclin-dependent kinase inhibitor-1	U09507	1260 ± 240	3900 ± 470	3.10*
Growth factors				
ER-α	M38651	2930 ± 690	6670 ± 520	2.27*
Epidermal growth factor	J00380	2640 ± 320	9680 ± 2800	3.67*
VEGE receptor-1	L07297	1100 ± 140	2630 ± 580	2.38*
IGF-binding protein-1 (IGFBP-1)	X81579	2830 ± 470	7060 ± 1620	2.50*
Nuclear hormone ROR-alpha	U53228	755 ± 130	1930 ± 480	2.55*
Apoptosis-related				
Caspase-1 (ICE)	L28095	5450 ± 420	2000 ± 1040	0.37*
Caspase-2	D28492	2930 ± 360	1760 ± 90	0.60*
Capase-11	U59463	3380 ± 960	1830 ± 410	0.54
BAD protein	L37296	4020 ± 620	2210 ± 490	0.55*
Tumor necrosis factor beta	M16819	4370 ± 1100	2670 ± 800	0.62
Stress-related				
Glutathione *S*-transferase	J03958	9480 ± 2500	24500 ± 5900	2.58*
Glutathione peroxidase-1	U13705	1180 ± 390	2490 ± 380	2.10*
Early response growth protein-1	M20157	870 ± 200	2460 ± 650	2.81*
ICAM-1	X52264	850 ± 220	2900 ± 820	3.42*
CD14 LPS receptor	M34510	1180 ± 410	4130 ± 900	3.48*
IL-4	M25892	4050 ± 250	980 ± 400	0.25*
IL-6 receptor	M83336	6530 ± 1500	14000 ± 2800	2.15*
NF-κB p65	M61909	5180 ± 1280	16250 ± 4460	3.16*
NF-κB p105	M57999	470 ± 50	1800 ± 520	3.84*
NADPH P450 reductase	D17671	12400 ± 460	8770 ± 1100	0.71*
Other categories				
Type-1 cytokeratin-18	M11686	12460 ± 2660	21720 ± 2900	1.74*
XPAC	X74351	19560 ± 5500	5690 ± 1930	0.30*
MmRad52	Z32767	5260 ± 230	2670 ± 600	0.51*
RXR interaction protein RIP15	U09419	790 ± 150	2460 ± 520	3.11*
Homobox protein D4	J03770	640 ± 120	2490 ± 330	3.93*
Tie-2 protooncogene	S67051	590 ± 80	2190 ± 670	3.73*

Data are mean ± SEM of five hybridizations using pooled RNA isolated from four mice.
*Significantly different from controls $P < 0.05$.

Global DNA hypomethylation is induced by chronic arsenic exposure in mouse liver

Our prior work indicated *in vitro* chronic arsenite exposure induced global DNA hypomethylation and aberrant gene expression in rodent liver cells concomitantly with malignant

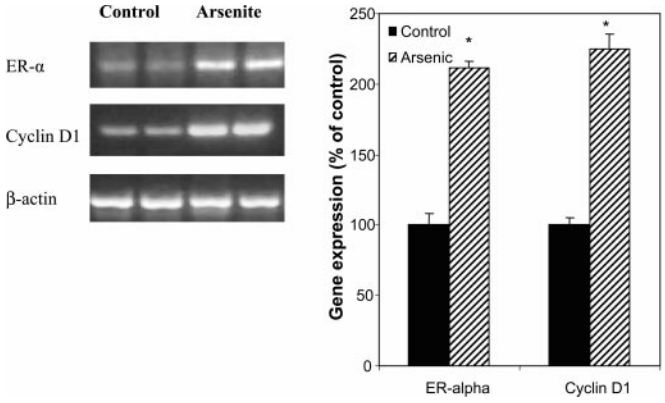

Fig. 2. RT–PCR analysis of the expression of ER-α and cyclin D1 in control and chronic arsenic exposed liver. In liver treated with arsenic, mRNA level of ER and cyclin D1 were increased after chronic exposure to arsenic. Data are the mean ± SEM ($n = 4$ mice). *Significantly different from control, $P < 0.05$.

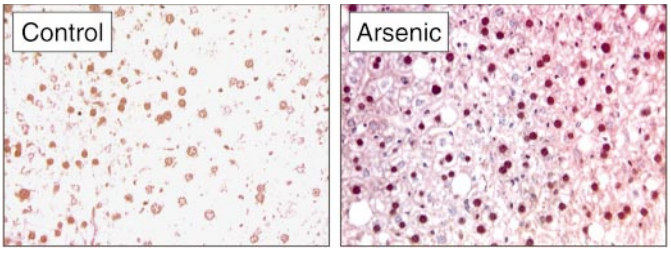

Fig. 3. Immunohistochemical localization of ER-α in the mice liver after chronic exposure to arsenic in drinking water for 48 weeks. Note prominent nuclear staining (brown) in a representative section of arsenic exposed liver and minimal staining in control liver.

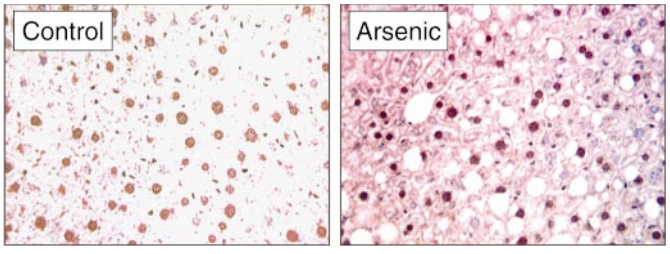

Fig. 4. Immunohistochemical localization of cyclin D1 in the mice liver after chronic exposure to arsenic in drinking water for 48 weeks. Note prominent nuclear staining (brown) in a representative section of arsenic exposed liver and minimal staining in control liver.

transformation (9–11). This study attempted to extend these findings to an *in vivo* system by examining DNA methylation status in mouse liver after chronic oral inorganic arsenic exposure. Global DNA methylation status was determined in purified genomic DNA by assessing methylated cytosine residues (as 5 mdC) using HPLC or by the methyl acceptance assay. Both methods revealed that a significant reduction in hepatic genomic DNA methylation occurred after chronic arsenite exposure (Figure 5). The proportion of cytosines found as 5 mdC was significantly reduced by 30% after chronic arsenite exposure. Similarly, a significant increase in

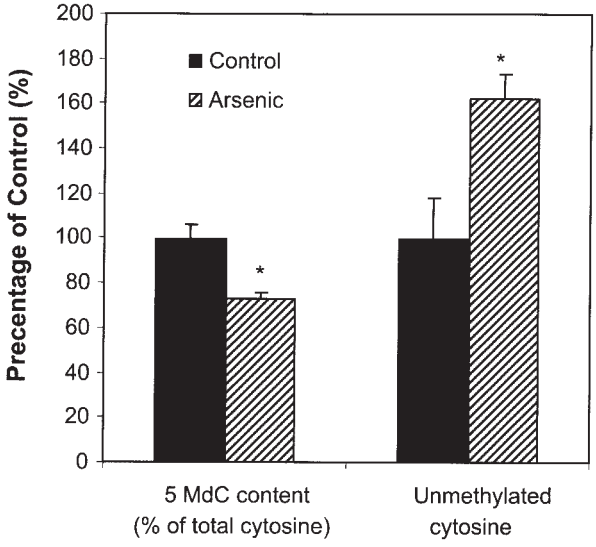

Fig. 5. Arsenic-induced alteration in hepatic global DNA methylation after chronic exposure for 48 weeks. HPLC assessment of methylated cytosine residues (as 5 mdC) in DNA expressed as a percent of total cytosine residues (5 mdC plus 2′-dC) (left). The methyl acceptance assay in which all unmethylated cytosines in DNA samples are methylated by a bacterial DNA methyltransferase using radioisotopic [^{3}H]SAM as the methyl donor with the result expressed as percent of control (right). Data are given as the mean ± SEM and are based on individual DNA samples isolated from 5 mice/group. An asterisk indicates a significant difference from appropriate control ($P < 0.05$).

unmethylated cytosines in genomic DNA was detected by the methyl-acceptance capacity. Together both methods provide consistent evidence that global DNA hypomethylation occurred with chronic oral arsenic exposure in the mouse liver.

Methylation status of the promoter region of the ER-α and cyclin D1

Methylation of DNA, especially when found in the promoter region of a gene, can modify expression, with reduced methylation typically associated with enhanced activity (24,25). In order to define the effect of arsenic exposure on the methylation status of ER-α or cyclin D1, a 341-bp DNA fragment including 13 CpG sites in the promoter region of ER-α, and a 211-bp DNA fragment including 16 CpG sites within the promoter of cyclin D1, were subjected to a high sensitivity mapping of methylated cytosines via a genomic sequencing technique involving sodium bisulfite-modification. The methylations status of CpG sites in the promoter of the ER-α gene from hepatic DNA is shown in Figure 6. Of the 13 CpG sites in the ER-α gene promoter all showed less methylation in the liver of mice exposed to arsenic, and this reduction was significant in 8 of 13 sites (Figure 6). Overall, the methylation of the CpG sites within ER-α promoter region was reduced nearly 90% from a control rate of 28% of all available sites to 2.9% after chronic arsenic exposure. In marked contrast to the ER-α gene, the methylation status of cyclin D1 gene promoter region was unchanged by arsenic treatment (Table II). In fact, the 16 CpG sites in promoter region of cyclin D1 gene from control liver were essentially unmethylated. Thus, the activation of cyclin D1 by arsenic in this case probably occurs by a mechanism other than aberrant methylation at promoter region DNA.

Discussion

The present study demonstrates clearly that chronic oral inorganic arsenic exposure can produce global DNA hypomethylation in the mouse liver and specific hypomethylation of the promoter region of the ER-α gene. This is consistent with our prior *in vitro* work showing genome-wide hypomethylation and concurrent aberrant gene expression in liver cells malignantly transformed by arsenic (9,10). Alteration of hepatic gene expression was also evident in the present study, and some, but not all of these may be a result of alteration in DNA methylation status. For instance, loss of promoter region methylation is associated with increased expression of ER-α (26,27). RT–PCR and immunohistology analysis confirmed the over-expression of ER-α and cyclin D1 at the mRNA and the protein levels. Although tumors did not develop in the mice exposed to arsenic in the present study, cellular hypertrophy and steatosis were common. Steatosis is associated with consumption of methyl-deficient diets that can lead to liver tumor formation (28,29). Thus, alterations in DNA methylation status together with the aberrant gene expression patterns observed in the present work may provide novel insight into the mechanisms of arsenic hepatocarcinogenesis.

Methylation of DNA is an epigenetic modification that can play an important role in the control of gene expression in mammalian cells, and aberrant methylation frequently occurs during oncogenesis (15,30,31). In vertebrate DNA, ~3–5% of the total cytosine residues are present as 5 mdC (15,24,32). Generally this methylation occurs at cytosines that are contained in a symmetrical CpG nucleotide sequence (24). Alterations in global levels and regional changes in the patterns of DNA methylation are commonly observed in the early stages of neoplasia (15,33). Errors in DNA methylation begin to appear early in tumor development, prior to the appearance of overt neoplasia (34,35). In particular, it is thought that aberrant DNA methylation could be important in the development of liver cancers (36) and is a possible epigenetic mechanism that underlies the aberrant expression of genes involved in the mouse liver carcinogenesis (16,37). This fortifies the concept that the hepatic DNA methylation errors observed in the present study after arsenic exposure may have implications in tumor causation. The liver is clearly a target of arsenic carcinogenesis in humans (2), and proliferative liver lesions occur after inorganic arsenic exposure in mice ranging from hyperplasia to hepatocellular carcinoma (5,7,8). It is also of interest that methyl-deficient diets, which produce DNA hypomethylation and steatosis, also produce hepatocellular tumors (28,29). This is consistent with the present results where DNA methylation changes are associated with fatty changes in the liver. It is unclear if these hepatic changes would have resulted in eventual tumor formation, but this may have been the case had the exposure period been extended. However, the present results clearly indicate chronic arsenic exposure *in vivo* can cause generalized DNA methylation errors that could lead to liver tumor formation.

DNA hypomethylation can be induced by methyl-deficient diets or chemically induced methyl donor group depletion, including chronic depletion of the cellular SAM pool (29). Such depletion is thought to induce methylation errors by causing insufficient methyl groups for DNA methylation during replication (37,38). Indeed, feeding of methionine and choline-deficient (i.e. 'methyl-deficient') diets results in hepatocellular tumor formation in mice associated with SAM depletion and DNA hypomethylation (28,29,37). In fact, the combination of methyl-deficient diets and arsenic produced DNA hypomethylation in mice (38), although in this study arsenic alone was ineffective. It is noteworthy that steatosis associated with feeding methyl-deficient diets in this study was made more severe by arsenic exposure (38). In rats fed a choline-devoid diet for up to 14 months, hepatic lesions develop and progressed through two distinct stages, the first characterized by severe steatosis and an increase in cell turnover and the second by fibrosis and the development of hepatocellular carcinomas (39). Therefore, steatosis seen in this study may well be related to DNA hypomethylation, which in turn, may be a precursor of liver neoplasia. Chronic methyl depletion is probably not the only mechanism by which arsenic induces hypomethylation and factors such as direct inhibition of DNA methyltransferase (9) probably also contribute to this response.

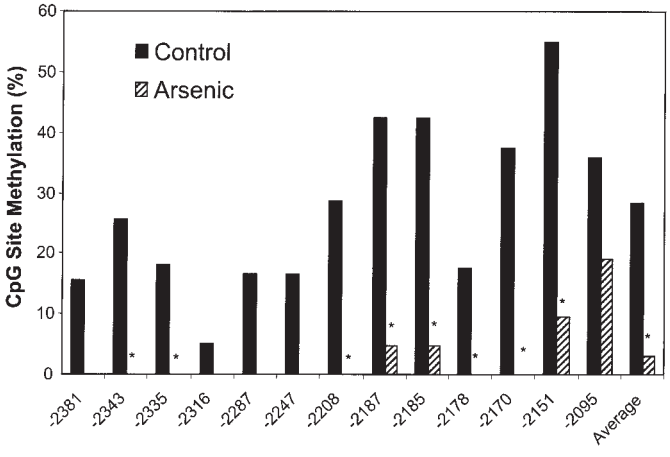

Fig. 6. Alteration of methylation status at each CpG site of ER-α in mouse liver after chronic arsenite treatment for 48 weeks. Arsenic-treatment resulted in decreased methylation in 8 of 13 CpG islands. Data represent the incidence (%) of methylation at the specific sites as assessed in 19–21 individual clones derived using individual DNAs isolated from 5 mice/group. Data designated 'average' are the average clonal incidence for all sites. An asterisk indicates a significant difference from control ($P < 0.05$).

Table II. The CpG site methylation of the cyclin D1 gene

CpG Site	−31	−41	−59	−61	−63	−71	−73	−84	−96	−98	−100	−125	−128	−185	−192	Total
Control	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	1/22 (5%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	1/352 (0.3%)
Arsenic	0/23 (0%)	0/23 (0%)	0/23 (0%)	1/23 (4%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	1/368 (0.3%)

Measured in the liver of mice after chronic exposure to arsenic (45 p.p.m. arsenic as sodium arsenite) in the drinking water for 48 weeks. Data represent the incidence (%) of methylation at the specific sites as assessed in 22–23 clones derived using individual DNAs isolated from 5 animals/group.

Hypomethylation in the promoter region of various genes can control their expression via control of transcription (24,25). The presence of methylated CpG islands in promoter regions typically suppresses expression, while hypomethylation leads to over-expression (24). This process is thought to be due to the presence of 5-methylcytosine that interferes with the binding of transcription factors or other DNA-binding proteins thereby blocking transcription. In different types of tumors, aberrant methylation of CpG islands in the promoter region has been observed for many cancer-related genes, such as *c-myc*, and Ha-*ras* (38,40). Decreases in the 5-methylcytosine content of DNA and the hypomethylation of individual genes are common early events found in many human and animal tumors (29,34,35,41). In the present study, when the genomic sequencing technique to map methylation of the 13 CpG rich sites within ER-α promoter region, they all showed much less methylation after arsenic exposure. Overall, there was almost a 90% decrease in the methylation of the ER-α promoter region after arsenic exposure. These results clearly indicated that DNA hypomethylation of ER-α gene occurred after chronic arsenic exposure, and thus was potentially responsible for ER-α over-expression, since several studies indicate ER expression is inversely associated with methylation (26,27). Thus, chronic arsenic exposure can introduce DNA methylation errors that may activate critical genes in oncogenesis, at least in the liver.

In the present work, microarray analysis showed alterations in expression of $\approx 5\%$ of the genes investigated including steroid-related genes, cytokines, apoptosis genes and cell cycle-related genes. Especially noteworthy are the over-expression of ER-α gene and cyclin D1. Prior work has shown that chronic exposure to inorganic arsenic in mice can produce hepatocellular proliferative lesions together with over-expression of the ER-α and cyclin D1 (7). In mice, transplacental arsenic exposure, which produces hepatocellular carcinoma when animals reach adulthood, also causes over-expresssion of ER-α and cyclin D1 (42). ER-α, a member of the steroid hormone receptor superfamily, is a hormone-activated transcription factor that mediates the biological effects of estrogen in a variety of responsive tissues (43). The expression of ER-α can be affected by changes in methylation of the ER gene (26,27,44), and is often over-expressed in early proliferative lesions in the liver or elsewhere (45–47). Estrogens are unequivocal liver carcinogens in rodents and suspected human carcinogens (48). It has been proposed that they act as epigenetic carcinogens probably via ER-mediated mechanisms (22,45,48). There is strong evidence that aberrant mitogenesis and hepatocellular proliferation observed during estrogen-induced hepatocarcinogenesis is mediated through ER (48). We have reported that over-expression of the ER-α from arsenic exposure is associated with proliferative lesions and hepatocellular carcinogenesis (7,8,42) and this over-expression is associated marked loss of methylation in the ER-α promoter region (42). In human hepatocellular carcinoma recent evidence indicates that in ~37% of cases the ER gene appears to be hypomethylated (49). Thus, the over-expression of ER-α observed in the present study in the mouse liver after chronic arsenic exposure supports a hypothesis that arsenic might somehow act through an estrogenic mechanism. Presumably, the over-expressed ER-α would create hepatic hypersensitivity to endogenous steroids.

Microarray analysis also revealed arsenic-induced over-expression of the cell cycle regulating genes cyclin D1, cyclin D2 and cyclin D3. Cyclin D1, an important cell-cycle regulating oncogene (23), is transcriptionally up-regulated by numerous growth factors, including potentially estrogens (50). Cyclin D1 is also over-expressed in liver cells malignantly transformed with arsenic (11) and is considered a hepatic oncogene (51). Targeted over-expression of cyclin D1 in the liver is alone sufficient to initiate hepatocellular carcinogenesis (51), indicating it is a cause, and not a consequence, of malignant transformation of liver cells. In addition, co-over-expression of cyclin D1 and ER-α has been reported after chronic arsenic exposure (7,42), especially in estrogen responsive tissues where arsenic-induced proliferative lesions and cancers occur, such as the uterus and liver (7,8,42,52). Aberrant expression of cyclin D1 would also be expected to cooperate with other oncogenes in carcinogenic transformation (23). In the present study, the 16 CpG sites in promoter region of cyclin D1 gene from control liver were essentially unmethylated, and thus no significant arsenic-induced changes occurred in cyclin D1 methylation. Since cyclin D1 is potentially an ER-α-linked gene, the activation of cyclin D1 by arsenic in this case may occur secondarily to ER-α over-expression. In any event, over-expression of cyclin D1 has been observed after arsenic exposure in a number of both *in vitro* (10,11,53,54) and *in vivo* (7,41,55,56) model systems of arsenic carcinogenesis, including skin and bladder cancers in rodents associated with arsenic exposure. Thus, it appears cyclin D1 over-expression may be a consistent event in arsenic carcinogenesis.

Although at one time, arsenic was thought to be an equivocal carcinogen, that was clearly carcinogenic in humans but not active in rodents, there is accumulating evidence that arsenicals can be carcinogenic in animals (8,52,55,56,57). Indeed, animal models of arsenic carcinogenesis have been developed that show activity in skin, urinary bladder, liver and lung (8,52,55,56,57), all of which are known human target tissues (2). This target-site concordance provides support to the concept that similar carcinogenic mechanisms may apply in humans and rodents.

In summary, the present study indicates clearly that chronic arsenic exposure *in vivo* induces hepatic DNA hypomethylation, and inappropriate expression of genes potentially important in oncogenesis. The induction of DNA hypomethylation after chronic arsenic exposure in mice is consistent with cell systems associating arsenic-induced malignant transformation with DNA hypomethylation in liver cells. Thus, arsenic-induced errors in DNA methylation could be an early molecular lesion with the potential for impacting oncogenic growth in the liver, and therefore a plausible mechanism of hepatocarcinogenesis. This should not be seen as the only potential mechsnism of arsenic carcinogenesis and it is quite likely arsenic acts through multiple mechanisms in a tissue-specific manner.

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