Urogenital Carcinogenesis in Female CD1 Mice Induced by In utero Arsenic Exposure Is Exacerbated by Postnatal Diethylstilbestrol Treatment

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Abstract

Transplacental inorganic arsenic carcinogenicity, together with postnatal exposure to diethylstilbestrol or tamoxifen, was studied. Pregnant CD1 mice received 85 ppm arsenic in the drinking water from gestation days 8 to 18 and were allowed to give birth. Groups (n = 35) of female offspring were injected s.c. on postpartum days 1 through 5 with diethylstilbestrol (2 µg/pup/d) or tamoxifen (10 µg/pup/d) and observed for 90 weeks. Arsenic alone induced some urogenital system tumors, including mostly benign tumors of the ovary and uterus, and adrenal adenoma. Diethylstilbestrol alone induced some tumors (primarily cervical) but when given after in utero arsenic, it greatly enhanced urogenital tumor incidence, multiplicity, and progression. For instance, compared with the incidence of urogenital malignancies in the control (0%), arsenic alone (9%), and diethylstilbestrol alone (21%) groups, arsenic plus diethylstilbestrol acted synergistically, inducing a 48% incidence of malignant urogenital tumors. Of the urogenital tumors induced by arsenic alone plus diethylstilbestrol, 80% were malignant, and 55% were multiple site. Arsenic plus diethylstilbestrol increased ovarian, uterine, and vaginal tumors, and urinary bladder proliferative lesions, including three transitional cell carcinomas. Tamoxifen alone did not increase urogenital tumors or affect arsenic-induced neoplasia but did increase arsenic-induced uroepithelial proliferative lesions. Uterine and bladder carcinoma induced by arsenic plus diethylstilbestrol greatly overexpressed estrogen receptor-α (ER-α) and pS2, an estrogen-regulated gene. In neonatal uteri, prenatal arsenic increased ER-α expression and enhanced estrogen-related gene expression induced by postnatal diethylstilbestrol. Thus, arsenic acts with estrogens to enhance production of female mouse urogenital cancers.

Introduction

Inorganic arsenic is a human carcinogen and environmental exposure through contaminated drinking water is a major concern throughout the world (1). Inorganic arsenic is causally linked to a variety of cancers in humans (1–4) and seems to show a predilection for the urogenital system. Arsenic exposure is linked to urinary bladder and renal cancer and is potentially associated with cancer of the prostate and ureter (1). There is limited evidence linking arsenic poisoning and uterine cancer (2, 3). Oral inorganic arsenic exposure is also linked with dermal, hepatic, and pulmonary tumors in humans (1, 4). In mice, oral inorganic arsenic acts as a cocarcinogen or copromoter in skin (5, 6), and as a complete transplacental carcinogen in several tissues (7, 8). In rats, dimethylarsinic acid, a methylated metabolite of inorganic arsenic, induces urinary bladder tumors (9). Dimethylarsinic acid is also a urinary bladder and renal tumor promoter in rats after initiation with tissue-specific organic carcinogens (10, 11). Thus, arsenical carcinogens can also target the urogenital system in rodents. Embryologically, the urinary and genital systems are closely related in mammalian development and conceptualizing them as distinct systems is somewhat arbitrary (12). Indeed, the sex ducts, gonads, and kidneys in mice are derived from common intermediate mesodermal cells during early in utero development (13). The fact that arsenical carcinogens attack various sites in the human and rodent urogenital tract may indicate a heightened sensitivity of cells within this system.

Gestation is a period of high sensitivity to chemical carcinogenesis because of factors such as organogenesis coupled with global proliferative growth (14, 15). In this regard, oral inorganic arsenic exposure of pregnant C3H mice during gestation reproducibly induces tumors in the offspring as adults (7, 8). This response to transplacental arsenic includes induction of hepato-cellular carcinoma and induction or initiation of lung cancers (7, 8). The liver and lung are targets of arsenic carcinogenesis in humans (1, 3, 4). In utero arsenic exposure in mice also induces ovarian tumors and uterine and oviduct preneoplastic lesions (7, 8). The C3H mouse is considered to be sensitive to chemical carcinogens but because it had proven difficult to induce tumors in rodents with inorganic arsenic at the time of our original transplacental study (7), this was considered an appropriate rationale for the selection of mouse test strain. The C3H mouse also has a significant spontaneous rate of tumor formation in some of the target tissues of arsenic carcinogenesis (7, 8). Nonetheless, the remarkable multiorgan carcinogenic sensitivity seen with in utero arsenic exposure in C3H mice (7, 8) would be extremely alarming if it held true for similar life-stage exposures in humans.

The proliferative lesions and tumors seen with inorganic arsenic as a transplacental carcinogen in mice, including tumors of the ovary, liver, and adrenal, and hyperplasia of the uterus and oviduct, are all potential targets of carcinogenic estrogens in humans and/or rodents (14–22). Estrogenic compounds can have profoundly adverse effects during development (14, 15, 23–25),
including the synthetic estrogen diethylstilbestrol, which is considered to be a human and rodent transplacental carcinogen (14, 15, 23, 24). Because arsenic showed an "estrogen-like" tumor spectrum, we hypothesized that aberrant estrogen signaling may play a role in transplacental arsenic carcinogenesis (7, 8). In fact, marked overexpression of hepatic estrogen receptor-α (ER-α), a critical factor in estrogen signaling pathways, as well as activation of estrogen-related genes potentially important in carcinogenesis, were observed in adult mice bearing transplacental arsenic-induced hepatocellular carcinoma (26). The overexpression of ER-α is clearly associated with sensitivity to estrogen-induced tumors in mice (27). Furthermore, in an arsenic-exposed human population that shows an elevation in liver cancers, hepatic ER-α was clearly overexpressed (26). In addition, in adult mice, repeated exposure to inorganic arsenic induces uterine proliferative lesions that show marked overexpression of ER-α (28) and chronic arsenic exposure activates the hepatic ER-α gene (29). Thus, ER-α overexpression seems to be associated with the carcinogenicity of arsenic in mice in several tissues (26, 28).

The actions of inorganic arsenic as a transplacental carcinogen deserve additional study. Thus, a primary goal of the present study was to extend our initial findings in C3H mice (7, 8) to another mouse strain with lower spontaneous tumor rates. In addition, as a further test of the hypothesis that aberrant stimulation of estrogen response pathways may play a role in transplacental arsenic carcinogenesis in some tissues (26, 28, 29), the effects of postnatal exposure to the synthetic estrogen, diethylstilbestrol, and the selective ER modulator, tamoxifen, on the carcinogenic effects of in utero arsenic exposure were studied. For this study, CD1 mice were selected because of a low spontaneous tumor rate (30, 31) and because estrogen carcinogenesis has been extensively studied in this strain (14, 15, 23).

Materials and Methods

Chemicals. Sodium arsenite (NaAsO₂), diethylstilbestrol, and tamoxifen were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and treatments. Animal care was provided in accordance with the U.S. Public Health Policy on the Care and Use of Animals as defined in the Guide to the Care and Use of Animals (NIH Publication 86-23). Mice were housed in a barrier facility, at a temperature of 68 °F to 72 °F and with a relative humidity of 50 ± 5% and a 12-hour light/dark cycle. A basal diet (NIH 31; Charles River Laboratory, Bowley, NC) and water (unmodified or modified as below) were provided ad libitum. The National Cancer Institute-Frederick animal facility, where the study was conducted, and its animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. CD1 mice were obtained from Charles River Laboratory.

A total of 72 primigravid females randomly divided into six groups of 12 each and given sterile drinking water containing sodium arsenite (NaAsO₂) at 0 (control, diethylstilbestrol alone, and tamoxifen alone) or 85 ppm arsenic (arsenic alone, arsenic plus diethylstilbestrol, and arsenic plus tamoxifen) ad libitum from days 8 to 18 of gestation. The arsenic dose was based on prior work in C3H mice showing transplacental carcinogenic potential (7, 8). After birth, litters were culled 8 or less. On postpartum days 1, 2, 3, 4, and 5, mice received s.c. injections of diethylstilbestrol (2 μg/pup/d), tamoxifen (10 μg/pup/d), or vehicle (corn oil) after the protocols developed by Newbold et al. (18, 25). Mice were weaned at 4 weeks postpartum and offspring were grouped (n = 35) according to maternal and postpartum treatments. The offspring were observed for a total of 90 weeks (including preweaning). This work with females was done contemporaneously with male offspring from the same mothers, which will be reported separately.

Dam body weights were recorded between days 8 and 18 of gestation. Maternal water consumption was recorded between gestation days 11 to 12 and 15 to 16. Neonatal weights were recorded at birth (time 0), then weekly until weaning and every 5 weeks thereafter. Clinical signs were checked daily and mice were sacrificed when significant signs developed or at 90 experimental weeks.

Pathology. In the 2-year study, a complete necropsy was done on all moribund animals, animals found dead, or on mice at terminal sacrifice. The components of the urogenital system (ovary, oviduct, uterus, cervix, vagina, kidney, and urinary bladder) and liver, lung, adrenal, spleen, thyroid, thymus, skin, and grossly abnormal tissues were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 5 μm, and stained with H&E. Pathologic assessment was done without knowledge of treatment group. The designation of urogenital tumors of "any type" includes both mesenchymal tumors (primarily hemangiomas and hemangiosarcomas) and epithelial tumors (adenomas and carcinomas).

Immunohistochemistry. Uterine adenocarcinoma and bladder transitional cell carcinoma from mice exposed to arsenic plus diethylstilbestrol were studied immunohistochemically for localization and intensity of ER-α and pS2. The sections were treated and the reactions were visualized as described (26, 28), using a polyclonal rabbit anti-ER-α antibody and goat polyclonal anti-pS2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Vector Elite kits (Vector Labs, Burlingame, CA). The primary pS2 antibody was used at a 1:100 dilution. To define specificity, the primary antibodies were omitted from each staining series as a control.

ER-α and estrogen-related gene expression. A preliminary study was done before the tumor end point study, using the same doses and treatment schedule. To see if the various treatments showed any early toxicity. At its termination, uteri were collected (n = 4-8; from 3-5 litters) on day 12 postpartum 1 week after diethylstilbestrol or tamoxifen treatments had ended. These samples were weighed and snap frozen for molecular analysis. In these uteri, the transcript levels of the ER-α, pS2, CYP2A4, and lactoferin were quantified using real-time reverse transcription-PCR (RT-PCR) analysis as described previously (26). Total RNA was isolated from frozen samples and real-time RT-PCR was done using SYBR green master PCR mix (Applied Biosystems, Foster City, CA). Data were normalized with β-actin and expressed as percent control.

Data analysis. Data are given as incidence or as mean ± SE, as appropriate. A probability level of P ≤ 0.05 was considered to indicate a significant difference. Separate benign and malignant tumors in the same tissue of an individual animal were considered to constitute separate cases when tumors were considered by stage but were treated as a single case when determining total tumors in that tissue in a given group. Total tumor incidence is defined as those mice bearing at least one benign or malignant tumor in a given tissue. Total proliferative lesions are defined as the incidence of mice bearing either a benign or malignant tumor or hyperplasia in a given tissue. In pair-wise comparison of lesion incidence, a one-sided Fisher's exact test was used. Tumor incidence is based on numbers of animals available for observation, and losses were due to autolysis that was considered too advanced for diagnosis. For multiple comparisons of average survival, body weight, and gene transcript data, two-sided Dunnett's t tests afterANOVA were used. Data for gene expression levels were log transformed before statistical analysis. Survival rates were also compared at several time points by Fisher's exact test.

A synergistic response in tumor formation is here defined as the joint actions of agents such that their combined effect is greater than the mathematical sum of their individual effects. Synergy with combined treatments was examined statistically by calculating the number of excess cases of a given lesion (e.g., number of cases in the arsenic plus diethylstilbestrol group minus the number of cases in arsenic alone group and minus the number of cases in diethylstilbestrol alone group = number of excess cases) and comparing this to the hypothetical purely additive scenario (zero excess cases) using the average group size (rounded to the nearest whole number) of the arsenic alone and diethylstilbestrol alone groups or the arsenic alone and tamoxifen alone groups, as appropriate. The incidence of excess cases was then compared with the hypothetical additive by Fisher's exact test. Using these criteria, in both cases with combined treatments (arsenic plus diethylstilbestrol or plus tamoxifen), five or more excess cases were statistically significant.
Results

Body weights, water consumption, and survival. Pregnant CD1 mice were treated with arsenic in the drinking water, the female offspring were subsequently treated with diethylstilbestrol or tamoxifen, and carcinogenic response was evaluated. Arsenic in the water did not alter maternal body weight gain or water consumption and neonatal weights were not altered by the prenatal or postnatal treatments (data not shown). Survival relative to control was not altered by the various treatments. The mean (n = 35) survival values in weeks (±SE) were as follows: control, 82.2 ± 2.1; arsenic alone, 77.7 ± 3.5; diethylstilbestrol alone, 74.3 ± 4.1; tamoxifen alone, 85.1 ± 2.4; arsenic plus diethylstilbestrol, 73.7 ± 3.7; arsenic plus tamoxifen, 78.8 ± 3.6. Body weights of female offspring were not suppressed by the various treatments. For instance, at 55 weeks, the mean body weights (n = 27 to 31) were as follows: control, 45.7 ± 1.5 (mean ± SE in grams); arsenic alone, 47.6 ± 1.6; diethylstilbestrol alone, 48.4 ± 1.9; tamoxifen alone, 50.1 ± 1.4; arsenic plus diethylstilbestrol, 50.2 ± 1.8; and arsenic plus tamoxifen, 51.3 ± 1.4.

Tumor pathology. Transplacental arsenic exposure alone induced a significant increase in tumors of the urogenital system compared with control (Table 1), which were largely benign in nature. Diethylstilbestrol alone also increased urogenital tumor incidence. However, when arsenic exposure was combined with diethylstilbestrol, there was a pronounced, often synergistic (see Materials and Methods) increase in incidence and severity of urogenital tumors. Thus, arsenic plus diethylstilbestrol increased urogenital system total malignancies of any type (mesenchymal and epithelial), total tumors of any type, carcinomas, and total epithelial tumors compared with control, arsenic alone, or diethylstilbestrol alone. Marked increases in multiple-site urogenital system tumors occurred with arsenic plus diethylstilbestrol compared with control, arsenic alone, or diethylstilbestrol alone. A synergistic response in mice treated with arsenic plus diethylstilbestrol was clearly seen in urogenital malignancies of any type, where six excess cases occurred in the arsenic plus diethylstilbestrol group (6 excess cases of 33 mice versus 0 of 34 for purely additive; P<0.05) compared with the treatments alone. Similarly, synergy occurred with arsenic plus diethylstilbestrol in urogenital carcinomas (six excess cases), multiple-site urogenital tumors of any type (nine excess cases), and multiple-site urogenital epithelial tumors (eight excess cases). Tamoxifen alone did not significantly increase urogenital tumors. Arsenic plus tamoxifen induced increases in malignant urogenital tumors of any type and urogenital carcinoma compared with controls that were not observed with arsenic or tamoxifen alone.

For specific tumors of the genital system (Table 2), arsenic plus diethylstilbestrol induced increases in uterine carcinoma compared with control not observed with arsenic or diethylstilbestrol alone. Combined arsenic and diethylstilbestrol synergistically increased uterine carcinoma (five excess cases). Significant increases in vaginal carcinoma occurred with arsenic plus diethylstilbestrol compared with controls that were not observed with the separate treatments. Arsenic alone increased total ovarian tumors of any type compared with control. Significant increases over control in ovarian adenoma and total epithelial tumors occurred after arsenic

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Group (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urogenital tumor Any type</td>
<td>Control (33)</td>
</tr>
<tr>
<td>Benign</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Malignant</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Multiple site</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Epithelial</td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total epithelial</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Multiple site</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

NOTE: Pregnant female mice were exposed to sodium arsenite from days 8 to 18 of gestation, female offspring were treated with diethylstilbestrol or tamoxifen on days 1 to 5 postpartum, and tumors were assessed in female offspring as adults (see Materials and Methods for treatment details). Samples size (n) is the number of mice at risk. The urogenital system was considered to include the tissues of the genital system, including ovary, oviduct, uterus, cervix, and vagina, and the urinary system, including urinary bladder and kidney. The designation of tumors of “any type” includes both mesenchymal and epithelial tumors. The incidence of benign tumors is given irrespective of more advanced tumors in the same tissue. Total tumor incidence is defined as those mice bearing at least one benign or malignant tumor. The incidence of tumors at multiple sites is the number of cases where two or more tumors occurred at separate sites in the urogenital system of the same animal.

Abbreviations: DES, diethylstilbestrol; TAM, tamoxifen.

*Significantly different (P < 0.05) from control.
†Significantly different (P < 0.05) from diethylstilbestrol alone.
‡Significantly different (P < 0.05) from arsenic alone.
§Significantly different (P < 0.05) from tamoxifen alone.
plus diethylstilbestrol that were not observed with arsenic or diethylstilbestrol alone. Cervical tumors (primarily squamous cell carcinoma) were increased by 50% with arsenic plus diethylstilbestrol compared with diethylstilbestrol alone and were not increased with arsenic alone. Tamoxifen alone did not increase tumors in specific components of the genital system. With arsenic plus tamoxifen, an increase over control occurred in ovarian tumors of any type that was similar to arsenic alone. All treatments induced oviduct hyperplasia.

In the urinary system, none of the treatments alone or in combination increased the incidence of specific tumors (Table 3). However, three transitional cell carcinoma of the urinary bladder occurred in the arsenic plus diethylstilbestrol group, which, although not statistically elevated \( (P = 0.119) \), are noteworthy because urinary bladder tumors in female mice are very rare. Arsenic plus diethylstilbestrol and arsenic plus tamoxifen treatments increased urinary bladder transitional cell hyperplasia compared with control. Bladder total proliferative lesions (combined hyperplasia, papilloma, and carcinoma) with arsenic plus diethylstilbestrol or arsenic plus tamoxifen were markedly increased compared with control. This increase in bladder lesions was synergistic after arsenic plus diethylstilbestrol or arsenic plus tamoxifen. A metastatic renal cell carcinoma occurred in a mouse treated with arsenic plus tamoxifen and preneoplastic lesions (cystic hyperplastic/dysplastic renal tubules) occurred in one mouse each from the arsenic alone and arsenic plus tamoxifen groups.

Two remarkable multisite urogenital tumors occurred in mice treated with arsenic plus diethylstilbestrol. These grossly appeared as large masses in the urogenital region, which proved to be tumors of multiple organs, including undifferentiated transitional cell carcinoma of the urinary bladder, adenocarcinoma of the uterus, and separate squamous cell carcinomas of the cervix and vagina. These were highly unusual and very aggressive multisite tumors.

Other tumors were impacted by the various treatments, including tumors of the liver and adrenal (Table 4). Arsenic treatment, regardless of other treatments, increased adrenal gland tumor incidence. The incidence of total liver tumors of any type was increased by arsenic plus diethylstilbestrol. Lung tumors were not altered by the treatments, whereas lymphoma incidence was reduced compared with control in the arsenic alone and diethylstilbestrol alone groups.

When all arsenic-treated animals (including the arsenic alone, arsenic plus diethylstilbestrol, and arsenic plus tamoxifen groups) were considered as a whole, there were 13 mice with hemangiomia or hemangiosarcoma in the 101 mice total compared with 3 cases in the 102 nonarsenic-exposed mice (control, diethylstilbestrol alone, and tamoxifen alone groups: \( P < 0.05 \)). In the pooled arsenic-exposed mice, eight hemangiomia or hemangiosarcoma occurred in the urogenital system compared with 0 in the 102 nonarsenic-exposed mice (control, diethylstilbestrol alone, and tamoxifen alone groups: \( P < 0.05 \)). Similarly, of 24 ovarian tumors total, 21 occurred in the 101 mice exposed to arsenic regardless of other treatments, compared with 3 in the 102 nonarsenic-exposed mice (\( P < 0.05 \)).

### Table 2. Site-specific urogenital system tumors induced by transplacental arsenic exposure together with postnatal diethylstilbestrol or tamoxifen treatment in female CD1 mice; ovary, oviduct, uterus, cervix, and vagina

<table>
<thead>
<tr>
<th>Tumor site and type</th>
<th>Group (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (33)</td>
</tr>
<tr>
<td>Ovary</td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total epithelial tumors</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total tumors any type</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Oviduct</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>5 (15%)</td>
</tr>
<tr>
<td>Adenoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Uterus</td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total epithelial tumors</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total tumors any type</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Cervix</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total tumors any type</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Vagina</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

NOTE: See Table 1 for details. There were no adenoma of the cervix or vagina and no carcinoma of the oviduct. Total tumors any type (both epithelial and mesenchymal) includes a leiomyosarcoma and a hemangiosarcoma of the uterus in the arsenic alone group and a leiomyosarcoma of the cervix also in the arsenic alone group. The ovarian carcinoma in the arsenic + diethylstilbestrol group had metastasized to the liver.

*Significantly different \( (P < 0.05) \) from control.

†Significantly different \( (P < 0.05) \) from diethylstilbestrol alone.

§Significantly different \( (P < 0.05) \) from arsenic alone.
This included seven ovarian hemangiomas with arsenic exposure compared with 0 in nonarsenic-exposed mice (\(P < 0.05\)). Of a total of 14 cases of liver tumors, 13 occurred in the 101 arsenic-exposed mice compared with 1 in the 102 nonarsenic-exposed animals (\(P < 0.05\)).

Tumors not associated with any treatments and are not previously discussed included the following: controls, a mammary gland carcinoma, a leukemia, and a skin sarcoma; arsenic alone, a mammary adenocarcinoma; diethylstilbestrol alone, a mixosarcoma and a sarcoma (skin); tamoxifen alone, a rhabdomyosarcoma (skin); arsenic plus diethylstilbestrol, a rhabdomyosarcoma (skin), a salivary gland malignant myoepithelioma, a leukemia, a thyroid adenocarcinoma; arsenic plus tamoxifen, a leukemia, a skin osteosarcoma, and a small intestine adenoma.

**ER-\(\alpha\) and estrogen-related gene expression in newborn uterus.** Because there was a distinct increase in tumors with

<table>
<thead>
<tr>
<th>Tumor site and type</th>
<th>Group ((n))</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (33)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Papilloma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total epithelial tumors</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total proliferative lesions</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

NOTE: See Table 1 for details. Urinary bladder total proliferative lesions is the incidence of mice bearing at least one uroepithelial preneoplasia, papilloma, or carcinoma. All urinary bladder lesions were of the transitional cell type. The kidney lesions included a renal cell carcinoma and one case of renal cystic tubular hyperplasia both in the arsenic + tamoxifen group. The kidney carcinoma had metastasized to the lung.

*Significantly different \((P < 0.05)\) from control.

†Significantly different \((P < 0.05)\) from diethylstilbestrol alone.

‡Significantly different \((P < 0.05)\) from tamoxifen alone.

§Significantly different \((P < 0.05)\) from arsenic alone.

This included seven ovarian hemangiomas with arsenic exposure compared with 0 in nonarsenic-exposed mice \((P < 0.05)\). Of a total of 14 cases of liver tumors, 13 occurred in the 101 arsenic-exposed mice compared with 1 in the 102 nonarsenic-exposed animals \((P < 0.05)\).

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**ER-\(\alpha\) and estrogen-related gene expression in newborn uterus.** Because there was a distinct increase in tumors with
arsenic plus diethylstilbestrol, expression of ER-α and selected estrogen-related genes was assessed in neonatal uteri on postpartum day 12 in female mice exposed to arsenic and/or diethylstilbestrol. ER-α expression was increased by 56% by arsenic treatment alone compared with control (Fig. 1), indicating up-regulation of this steroid receptor. Diethylstilbestrol with or without prior arsenic treatment did not increase uterine ER-α expression. The increase noted in uterine weight with diethylstilbestrol (186%) compared with control or arsenic alone may have diluted any gene overexpression.

Expression analysis of three estrogen-regulated genes (pS2, CYP2A4, and lactoferrin) showed arsenic alone did not increase expression (Fig. 2). Diethylstilbestrol alone greatly increased expression of CYP2A4 (3.2-fold), pS2 (96-fold), and lactoferrin (234-fold) compared with control. Combined arsenic and diethylstilbestrol treatments increased expression of these genes even further, increasing expression compared with diethylstilbestrol alone 7.8-fold for CYP2A4, 3.0-fold for pS2, and 47% for lactoferrin. Compared with control, arsenic plus diethylstilbestrol increased CYP2A4 expression 25-fold, pS2 expression 288-fold, and lactoferrin 344-fold.

ER-α and estrogen-related gene expression in urogenital tumors. To determine if ER-α and estrogen-related gene overexpression continued into adulthood, urogenital malignancies from the arsenic plus diethylstilbestrol group were assessed for ER-α and pS2 expression (Fig. 3). Uterine adenocarcinoma from the arsenic plus diethylstilbestrol consistently showed intense and widespread staining for ER-α, particularly in tumor cell nuclei (Fig. 3A). Similarly, pS2 staining was intense, widespread, and primarily nuclear in uterine carcinoma induced by arsenic plus diethylstilbestrol (Fig. 3B). Urinary bladder transitional cell carcinomas associated with arsenic and postnatal diethylstilbestrol also showed widespread, intense, and primarily nuclear ER-α staining (Fig. 3C) as well as intense nuclear pS2 expression (Fig. 3D).

Discussion

The present results clearly show that maternal exposure to inorganic arsenic in CD1 mice is a complete transplacental carcinogen in the female offspring that targets the urogenital system in general and the ovary and uterus in particular. In utero arsenic exposure also induced adrenal tumors. The background rate of tumors in these tissues in the control CD1 mice in the present study was either at zero (urogenital system, ovary, and uterus) or very near zero (adrenal; 3%). These results are consistent with prior work in C3H mice where transplacental arsenic exposure induced ovarian tumors and uterine hyperplasia in female offspring and adrenal tumors in male offspring (7, 8). Perhaps, more importantly, the present results indicate that in utero arsenic exposure and postnatal diethylstilbestrol treatment resulted in a marked, often synergistic, exacerbation of urogenital tumor development and progression. This included increased urogenital malignancies, as well as increases in mice with multiple-site urogenital tumors. In fact, two cases of large masses occurred in mice treated with arsenic plus diethylstilbestrol that proved to be highly unusual and very aggressive, multiorgan urogenital tumors that included undifferentiated transitional cell carcinoma of the urinary bladder, adenocarcinoma of the uterus, and separate squamous cell carcinomas of the cervix and vagina. Similarly, combined arsenic and diethylstilbestrol increased vaginal carcinoma incidence above control rates, although alone neither arsenic nor diethylstilbestrol treatment had this effect. Combined arsenic and diethylstilbestrol or tamoxifen markedly increased proliferative lesions of the bladder, a clear target of arsenic carcinogenesis in humans (1). This included three separate cases of urinary bladder transitional cell carcinoma in female CD1 mice treated with arsenic plus diethylstilbestrol, which, although not statistically significant when compared with control (0%), is noteworthy because spontaneous bladder tumors are exceedingly rare in female mice (30–32). Indeed, data compiled from 2-year carcinogenesis studies done at the Institute for Environmental Toxicology show that in 890 negative control female CD1 mice, not a single urinary bladder neoplasm of any type occurred (30). Together, the present data provide compelling evidence that arsenic can act alone to initiate or induce urogenital tract cancers in mice and that this response can be exacerbated by the synthetic estrogen, diethylstilbestrol.

The tumors and proliferative lesions seen with inorganic arsenic as a transplacental carcinogen in female mice, including lesions of the ovary, uterus, vagina, oviduct, and adrenal, are all potential targets of carcinogenic estrogens in humans and/or rodents (14–21, 23, 24). Similarly, estrogens can be hepatocarcinogenic (22) and liver cancers have been consistently observed in male mice exposed to arsenic in utero (7, 8). There is some evidence of an estrogen-related, estrogen increased response in the female liver in the present study. Because in utero arsenic consistently shows an estrogen-like tumor spectrum, we tested the hypothesis that aberrant estrogen signaling may play a role in transplacental arsenic carcinogenesis (26, 28, 29). Clearly, diethylstilbestrol exacerbated arsenic-induced tumor formation and progression in the urogenital system, often in a synergistic fashion. At the
molecular level in the neonatal uterus, arsenic alone up-regulated ER-α, and arsenic pretreatment caused very large elevations in diethylstilbestrol-induced, ER-related gene expression, including CYP2A4, pS2, and lactoferrin. In fact, arsenic plus diethylstilbestrol caused the expression of these genes to increase an additional 22-fold (CYP2A4), 110-fold (pS2), and 178-fold (lactoferrin) over control when compared with diethylstilbestrol alone. Estrogen-related expression of both pS2 and lactoferrin occurs through ER-α (33, 34), whereas expression patterns of CYP2A4, a female dominant cytochrome, seems to be imprinted in an ER-α-dependent fashion during development (35). Transgenic mice that overexpress ER-α show an increased sensitivity to diethylstilbestrol carcinogenesis (27), and many of the detrimental effects of neonatal diethylstilbestrol are mediated by ER-α (25). Thus, early molecular events indicate that arsenic precipitates and can further facilitate aberrant estrogen signaling in some target tissues.

Furthermore, in uterine carcinoma formed in adults after gestational arsenic exposure and postnatal diethylstilbestrol, both ER-α and pS2 were highly overexpressed. Diethylstilbestrol is considered to be a potent transplacental carcinogenic estrogen in both humans and rodents, associated with uterine, cervical, and vaginal carcinoma (22, 23, 25) that likely acts through ER-α (25). Thus, it seems that arsenic predisposed tissues of the female urogenital tract to estrogen carcinogenesis. Marked overexpression of hepatic ER-α, as well as activation of estrogen-related genes potentially important in carcinogenesis, occurs in adult male mice bearing hepatocellular carcinoma induced by transplacental arsenic exposure with no additional treatment (26). Preliminary data indicate hepatic ER-α is overexpressed in an arsenic-exposed human population that shows an elevation in liver cancers (26). In addition, repeated inorganic arsenic exposure in adult female mice induces uterine hyperplastic lesions that markedly overexpress ER-α (28). In the present study, urinary bladder carcinoma associated with arsenic plus diethylstilbestrol greatly overexpressed ER-α and pS2. Thus, functional ER-α overexpression seems to be associated with the carcinogenicity of arsenic in several tissues. Based on these data, we hypothesize that arsenic in utero may attack a critical pool of progenitor cells in urogenital system and induce aberrant genetic "reprogramming" as part of its carcinogenic mechanism, in a fashion similar to that thought to occur in early life exposure to diethylstilbestrol (36). An intrauterine component of estrogen carcinogenesis involving stem cells has been long suspected (37). Estrogen concentrations are at least 10 times higher during pregnancy than in other periods of adult life (37), which could provide an endogenous stimulus for in utero arsenic carcinogenesis.

The present results verify that the fetal period is a time of very high sensitivity to arsenic carcinogenesis in mice (7, 8). This has now been shown in three separate tumor end-point studies using two different mouse strains, namely C3H (7, 8) and CD1 (present work). The significance of the results of the first two studies (7, 8) have been questioned because the C3H mouse shows substantial levels of spontaneous tumor formation in some of the tissues that were also targets of arsenic carcinogenesis (38). However, the urogenital tract tumors seen in the present work in adult females after in utero arsenic exposure alone or after arsenic in combination with diethylstilbestrol occurred in a strain that showed no spontaneous urogenital tumors of any type. Thus, a propensity towards spontaneous tumor formation cannot explain the present results with arsenic-induced urogenital tract cancer.

The current findings have important public health implications. Gestation is clearly a period of high sensitivity to arsenic carcinogenesis in mice and a comparable sensitivity in humans would be cause for great alarm. Although arsenic readily crosses the rodent or human placenta (1), a transplacental component of arsenic carcinogenesis in the human may be difficult to prove. Unlike the human data supporting diethylstilbestrol as a transplacental carcinogen (14, 15, 24), populations that were exposed to arsenic only during gestation do not seem to exist. Indeed, in areas where chronic exposure to elevated environmental inorganic

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**Figure 2.** Expression of estrogen-related genes in 12-day-old CD1 mouse uteri after transplacental arsenic exposure and postnatal exposure to diethylstilbestrol. Uteri were excised and CYP2A4, pS2, and lactoferrin transcript levels were determined by real-time RT-PCR (see Materials and Methods). Columns, mean SE (n = 4-8; from three to five litters), expressed as percent control expression that is set as 100%. *, P < 0.05, significant difference from control.
arsenic is common, all life stages would be involved and it is likely that significant *in utero* exposure occurs. Because of this, protection of pregnant women from excessive arsenic exposure could prove to be an important intervention strategy in cancer prevention. Beyond this, there is the possibility that coexposure to pharmacologic or environmental estrogens could enhance development of arsenic-initiated cancer. Conversely, there may be a possibility that prenatal exposure to arsenic would predispose people to the development of estrogen-related cancers. Indeed, recent data provide compelling evidence that susceptibility to endocrine-related cancers may be a result of developmental exposures (24).

In summary, gestational arsenic exposure is a complete carcinogen in the female CD1 mouse that targets the urogenital system and exposure to the synthetic estrogen, diethylstilbestrol, after arsenic increased tumor incidence, multiplicity, and progression. This included proliferative lesions of the urinary bladder, an important target of arsenic carcinogenesis in humans. Furthermore, molecular and immunohistochemical data provide strong evidence of exaggerated estrogen signaling in neonatal tissue and in urogenital tumors in adults induced by arsenic plus diethylstilbestrol. Thus, arsenic can act together with estrogens to stimulate oncogenesis in the female mouse urogenital tract. Because it is unreasonable to expect to eliminate arsenic in the environment, intervention by reduction of arsenic exposure during pregnancy could prove to be a valid strategy in prevention of human cancer.

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**Figure 3.** Immunohistochemical analysis of ER-α and pS2 expression in urogenital tumors formed in adult female CD1 mice after transplacental exposure to arsenic and postnatal exposure to diethylstilbestrol. By the method used, brown staining indicates the presence of the particular protein. The overexpression of ER-α and pS2 were primarily nuclear in nature. A, a uterine adenocarcinoma associated with arsenic plus diethylstilbestrol exposure stained for ER-α showing widespread and intense nuclear and cytoplasmic expression (×400). B, a uterine adenocarcinoma associated with arsenic plus diethylstilbestrol exposure stained for pS2 showing widespread and intense nuclear and cytoplasmic expression (×40). C, a urinary bladder transitional cell carcinoma associated with arsenic plus diethylstilbestrol exposure stained for ER-α showing widespread and intense nuclear and cytoplasmic expression (×400). D, a urinary bladder transitional cell carcinoma associated with arsenic plus diethylstilbestrol exposure stained for pS2 showing widespread and intense nuclear and cytoplasmic expression (×40).
References


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