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Induction of Proliferative Lesions of the Uterus, Testes, and Liver in Swiss Mice Given Repeated Injections of Sodium Arsenate: Possible Estrogenic Mode of Action

Michael P. Waalkes,*,[1] Larry K. Keefer,† and Bhalchandra A. Diwan‡

Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709; and †Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute and ‡Intramural Research Support Program, SAIC Frederick, NCI–Frederick Cancer Research and Development Center, Frederick, Maryland 21702

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Inorganic arsenic (As) is a human carcinogen but has not been unequivocally proven carcinogenic in rodents. For instance, one older study indicates that repeated iv injections of sodium arsenate might induce lymphomas in Swiss mice (58% incidence) (Osswald and Goerttler, *Verh. Dtsch. Ges. Pathol.* **55, 289–293, 1971), but it was considered inadequate for critical evaluation of carcinogenic potential largely because of issues in experimental design. Therefore, we studied repeated iv sodium arsenate injection and neoplastic response in male and female Swiss mice. Groups ($n = 25$) of mice received sodium arsenate (0.5 mg/kg, iv) or saline (control) once/week for 20 weeks and were observed for a total of 96 weeks when the study ended. Differences in survival and body weights were unremarkable. In females, arsenate induced marked increases in the incidence and severity of cystic hyperplasia of the uterus compared against controls. Arsenate also was associated with a rare adenocarcinoma of the uterus. Hyperplastic uterine epithelium from arsenate-exposed animals showed strong positive immunostaining for the proliferating cell nuclear antigen (PCNA). There was also an upregulation of estrogen receptor (ER) immunoreactive protein in the early lesions of uterine luminal and glandular hyperplasia, although a progressive decrease in its expression was seen in the severe hyperplastic or neoplastic epithelium. In common with the preneoplastic and neoplastic gynecological lesions in humans, the levels of immunoreactive inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine-containing proteins were greater in the uterine hyperplastic epidermis and their intensity was positively correlated with the severity of the lesions. Arsenate-induced uterine hyperplastic lesions also showed a strong upregulation of cyclin D1, an estrogen-associated gene product essential for progression through the G1 phase of the cell cycle. In other tissues, arsenate increased testicular interstitial cell hyperplasia incidence and severity over control but without affecting the incidence of tubular degeneration. Arsenate also induced increases in hepatic proliferative lesions (HPL; foci of alteration + neoplasia), but only in females. Significant skin changes (incidence of hyperkeratotic lesions) and renal lesions (severity of nephropathy) also occurred in arsenate-treated females. Thus, repeated arsenate exposure, though not outright tumorigenic in the present study, was associated with proliferative, preneoplastic lesions of the uterus, testes, and liver. Estrogen treatment has been associated with proliferative lesions and tumors of the uterus, female liver, and testes in other studies, supporting a hypothesis that arsenate might somehow act through an estrogenic mode of action.**

Key Words: **arsenic; uterus; testes; liver; proliferative lesions; mice.**

Inorganic arsenicals, in the form of arsenite (3+) and arsenate (5+), are widely dispersed and common environmental toxicants and known human carcinogens (IARC, 1980, 1987; NRC, 1999; Goering *et al.*, 1999). Arsenic is a multisite carcinogen in humans, causing tumors in a variety of tissues including lung, skin, and bladder (IARC, 1980, 1987; NRC, 1999). Other studies in humans indicate that the kidney, liver, and prostate may also be target sites of arsenic carcinogenesis in humans (NRC, 1999). Although chronic dermal toxicity, nephrotoxicity, and hepatotoxicity all occur with arsenic exposure, a primary concern for chronic exposure in humans is its carcinogenic potential (NRC, 1999). Inorganic arsenicals can be carcinogenic in humans after either occupational or environmental exposure (IARC, 1980, 1987; NRC, 1999), fortifying the rationale for making arsenic consistently one of the top priority hazardous substances to the population of the United States (ATSDR, 1997; Goering *et al.*, 1999).

Although clearly a human carcinogen, inorganic arsenic to date has not been unequivocally demonstrated to be, by itself, carcinogenic in rodents (NRC, 1999). In fact, it is considered that, in general, long-term studies on the oral carcinogenicity of inorganic arsenicals with appropriate experimental design have provided consistently negative results (NRC, 1999). This cre-

[1] To whom correspondence should be addressed at National Cancer Institute at NIEHS, Inorganic Carcinogenesis Section, P.O. Box 12233, Mail Drop F0-09, 111 Alexander Drive, Research Triangle Park, NC 27709. Fax: (919) 541-2328. E-mail: waalkes@niehs.nih.gov.

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TABLE 1
Study Design and Survival of Male and Female Swiss Mice Given Repeated Injections of Sodium Arsenate

Group	Initial *n*	Weekly dosage of sodium arsenate (iv, in mg/kg)	Weeks of treatment	Total weeks of observation	Animals surviving at 96 weeks[a]
Male control	25	0	20	96	10
Male arsenate	25	0.5	20	96	11
Female control	25	0	20	96	14
Female arsenate	25	0.5	20	96	11

Note. All groups were treated with one iv injection in the tail vein of 0.5 mg sodium arsenate/kg (at 10 ml/kg) or 10 ml saline/kg (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks.

[a] Given as number of animals surviving to the termination of the study. There were no significant differences when comparing treated mice to gender-matched controls by the Fisher exact test. Both the Kaplan–Meier and the Cox methods for defining differences in survival also indicated no significant differences between treated mice and gender-matched controls.

ates the alarming possibility that, for whatever reasons, humans may be one of the more sensitive species to inorganic arsenic-induced cancers (Goering *et al.*, 1999). Oral treatment with inorganic arsenic will increase the incidence of skin papillomas in Tg.AC mice (which carry the *v-Ha-ras* oncogene), but only after tetradecanoyl phorbol acetate (TPA) exposure and not when given alone (Germolec *et al.*, 1998). Inorganic arsenicals can undergo extensive metabolism and undergo reduction and/or methylation in many tissues (Goering *et al.*, 1999) and the organo-arsenical, dimethylarsinic acid (DMA; also known as cacodylic acid) has been shown to be a tumor promoter in rats in two-stage carcinogenesis studies using various organic carcinogens as the initiators (Yamamoto *et al.*, 1997) and can be a complete carcinogen in the male rat bladder (Wei *et al.*, 1999). DMA is much less acutely toxic than inorganic arsenicals and the high level of DMA used in most of these studies (Yamamoto *et al.*, 1997) raises the question of relevance of these results with regard to the actual attainable doses of inorganic arsenic (NRC, 1999).

The role of arsenic metabolism in carcinogenesis is also currently a matter of some controversy (Goering *et al.*, 1999). In any event, the carcinogenicity of inorganic arsenicals when given alone in animals is considered equivocal or limited (IARC, 1980, 1987; NRC, 1999). For instance, one study by Osswald and Goerttler (1971) gave repeated iv injections of sodium arsenate to female Swiss mice and compared the results to untreated mice over 24 months. Of 19 arsenate-treated mice having undergone postmortem examination, 11 had lymphoma or lymphocytic leukemia, compared to none in 16 examined controls (Osswald and Goerttler, 1971; IARC 1980). Unfortunately, this study was reported prior to its completion and a substantial number of the control animals were still alive at the time of its reporting (Osswald and Goerttler, 1971; IARC 1980) and no subsequent reporting has occurred. The early reporting while control animals were still alive was considered to make these results difficult to interpret and was appraised as a noteworthy inadequacy in study design (IARC, 1980). Additionally, this work lacks an appropriately treated vehicle control.

Thus, the purpose of the present study was to determine whether repeated iv injections of sodium arsenate are carcinogenic when compared to appropriate control groups under more rigorous conditions of modern bioassays and at a point at which all animals had been terminated and examined. Because prior study apparently associated iv arsenate injections with lymphoma or lymphocytic leukemia (Osswald and Goerttler, 1971), special attention was paid to the hematopoietic system tumors. The results for hematopoietic system tumors in our study proved negative. Surprisingly, however, several arsenate-treated mice showed reproductive tract abnormalities including severe hyperplastic lesions of uterus and testis, effects similar to those induced by estrogenic agonists, such as diethylstilbestrol and tamoxifen (Johnson, 1987; Newbold, 1995; Diwan *et al.*, 1997). Further study demonstrated the enhanced expression of estrogen receptor (ER) and various ER-associated gene products in arsenate-induced uterine lesions, suggesting that arsenate may exhibit estrogenic properties in mice as part of a carcinogenic mechanism.

MATERIALS AND METHODS

Animals and Treatment

A total of 50 male and 50 female Swiss [CR:NIH(S)] mice were obtained at 4 weeks of age from the Animal Production Area, NCI–FCRDC (Frederick, MD). Animals were housed five per hanging polycarbonate cage (size $11\frac{1}{2}'' \times 7\frac{1}{2}'' \times 5''$) with hardwood chip bedding and provided food (NIH-31 Block Open Formula 6% Modified; Zeigler Brothers, Gardners, PA) and water (acidified tap) *ad libitum*. Lighting (fluorescent) schedule was 12 h on (6:00 a.m. to 6:00 p.m.) and 12 h off (6:00 p.m. to 6:00 a.m.). Environmental temperatures were held between 68 and 72°F, with a relative humidity of 50 ± 5%. Animals were cared for and used humanely according to the U.S. Public Health Service Policy on the Care and Use of Animals and the Guide for the Care and Use of Laboratory Animals. The NCI–FCRDC animal facility, where the bioassay portion of this study was conducted, and its animal program are accredited by the Association for the Assessment of Laboratory Animal Care International.

The experimental design of the present study is shown in Table 1. For injection, sodium arsenate (dibasic; Na_2HAsO_4; J.T. Baker Chemical Co., Phillipsburg, NJ) solutions were prepared in sterile normal saline. At 8 weeks of age animals were randomly divided into gender-matched groups of 25 as designated in Table 1 and treated intravenously (iv; 10.0 ml/kg) with 0.5 mg arsenate/kg once weekly for 20 consecutive weeks. This injection schedule was based on a prior study (Osswald and Goerttler, 1971). Controls received saline iv at 10 ml/kg. Injections were given in the tail vein. The week of the first injection was designated as experimental Week 1. Body weights, survival, and clinical signs were recorded throughout the experiment. Body weights were recorded weekly for the first 26 weeks and biweekly thereafter. Clinical signs were checked daily. Animals were killed either when significant clinical signs developed or at 96 experimental weeks.

Pathology

An extensive necropsy was performed on all animals whether found dead, killed during the experiment when appropriate clinical signs developed, or killed at the conclusion of the experiment. Kidney, liver, lung, spleen, lymph nodes (mandibular, mesenteric), testes, coagulating gland, urinary bladder, seminal vesicles, uterus, ovaries, and all abnormal tissues from each animal were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histological examination. Skin was carefully inspected on all parts of the animal and any abnormal lesions were taken and processed as earlier. Upon the discovery of excess uterine proliferative lesions in the arsenate-treated mice, lesions were reevaluated using the criteria described earlier (Nagaoka *et al.*, 1994; Diwan *et al.*, 1997). Briefly, endometrial hyperplasias were subclassified into three categories on the basis of size and the presence of atypical cells. Mild hyperplasia referred to lesions that contained an increased number of glands with some atypical cells in focal and diffuse areas of the endometrium. Moderate to severe hyperplasias were composed of large cystic and dysplastic lesions with moderate/severe atypical glands, which were lined with hyperchromatic and pleomorphic epithelium. Diagnosis of adenocarcinoma was based on a combination of gross and microscopic features. All pathological assessments were performed in a blind fashion.

Immunohistochemical Analysis

Proliferating cell nuclear antigen (PCNA) was used as an indication of cellular proliferation. For immunohistochemical determination of the expression of PCNA, the primary antibody, MOUSE a/PCNA, clone PC10, was obtained from DAKO Corporation (Santa Barbara, CA) and representative formalin-fixed, paraffin-processed sections of uterus were prepared according to instructions provided by the manufacturer using the DAKO ARK system. The specimens were first incubated with the biotinylated primary antibody, followed by an incubation with streptavidin-peroxidase. This was then followed by reaction with diaminobenzidine/hydrogen peroxidase as chromagen substrate. The specimens included nine uterine hyperplastic lesions (three mild and six moderate-to-severe cases) from treated animals, five cases (two normal, two mild, and one moderate-to-severe) from control mice, and one adenocarcinoma (from an arsenate-exposed animal). Sections from these same cases were also used to evaluate the immunohistochemical localization and intensity of estrogen receptor (ER-α), cyclin D1, immunoreactive inducible nitric oxide synthase (iNOS), and 3-nitrotyrosine. The sections were microwaved after deparaffinization for 10 min in citrate buffer. The immunohistochemical detection was performed with polyclonal rabbit anti-ER antibody (MC-20, sc-542; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-human cyclin D antibody (Upstate Biotechnology, Lake Placid, NY), polyclonal rabbit anti-iNOS (N-20, sc-651; Santa Cruz Biotechnology), and polyclonal rabbit anti-3-nitrotyrosine antibody (clone 1A6; Upstate Biotechnology). Reactions were visualized with an avidin–biotin–peroxidase kit (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromagen. The nuclear counterstain was hematoxylin. To demonstrate the specificity of the immunostaining, primary antibodies were omitted from each staining series. Each slide was assessed for intensity of immunostaining, background, and percentage of cells expressing the target protein. The staining intensity of the cells was categorized as negative (−), weak (+), moderate (++), or strong (+++), while the extent of staining was scored as 1 if fewer than 25% of cells were positive, 2 if 25 to 75% of cells were positive, and 3 if more than 75% of the cells were positive.

Data Analysis

In all cases a one-sided probability level of $p \leq 0.05$ was considered to indicate a significant difference. In pairwise comparison of lesion incidence or survival at the study termination, Fisher's exact test was used. For severity ratings and body weight data a Student's t test was used to compare means. Survival during the study was examined with the Cox test and the generalized Kaplan–Meier test and was considered significantly different only if so indicated by both tests.

RESULTS

Effects of the repeated injections of arsenate on survival of male and female Swiss mice are shown in Table 1. In both the males and females the number of animals surviving at the termination of the study was not reduced by the arsenate treatments. In addition, both the Kaplan–Meier and the Cox methods for defining differences in survival also indicated no significant differences in survival between treated mice and gender-matched controls. Furthermore, body weights were unaffected by arsenate treatment during the study (not shown). For instance, the body weights measured in the weeks after the final arsenate injection (experimental Week 21 to Week 96) averaged 97.7% of control in arsenate-treated males and 104.9% in arsenate-treated females.

The incidence of total proliferative hepatocellular lesions (adenomas and foci of cellular alteration) after repeated arsenate injections in male and female mice is shown in Table 2. There were no differences between male controls and arsenate-treated animals. However, in females there was a definite increase in the incidence of total proliferative hepatocellular lesions in arsenate-treated female mice compared with that in gender-matched controls. The bulk of the increase was made up of foci of cellular alteration. These were primarily clear cell and eosinophilic foci.

The effects of repeated arsenate exposures on the incidence and severity of testicular interstitial cell hyperplasia and tubular degeneration in male mice are shown in Table 3. Repeated arsenate injections doubled the incidence of interstitial cell hyperplasia and significantly increased their average severity rating. This was not associated with an increase in the incidence or severity of testicular tubular degeneration, a lesion which can be associated with indirectly induced testicular interstitial cell hyperplasia.

Table 4 shows the effects of arsenate injections on lesions in the uterus of female mice. Arsenate markedly increased the incidence (2.8-fold) and severity (moderate/severe lesions; 2.9-fold) of cystic hyperplasia of the uterus when compared to control. Such lesions are considered to be precursors to adenocarcinoma of the uterus (Johnson, 1987; Newbold, 1995;

TABLE 2
Effect of Repeated Injections of Sodium Arsenate on the Incidence of Proliferative Hepatocellular Lesions of the Liver in Male and Female Swiss Mice

Group	Foci of alteration	Adenoma	Carcinoma	Total tumors	Total proliferative lesions
Male control	1 (4%)	0	0	0	1 (4%)
Male arsenate	1 (4%)	0	0	0	1 (4%)
Female control	1 (4%)	0	0	0	1 (4%)
Female arsenate	5 (20%)	1 (4%)	0	1 (4%)	6 (24%)*

Note. All groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals). An asterisk (*) indicates a significant difference from control by Fisher exact test.

Diwan *et al.*, 1997) and, indeed, in the arsenate-treated group a relatively rare adenocarcinoma of the uterus occurred. The greatly elevated incidence of preneoplastic lesions of the uterus points toward arsenate as a causative factor in the adenocarcinoma, although this cannot be definitively established by the present data. Photomicrographs of typical arsenate-induced uterine lesions and the adenocarcinoma are shown in Figs. 1–3.

Because of this marked increase in the incidence of preneoplastic lesions of the uterus with arsenate and the occurrence of an adenocarcinoma of the uterus in an arsenate-treated animal, further study sought to more completely characterize these uterine lesions using immunohistochemical techniques. In this regard, PCNA was used to gauge the level of cellular proliferation within uterine lesions and adjacent normal tissue. Staining for PCNA was nuclear in all the specimens observed. In normal uterine tissue, the intensity of PCNA staining was weak and less than 25% of the cells showed positive staining (Table 5). In hyperplastic epithelium of uterus in the control mice, PCNA staining generally showed less intensity than in arsenate-treated uterine hyperplasias. Staining was often higher in some moderate hyperplastic lesions (Fig. 1B) while some such areas exhibited moderate-to-weak staining (Fig. 1C). The neoplastic cells in the uterine adenocarcinoma showed a variable PCNA staining pattern, with some areas staining intensely while the others were totally negative.

ER-α was detected in both epithelial and stromal cell nuclei of uterine tissue. The ER is a hormone-activated transcription factor that mediates the biological effects of estrogens in a variety of target tissues and is often overexpressed in tumors of endocrine-sensitive tissue (Barton and Shapiro, 1988). The expression of ER-α was weak to moderate in epithelium of normal uteri (Table 5), but was extensive in hyperplastic epithelium of control uteri (Fig. 1D). The number of positive epithelial cells and staining intensity appeared to increase in the hyperplastic epithelium of arsenate-treated rats (Fig. 1E). However, in more advanced lesions in treated mice the multilayered hyperplastic epithelium showed weak ER-α staining

TABLE 3
Effect of Repeated Injections of Sodium Arsenate on the Incidence and Severity of Testicular Interstitial Cell Hyperplasia and Tubular Degeneration in Male Swiss Mice

	Hyperplasia		Tubular degeneration	
Group	Incidence (%)	Severity	Incidence (%)	Severity
Control	8 (32%)	0.72 ± 0.20	20 (80%)	2.48 ± 0.28
Arsenate	16 (64%)*	1.28 ± 0.18*	17 (68%)	2.20 ± 0.34

Note. Both groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals) for incidence and means ± SEM score for severity based on the following severity ratings: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. An asterisk (*) indicates a significant difference from control by Fisher exact test (incidence) or Student's *t* test (severity).

TABLE 4
Effect of Repeated Injections of Sodium Arsenate on the Incidence and Severity of Cystic Hyperplasia of the Uterus in Female Swiss Mice

	Hyperplasia	
Group	Incidence (%)	Severity
Control	5 (20%)	0.56 ± 0.24
Arsenate	14 (56%)*[a]	1.64 ± 0.32*

Note. Both groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals) for incidence and means ± SEM score for severity based on the following severity ratings: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. An asterisk (*) indicates a significant difference from control by Fisher exact test (incidence) or Student's *t* test (severity).

[a] The arsenate-treated group also had a malignant adenocarcinoma of the uterus. Total proliferative lesion (hyperplasia + adenocarcinoma) incidence would then equal 60%.

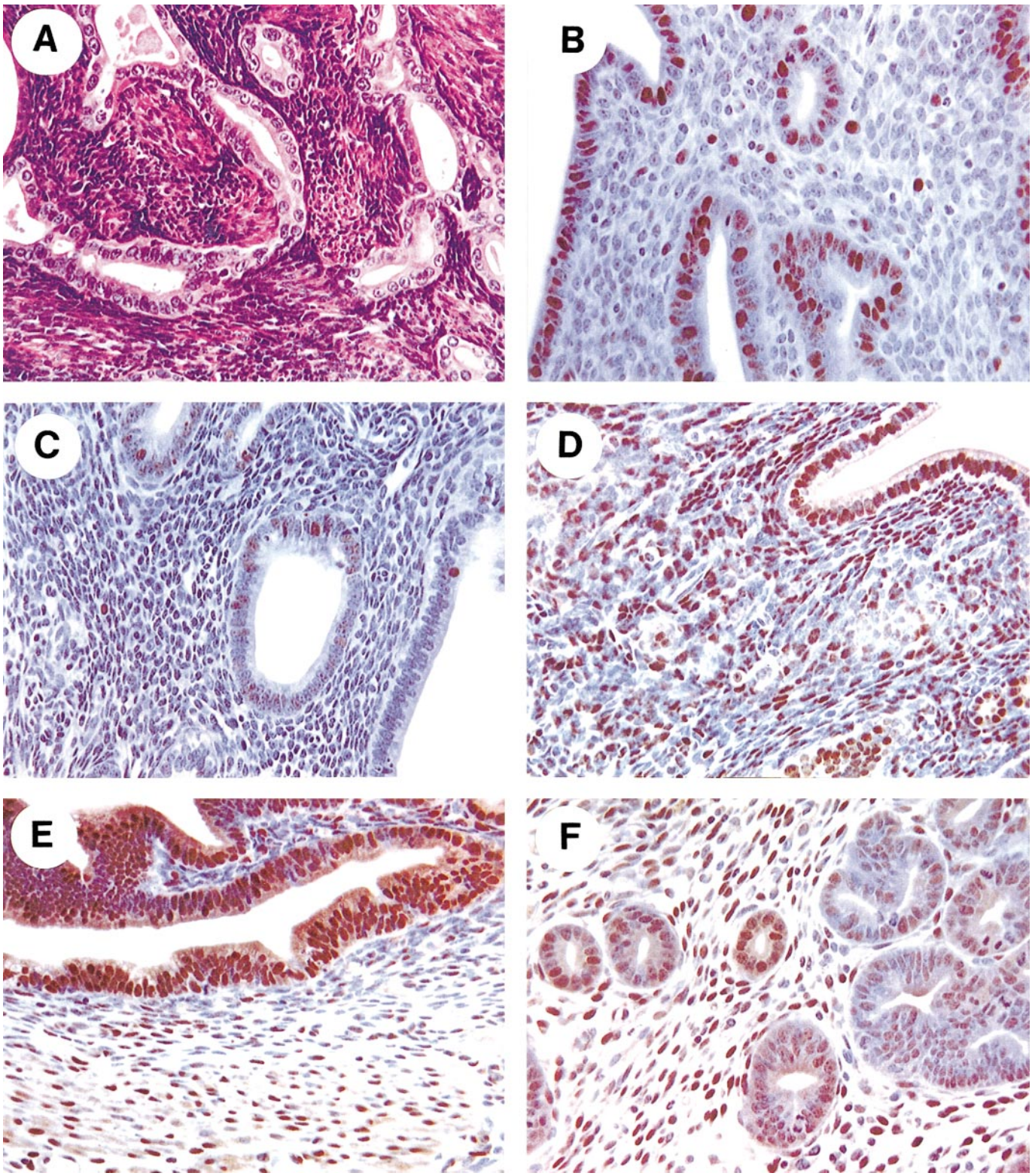

FIG. 1. Lesions of the uterus in arsenate-treated and control mice and presence of PCNA and ER-α in lesions. (A) Upper left: A section of a uterine adenocarcinoma induced by arsenate containing numerous nests of neoplastic epithelial cells extending through the muscularis to the serosa. The cells are smaller with basophilic cytoplasm and numerous mitotic figures (H & E; 300×). (B) Upper right: A section of uterus from an arsenate-treated mouse stained for PCNA showing moderate cystic hyperplasia. Note strong nuclear staining for PCNA (300×). (C) Middle left: A section of uterus from an arsenate-treated mouse stained for PCNA showing moderate/severe hyperplasia. Note moderate to weak nuclear staining for PCNA (300×). (D) Middle right: A section of uterus from a control mouse showing mild cystic hyperplasia stained for ER-α. Note fairly strong nuclear staining for ER-α (300×). (E) Lower left: A section of uterus from an arsenate-treated mouse showing a severe cystic hyperplastic lesion stained for ER-α. Note extensive nuclear staining for ER-α (300×). (F) Lower right: A section of uterus from an arsenate-treated mouse showing cystic hyperplastic glands stained for ER-α. Note weak nuclear staining for ER-α (300×).

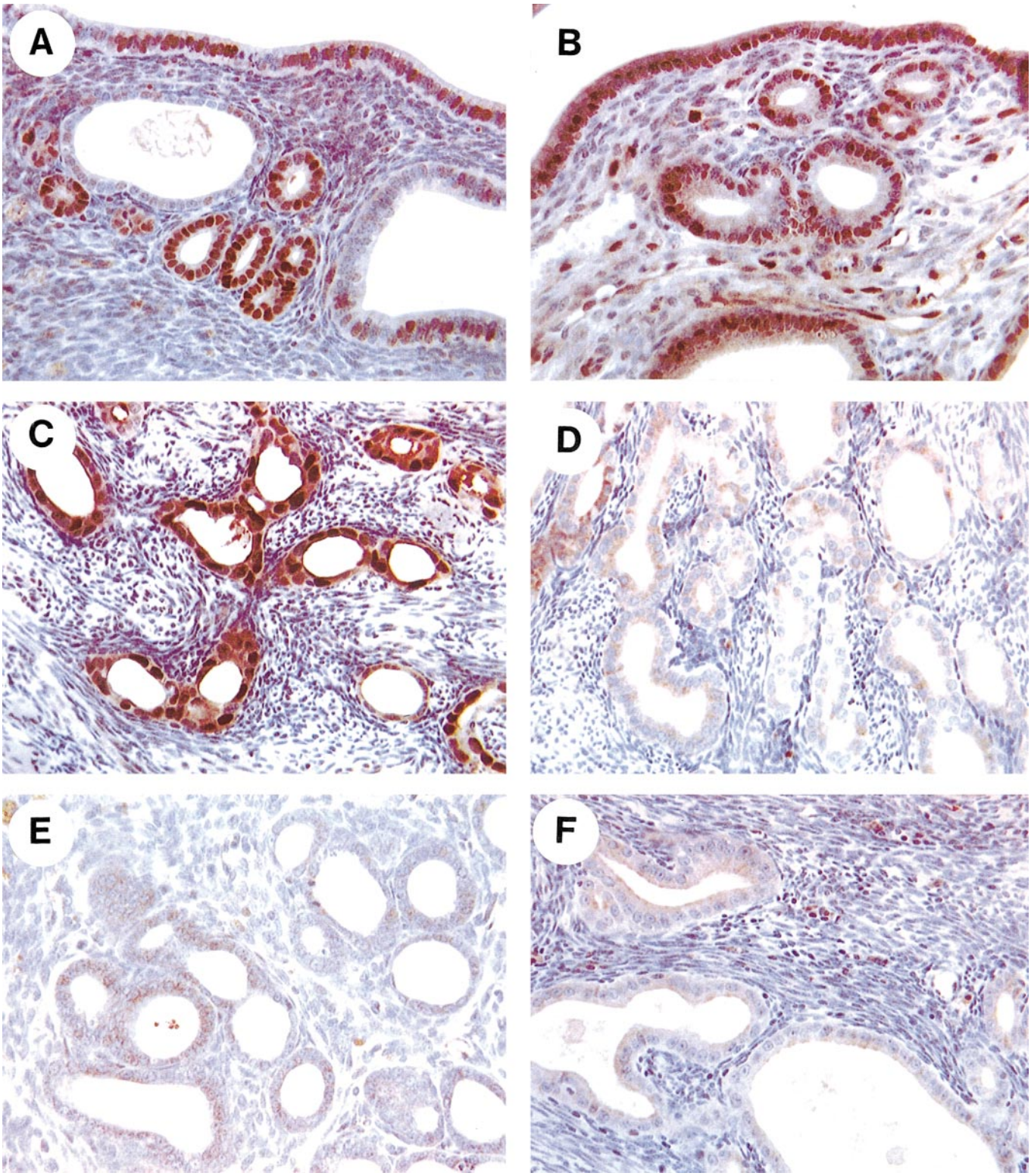

FIG. 2. Localization and staining intensity of cyclin D1 and iNOS in lesions of the uterus in arsenate-treated and control mice. (A) Upper left: A section of uterus from a control mouse showing a mild cystic hyperplastic lesion stained for cyclin D1. Note strong nuclear expression of cyclin D1 in some glands but moderate to low expression in other glands and in luminal epithelium (300×). (B) Upper right: A section of uterus from an arsenate-treated mouse showing a mild cystic hyperplastic lesion. Note strong nuclear expression of cyclin D1 in glandular and luminal epithelium (300×). (C) Middle left: A section of uterine adenocarcinoma from an arsenate-treated mouse showing strong nuclear expression of cyclin D1 in neoplastic glandular epithelium (300×). (D) Middle right: A section of uterus from a control mouse showing a moderate cystic hyperplastic lesion. Note weak expression of cytoplasmic iNOS in glandular epithelium and stromal tissue (300×). (E) Lower left: A section of uterus from an arsenate-treated mouse showing a moderate/severe cystic hyperplastic lesion. Note extensive expression of cytoplasmic iNOS in glandular epithelium and stromal tissue (300×). (F) Lower right: A section of uterine adenocarcinoma from an arsenate-treated mouse showing extensive expression of cytoplasmic iNOS, mostly in neoplastic glandular epithelium (300×).

TABLE 5
Immunoreactivity for PCNA, Cyclin D1, iNOS and 3-Nitrotyrosine in Uterine Epithelium of Swiss Mice Exposed to Sodium Arsenate

Treatment	Uterine hyperplasia	PCNA		Cyclin D1		iNOS		3-Nitrotyrosine	
		Intensity	Extent	Intensity	Extent	Intensity	Extent	Intensity	Extent
Arsenate	mild	+++	2	+++	3	++	3	++ (N)	2
Arsenate	mild	++	2	++	3	++	2	++ (N)	2
Arsenate	mild	+++	2	+++	2	++	2	+++ (C)	2
Arsenate	moderate to severe	++	2	++	3	++	3	+++ (C)	3
Arsenate	moderate to severe	+++	3	+++	3	++	2	++ (C)	3
Arsenate	moderate to severe	++	1	++	3	++	1	+++ (C)	2
Arsenate	moderate to severe	++	2	+++	2	++	2	+++ (C)	3
Arsenate	moderate to severe	++	1	++	3	++	2	++ (C)	3
Arsenate	moderate to severe	++	2	+++	2	+	2	+++ (C)	2
Arsenate	adenocarcinoma	++	2	+++	2	++	2	++	3
Control	absent	−	−	+	1	−	−	−	−
Control	absent	−	−	+	1	+	−	−	−
Control	mild	++	1	+	1	+	1	+	1
Control	mild	+	2	+	2	+	1	++	1
Control	moderate to severe	++	2	++	2	+	1	++	2

Note. Intensity: − = negative; + = weak; ++ = moderate; +++ = strong; Extent: 1 = 1–25% of cells per lesion stained; 2 = 25–75% of cells per lesion stained; 3 = 75–100% of cells per lesion stained; N = Nuclear; C = Cytoplasmic; PCNA and cyclin D1 were always nuclear and iNOS was always cytoplasmic.

(Fig. 1F). This was also true for the neoplastic epithelium in the adenocarcinoma associated with arsenate exposure.

The levels of cyclin D1 were also measured in uterine tissue. Such D-type cyclins are rate-limiting and essential for the progression through the G1 phase of the cell cycle, are considered indicators of cell proliferation, and can be expressed following estrogen stimulation (Musgrove and Sutherland, 1994; Sicinski *et al.*, 1995). Cyclin D1 protein was detected in both epithelial and stromal cell nuclei of uterine tissue. Immunohistochemical analysis in control uterine tissue showed minimal nuclear staining for cyclin D1 (Table 5). In contrast, hyperplastic uterine lesions in control (Fig. 2A) and arsenate-treated mice showed increased expression of cyclin D1 in nuclei of the uterine and glandular epithelium. Levels of nuclear staining for cyclin D1, however, were higher in the hyperplastic and neoplastic epithelium of arsenate-treated mice than in those of control mice (Table 5; Figs. 2B and 2C). These findings suggest that cyclin D1 overexpression precedes or is concurrent with cellular transformation.

The expression of iNOS and formation of the nitric oxide-derived protein adduct 3-nitrotyrosine were determined in normal, hyperplastic, and neoplastic lesions of uterus. Increased staining for both 3-nitrotyrosine and iNOS was seen in the hyperplastic epithelium of endometrium as well as in the inflammatory cells of stroma. Control sections of hyperplastic uterine epithelium showed little or no staining for 3-nitrotyrosine and iNOS (Table 5; Figs. 2D and 3A). In contrast, the presence of these proteins (as determined by the intensity of staining and the percentage of cells stained) increased with the severity of lesions in arsenate-treated mice (Figs. 2E and 2F; Figs. 3B and 3C; Table 5). Interestingly, in hyperplastic epithelium of some arsenate-treated mice nuclei were strongly positive for 3-nitrotyrosine (Fig. 3D; Table 5).

Hyperkeratotic skin lesions were also produced by the repeated injection of arsenate (Table 6). In males the incidence of these skin lesions was increased from 4% in controls to 20% with arsenate (not significant). whereas in females the incidence increased from 0% in controls to 38% with arsenate exposure (highly significant). One of the primary target sites in human arsenic carcinogenesis is the skin.

TABLE 6
Effect of Repeated Injections of Sodium Arsenate on the Incidence and Severity of Skin Hyperkeratosis in Male and Female Swiss Mice

Group	Incidence of hyperkeratotic skin lesions (%)
Control males	1 (4%)
Arsenate males	5 (20%)[a]
Control females	0 (0%)
Arsenate females	9 (38%)*

Note. All groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals). An asterisk (*) indicates a significant difference from control by Fisher exact test.

[a] The incidence of hyperkeratotic skin lesions in the arsenate-treated males approached significance when compared with that of gender-matched controls by Fisher exact test ($p = 0.095$).

TABLE 7
Effect of Repeated Injections of Sodium Arsenate on the Incidence of Proliferative Lesions of the Lung in Male and Female Swiss Mice

Group	Alveolar cell hyperplasia	Alveolar cell adenoma	Alveolar cell carcinoma	Total tumors	Total proliferative lesions
Male control	3 (12%)	6 (24%)	8 (32%)	14 (56%)	17 (68%)
Male arsenate	1 (4%)	2 (8%)	9 (36%)	11 (44%)	12 (48%)
Female control	5 (20%)	3 (12%)	8 (32%)	11 (44%)	16 (64%)
Female arsenate	2 (8%)	6 (24%)	8 (32%)	14 (56%)	16 (64%)

Note. All groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals).

Lung tumors are also associated with arsenic exposure in humans. However, repeated injections of arsenic had no effect on lung tumorigenesis, although lung tumors were quite common (Table 7). For the hematopoietic system there were five lymphomas (all follicle center cells [FCC]) in the 25 control female mice and three lymphomas (one each of lymphoblastic, FCC, and immunoblastic) in the 25 arsenate-treated female mice. In male mice there was one lymphoma in controls and one in arsenate-treated animals (both FCC). Other sites proved unremarkable with regard to proliferative lesions.

The effects of repeated arsenate exposures on chronic nephropathy are shown in Table 8. Although the incidence of chronic nephropathy was unaltered in female mice receiving arsenate, the severity of the renal lesions was markedly increased when compared with that in controls. In male mice arsenate had no effect on incidence or severity of chronic nephropathy.

TABLE 8
Effect of Repeated Injections of Sodium Arsenate on the Incidence and Severity of Chronic Nephropathy in Male and Female Swiss Mice

Group	Nephropathy incidence (%)	Severity of nephropathy
Control males	19 (76%)	1.12 ± 0.17
Arsenate males	18 (75%)	0.92 ± 0.14
Control females	13 (20%)	0.56 ± 0.13
Arsenate females	18 (56%)[a]	1.36 ± 0.24*

Note. All groups consisted of 25 mice and were treated with one iv injection of 0.5 mg sodium arsenate/kg or 10 ml/kg saline (control) per week for the first 20 weeks of the study. Animals were then observed for a total of 96 weeks. Values are given as number of animals bearing lesions (% total animals) for incidence and mean ± SEM score for severity based on the following severity ratings: 0 = absent; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe. An asterisk (*) indicates a significant difference from control by Fisher exact test (incidence) or Student's *t* test (severity).

[a] The incidence of chronic nephropathy in the arsenate-treated females approached significance when compared to gender-matched controls by Fisher exact test ($p = 0.122$).

DISCUSSION

The results of the present study indicate that repeated arsenate injections are associated with proliferative, preneoplastic lesions of reproductive tissues in Swiss mice, specifically the uterus and testis, and of the female liver. Additionally, an endometrial adenocarcinoma occurred in the arsenate-exposed mice in the present study. This latter event should be placed in the context of the rarity of spontaneous uterine adenocarcinomas in mice of Swiss background (~0.5%) (Percy and Jonas, 1971). Although not allowing a definitive conclusion to be drawn, a causal role for arsenate is implicated. Our study failed to confirm previous results of a possible association between repeated arsenate injections and hematopoietic tumors (Osswald and Goerttler, 1971) but the reasons for this are not immediately clear. Arsenate exposure was also associated with enhanced chronic nephropathy and hyperkeratotic skin lesions in female mice. Both the skin and kidney are recognized target sites of arsenic toxicity in humans (IARC, 1980; NRC, 1999) and rodents (Germolec *et al.*, 1998; Liu *et al.*, 2000).

The strong association between arsenate exposure in the current study and incidence and severity of uterine hyperplasia, and the possible association with neoplasia, are intriguing. Uterine hyperplasia, as seen in the present work, is considered to be a stage in a continuum of endometrial changes leading eventually, in some cases, to endometrial adenocarcinoma (Crum, 1994). Specifically, uterine hyperplasia is often related to an abnormally high, prolonged estrogenic stimulation, frequently in the absence of progestational activity (Crum, 1994). Thus, uterine hyperplasia can be associated with diminished ovarian function and anovulatory syndromes (Crum, 1994). Because these uterine lesions were unexpected in the present study, assessment of ovarian function, such as through menstrual cycling, was not performed. However, no overt lesions occurred in the ovaries of arsenate-treated female mice to indicate dysfunction, although this does not absolutely eliminate the possibility of diminished function. One study, in fact, indicates that arsenic treatment of freshwater fish (*Colisa fasciatus*) impairs ovarian function (Shukla and Pandey, 1984), but how this might apply to mammals is not known. On the

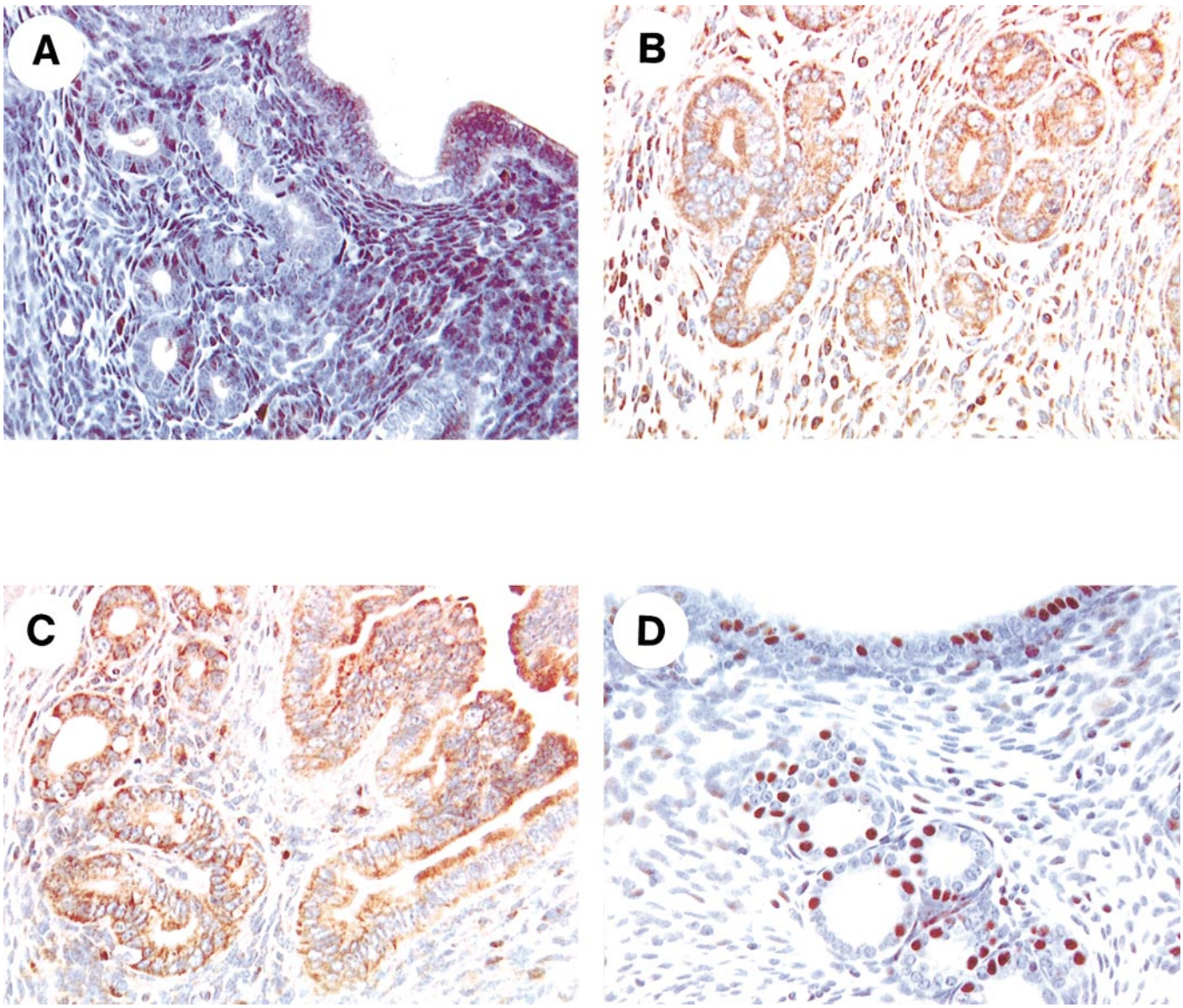

FIG. 3. Localization of 3-nitrotyrosine in the uterus in arsenate-treated and control mice. (A) Upper left: A section of uterus from a control mouse showing a moderate cystic hyperplastic lesion. Note weak expression of 3-nitrotyrosine in luminal and glandular epithelium as well as in stromal tissue (300×). (B) Upper right: A section of uterus from an arsenate-treated mouse showing a moderate/severe cystic hyperplastic lesion. Note extensive cytoplasmic staining for 3-nitrotyrosine in glandular epithelium and in stromal tissue (300×). (C) Lower left: A section of uterus from an arsenate-treated mouse showing a severe cystic hyperplastic lesion. Note extensive cytoplasmic expression of 3-nitrotyrosine in luminal and glandular epithelium as well as in stromal tissue (300×). (D) Lower right: A section of uterus from an arsenate-treated mouse showing a moderate cystic hyperplastic lesion. Note nuclear staining for 3-nitrotyrosine in the luminal and glandular hyperplastic epithelium (300×).

other hand, exogenous estrogen can supply the impetus for proliferative progression in the uterus and several studies have shown that steroid hormones and xenoestrogens can produce preneoplastic and neoplastic lesions in estrogen-responsive tissues, including uterus and breast, in humans and experimental animals (Johnson, 1987; Crum, 1994; Davis and Bradlow, 1995; Newbold, 1995; Diwan *et al.*, 1997; Korach *et al.*, 1997; Zava *et al.*, 1997; Persson *et al.*, 1999; Weiderpass *et al.*, 1999). Under this hypothesis arsenate would somehow be acting directly or indirectly to stimulate estrogen activity in the uterus.

To investigate whether an estrogenic mechanism might apply in these arsenic-induced hyperplastic lesions of the uterus, we determined the expression of ER-α and various estrogen-associated gene products in the uterine lesions. The ER-α, a member of the estrogen receptor superfamily, is a hormone-activated transcription factor that mediates the biological effects of estrogens in a variety of target tissues (Beato *et al.*,

1995; Musgrove and Sutherland, 1994). In responsive tissues, the ER stimulates the expression of specific estrogen-regulated genes (Ignar-Trowbridge *et al.*, 1992; Musgrove and Sutherland, 1994; Newbold, 1995; Korach *et al.*, 1997). The sensitivity of a given tissue to estrogens varies with the levels of estrogen receptor (Korach *et al.*, 1997). In the present study early hyperplastic lesions induced by arsenate showed moderate-to-strong expression of ER-α, in comparison with that in control lesions, in both epithelial and stromal cell nuclei. All these uterine lesions, as well as more severe hyperplastic lesions of arsenate-treated mice, were also strongly positive for cyclin D1. The D-type cyclins are thought to be rate-limiting and essential for the progression through the G1 phase of the cell cycle (Sherr 1993, 1994). Several studies have suggested that cyclin D1 may be involved in mediating steroid-dependent growth of both normal and malignant mammary epithelial cells (Buckley *et al.*, 1993; Sicinski *et al.*, 1995). Moreover, a strong expression of increased levels of cyclin D1 mRNA with ER overexpression has also been noted in malignant breast cells (Musgrove and Sutherland, 1994). Thus, estrogen may play a direct role in regulation of cyclin D1 expression. In any event, it appears that arsenate may be associated with estrogen-related genetic events in the uterus and that these events may lead to hyperplasia.

The arsenate-treated female mice in our study showed an increased incidence of hepatic proliferative lesions including preneoplastic hepatocellular foci and a hepatocellular adenoma. Experimental arsenic exposure can induce liver hyperplasia in rainbow trout (Kotsanis and Iliopoulou-Georgudaki, 1999) and there is some evidence linking arsenic and liver cancer in humans (NRC, 1999), which would be consistent with present findings of proliferative lesions with arsenate in the female mouse liver. The occurrence of such liver lesions is noteworthy because Swiss mice are refractory to spontaneous or chemical-induced liver tumor development (Diwan and Meier, 1976). Although the mechanism by which arsenate induces hepatocellular lesions is not known, it may be related to its estrogenic action, since estrogenic hormones are well-established hepatocellular carcinogens and liver tumor promoters (Schuppler and Günzel, 1979; Moolgavkar, 1986; Metzler and Degen, 1987). Thus, the finding of liver proliferative lesions in the present study is also indirect evidence of a potential estrogenic mechanism of action for arsenate, although such liver lesions can be induced by many other mechanisms.

The hyperplastic interstitial cell lesions seen with arsenic treatment in the present work could also be consistent with an estrogen-related mechanism. It is quite clear that chronic estrogenization in adult mice can induce proliferative interstitial cell lesions of the testes (Huseby, 1976; Bosland, 1994, 1996; Thomas, 1997). Other inorganic compounds, specifically compounds of cadmium, will induce interstitial cell proliferative lesions in mice and rats but do so in the presence of severe testicular degeneration (Bosland, 1994) and, at least in rats, with loss of androgen production and apparent subsequent hypergonadotrophic stimulation of remnant testicular tissue (Waalkes *et al.*, 1997). This is not consistent with the observed absence of degenerative testicular lesions in the present study with arsenic in mice. Furthermore, hypergonadotrophic stimulation by itself does not appear to lead to proliferative testicular interstitial cell lesions in mouse testes, as evidenced by a lack of correlation with gonadotrophin-secreting pituitary tumors (Bosland, 1994). Various experiments indicate that factors localized within the testes are critical to testicular proliferative lesion formation induced by estrogens (Huseby, 1976; Bosland, 1994). Under these circumstances arsenic could be either: (1) enhancing estrogen production or otherwise enhancing its actions; (2) reducing estrogen degradation or otherwise prolonging its actions; or (3) acting as an estrogen itself. Arsenic can interact with certain steroid receptors, although this leads to reduced activity (Simons *et al.*, 1990), so direct action as a steroid mimic seems unlikely. Otherwise, little is known about how arsenic might enhance or prolong the effects of estrogenic compounds. The activity of arsenic methyltransferase, the enzyme that methylates arsenic, is quite high in the mouse testis (Healy *et al.*, 1998), but how this might relate to hyperplasia development is unknown. Additional study clearly is required to define the possible role of arsenic in any estrogenic stimulation of testis and uterus.

NO is a messenger molecule serving a variety of functions in many different tissues (Lala, 1998). NO is synthesized in mammalian cells from L-arginine by the nitric oxide synthase (NOS) enzyme family. Three forms of this enzyme are known: the constitutive form of NOS, which includes endothelial and neuronal isoforms, and the calcium-independent inducible form of NOS, which is released by cells in response to cytokines and endotoxins. The long-term endogenous production of NO has been suspected of causing adverse effects *in vivo* (Wink *et al.*, 1998; Felley-Bosco, 1998; Wink and Mitchell, 1998). A by-product of NO production, 3-nitrotyrosine, which is used as a biomarker of such production, has been used as a signature of interaction of tissues with peroxynitrite and other reactive nitrogen intermediates (MacMillan-Crow and Thompson, 1999). Inducible NOS (iNOS) and 3-nitrotyrosine residues in proteins have been detected in a variety of human cancers including human gynecological cancers (ovarian, uterine, cervical), central nervous system tumors, breast tumors, gastric cancer, squamous cell carcinomas of the head and neck, prostatic cancer, and lung cancer (Thomsen *et al.*, 1994, 1995; Cobbs *et al.*, 1995; Takahashi *et al.*, 1997; Ambs *et al.*, 1998; Gallo *et al.*, 1998). Steroid hormones are known to modulate the L-arginine–NO synthetic pathway in the uterus (Chwalisz and Garfield, 1998). NO has also been demonstrated to play a role in the uterotrophic effects (hyperplasia) induced by estradiol in infant rats, as evidenced by the observation that the NO inhibitor L-NAME given before estradiol treatment abolished the uterine growth (Rao *et al.*, 1995). In the present investigation both iNOS and 3-nitrotyrosine stain strongly in the majority of the hyperplastic uterine lesions induced by arsenate,

further supporting a possible role of estrogen in the development of arsenate-induced lesions. To our knowledge, this is the first study to show the nuclear location of 3-nitrotyrosine in some of the uterine lesions induced by arsenate. Nuclear localization was prominent in hyperplastic glandular and luminal epithelium. The significance and function of nuclear 3-nitrotyrosine remains unclear at this stage.

In summary, the results of the present investigation clearly show that ER-α and receptor-associated gene products, including cyclin D1 and iNOS, are overexpressed in arsenate-induced hyperplastic and neoplastic uterine lesions. These results suggest, but do not establish, that the high incidence of uterine proliferative lesions observed in the present study may be caused by arsenic-mediated modulation of estrogen functions, by some as yet unknown mechanism. Our results showing interstitial cell hyperplasia in a large percentage of arsenate-treated animals tend to support this argument, since estrogens are known testicular tumorigens (Newbold *et al.*, 1986, 1987). Induction of proliferative lesions of the female liver is also consistent with estrogen exposure (Schuppler and Günzel, 1979; Moolgavkar, 1986), although many mechanisms can lead to liver hyperplasia. The precise fashion in which arsenic might alter the endogenous level and/or half-life of estrogenic steroid hormones is undefined. However, the possible action of arsenic as a xenoestrogen deserves additional study.

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