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Decreased Urinary Beta-Defensin-1 Expression as a Biomarker of Response to Arsenic

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Ingestion of arsenic (As) through contaminated drinking water results in increased risks of skin, lung, kidney, and bladder cancers. Due to its association with kidney and bladder cancers, we hypothesized that analysis of the urinary proteome could provide insight into the mechanisms of As toxicity. Urine from participants in a cross-sectional As biomarker study conducted in Nevada, classified as having either high (≥ 100 μg total urinary As/l) or low exposure (< 100 μg total urinary As/l) was analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Two polypeptides, 2.21 and 4.37 kDa, were significantly decreased in the high exposure group ($p < 0.05$) and were limited to men when stratified by sex. To replicate these findings, urine from participants in a second As study in Chile was analyzed and results confirmed the decrease of the 4.37 kDa polypeptide as well as a 4.76 kDa polypeptide among highly exposed men. These peaks were identified and confirmed as human β-defensin-1 (HBD-1) peptides. In a separate *in vitro* experiment, gene expression analysis of As-treated cell lines demonstrated reduced *HBD1* mRNA confirming that the observed decrease in HBD-1 resulted from As exposure. HBD-1 is an antimicrobial peptide constitutively expressed in multiple tissues including epithelial cells of the respiratory and urogenital systems. Recent studies support its role as a tumor suppressor gene for urological cancers suggesting that decreased HBD-1 levels may play a role in the development of cancers associated with As exposure. Further studies are warranted to investigate the role of HBD-1 in As-related toxicity.

Key Words: **arsenic; human beta-defensin-1; proteomics; SELDI-TOF MS.**

Arsenic (As) is classified as a group 1 carcinogen by the International Agency for Research on Cancer based on its association with cancers of the skin, lung, and bladder (IARC, 2004). The main source of As ingestion is through naturally contaminated drinking water, and occupations such as gold and uranium mining and copper smelting can also result in high As exposure (Tomasek and Darby, 1995; Viren and Silvers, 1994). Populations exposed to high levels of As through contaminated drinking water are found in many countries, including Bangladesh, India, Chile, and the United States. Thus, As exposure from contaminated drinking water is a major public health concern.

As is genotoxic and exposure has been associated with inhibition of DNA repair and cytogenetic alterations such as chromosomal aberrations, sister-chromatid exchanges and micronuclei formation (NRC, 2001). Increased micronuclei in exfoliated bladder cells are a marker of chronic As ingestion and genotoxicity, with higher prevalence in men than in women (Moore *et al.*, 1996, 1997). Additionally, As can modify cellular signaling through altered activation, expression, and DNA binding activity of transcription factors such as nuclear factor kappa B and activator-protein 1 (IARC, 2004; NRC, 2001). Despite this body of evidence and the large number of epidemiological studies investigating adverse health outcomes related to As exposure, the mechanisms of As-induced carcinogenesis and toxicity are not clearly understood.

Urinary proteome analysis has proven useful for the discovery of potential biomarkers of urological malignancies (Irmak *et al.*, 2005; Saito *et al.*, 2005). Urine excreted from healthy individuals contains measurable amounts of protein (~22 mg/day) in the mass range between 750 and 10,000 Da (Norden *et al.*, 2004). Because As is associated with cancers of the bladder and the kidney (Bates *et al.*, 1992; Chen and Wang, 1990; Chen *et al.*, 1992; IARC, 2004; NRC, 2001), and the urinary proteome includes soluble proteins that pass through the glomeruli in addition to protein components of solid phase elements such as exosomes derived from cells exposed to the bladder cavity (Pisitkun *et al.*, 2006), we hypothesized that analysis of the urinary proteomes of individuals with high

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and low urinary As levels would provide insight into the mechanism of As toxicity and carcinogenesis, as well as lead to the discovery of new biomarkers of exposure and early effect. To our knowledge, this is the first study of the urinary proteome in a human population exposed to a common environmental contaminant. We report differentially expressed proteins associated with As exposure, including decreased expression of human β-defensin-1 (HBD-1) peptides, in the urine of men with high As exposure from Nevada. These findings were replicated in a second, independent As exposed population from Chile, and the relationship between As exposure and HBD-1 was validated *in vitro*. We demonstrate that this decrease in urinary HBD-1 is most likely the result of a decrease in *HBD1* mRNA expression suggesting that HBD-1 may be a useful biomarker of As exposure and/or early effect.

MATERIALS AND METHODS

Nevada Study Population

Urine was obtained from a previous As biomarker study among individuals ingesting As contaminated water in Nevada. Details of the study have been presented elsewhere (Warner *et al*., 1994). Briefly, the original study population included individuals with private water supply wells that were sometimes contaminated with high As concentrations. Exposed subjects had well water As levels > 500 μg As/l ($n = 18$, mean = 1312 [± 350] μg As/l). They were individually matched on age, sex, and smoking status to subjects with well water As levels thought to be < 10 μg As/l ($n = 18$, actual mean after measurements = 16 [± 7] μg As/l). Enrollment was restricted to individuals who had resided in their homes for at least 1 year and used no additional water sources. Second and third morning urine voids were collected, stored at −80°C and thawed once to measure urinary concentrations of inorganic As (iAs) and its major metabolites, monomethyl As (MMA) and dimethyl As (DMA) as previously described (Kalman, 1988) for biomarkers of internal dose. Menstruation status was not known for the women.

Bladder cell micronuclei frequency were previously measured in this study as described (Warner *et al*., 1994). Briefly, urine was centrifuged and slides were prepared. Slides were preheated to allow cells to fully adhere to the slides, treated with pepsin to permeabilize the cells, washed, and fixed with 4% paraformaldehyde. They were then hybridized with a biotin labeled alpha-satellite probe for all human centromeres and propidium iodide was used to counterstain the DNA. Between 500 and 2900 cells were scored per subject and the frequency of micronuclei was based on the total number of cells scored. Micronuclei had to be less than one-third the diameter of the main nucleus, be in the same plane of focus, have the same color, texture, and refraction as the main nucleus, have a smooth oval on round shape, and be clearly separated from the main nucleus. All micnonuclei were photographed and cross-checked by two observers with questionable micronuclei disregarded.

Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF MS) analysis was performed on 31 urine samples from the Nevada study classified as high exposure (> 100 μg total As/l urinary level, 9 females, 8 males) or low exposure (< 100 μg total As/l urinary level, 8 females, 6 males) (Table 1) based on the median total As urinary level for male and female subjects combined. Samples were frequency matched for sex, age (± 5 years), and smoking status (ever, never). Samples without enough quantity to allow for multiple runs to ensure reproducibility and protein identification were not included. Total As was defined as the sum of iAs, MMA, and DMA.

Chile Study Population

Urine was obtained from a previous study of families from Chilean villages with water supplies containing high (Chiu Chiu) and low (Caspana) As levels. Details of the study have been presented elsewhere (Chung *et al*., 2002; Smith *et al*., 2000). Briefly, the study population included highly exposed families from Chiu Chiu in which one spouse had resided ≥ 20 years and the other spouse for ≥ 10 years (11 families, 44 participants). Children had to have been born in the village and have resided there since birth. The principal source of drinking water was the village water supply with reported As values ranging from 750 to 800 μg/l. Families with low exposures were selected from Caspana (8 families, 31 participants; drinking water As levels from 9 to 13 μg/l) with similar inclusion criteria. Spot urine samples were collected and urinary iAs, MMA, and DMA were measured as in the Nevada study. Menstruation status was not known for the women. All studies were approved by the University of California, Berkeley, Committee for Protection of Human Subjects.

SELDI-TOF MS analysis was performed on 60 urine samples from the Chilean study classified as high (≥ 100 μg total As/l urinary level, 15 female, 21 male) or low exposure (< 100 μg total As/l, 11 female, 13 male) (Table 1) based on the median As urinary level for male and female subjects combined from the Nevada study. As with the Nevada study, samples without enough quantity to allow for multiple runs and protein identification were not included.

Cell Culture and As Treatment Conditions

293T and HeLa cells (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone Laboratories, Inc., Logan, UT) with 10% fetal bovine sera, 4.5 mg/ml glucose, and 1% penicillin/streptomycin. Confluent cells were removed from plates with trypsin-ethylenediaminetetraacetic acid (0.25%) (Invitrogen, Carlsbad, CA) and subcultured at a ratio of 1:5.

Trivalent inorganic As (iAs^{III}) in the form of sodium arsenite ($NaAsO_2$) was obtained from Sigma (St Louis, MO) and methylarsine oxide ($MAs^{III}O$) was donated by Professor Miroslav Styblo at University of North Carolina, Chapel Hill. Both forms of As were dissolved in water to 10mM and diluted to the indicated concentrations in DMEM cell culture media. Subconfluent cell cultures were replated at 2×10^5 cells/well and the media was replaced with fresh media containing the indicated concentrations of As on the following day. At the end of 24 or 48 h, cells were washed with phosphate-buffered saline (PBS) and harvested for total RNA isolation. Noncytotoxic doses in a range relevant to urinary levels for the two studies were chosen as comparable effective low and high doses for each As form after performing several dose-response curves (data not shown). All experiments were performed in duplicate.

TABLE 1
Subjects Included in SELDI-TOF MS Analysis

	High exposure	Low exposure
Nevada		
Number of subjects	16	14
Number of males (current/former smokers)	8 (0/2)	6 (0/4)
Number of females (current/former smokers)	8 (4/0)	8 (4/0)
Average age	37	39
Average urinary As levels (μg/l) (range μg/l)	670 (114–2500)	33 (11–80)
Chile		
Number of subjects	34	23
Number of males (current/former smokers)	19 (1/3)	12 (0/2)
Number of females (current/former smokers)	15 (1/1)	11 (0/0)
Average age	24	27
Average urinary As levels (μg/l) (range μg/l)	454 (105–1025)	37 (8–64)

Note. This table indicates subjects in final analysis, excluding samples with poor reproducibility or outliers.

RNA Extraction and RT-PCR Analysis

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA) and treated with DNase 1, Turbo DNA-free (Ambion, Austin TX). RNA (2 μg) was reverse transcribed into cDNA using the Superscript III system (Invitrogen) following the manufacturer's instructions. cDNA was stored at −20°C until use.

Relative quantitative-reverse transcription-PCR (Q-RT-PCR) was performed using the Power SYBR Green PCR Master Mix in an ABI Prism 7300 thermocycler (Applied Biosystems, Foster City, CA) in the presence of 0.5μM primers, in a total volume of 10 μl. In all real-time PCR experiments, the final dissociation stage was run to generate a melting curve for the verification of amplification product specificity. All primers were designed and tested for their specificity using the Primer Express v. 1.5 (Applied Biosystems). For RNA analysis, primers were designed to span two adjacent exons to avoid amplification of contaminating genomic DNA sequences. The target amplicons were 120 bp for *HBD1* and 85 bp for beta actin (*ACTB*). Oligonucleotide sequences used as primers in the Q-RT-PCR experiments were the following: *HBD1* forward, 5′-CCT ACC TTC TGC TGT TTA CTC-3′; *HBD1* reverse, 5′-CAA TGT CTC CCT TCC GGT TCA-3′; B-actin forward, 5′-TGCGT GACATTAAGGA-GAAG-3′ and *ACTB* reverse, 5′-GCTCGTAGCTCTTCTCC-3′. Prequalified primer pairs for growth arrest and DNA damage protein 45 alpha (*GADD45A*) were purchased from Invitrogen. Each sample was run in triplicate and each PCR experiment included two nontemplate control wells. The relative amounts of mRNA were calculated by the comparative cycle threshold method (Livak and Schmittgen, 2001) and subsequently normalized by *ACTB* expression.

Protein Array Preparation

Urine samples selected for SELDI-TOF MS analysis were thawed and aliquoted into smaller volumes. Samples were monitored to ensure that they were not thawed more than four times based on previous studies showing no change in SELDI-TOF MS detected peaks following four freeze/thaw cycles (Papale *et al*., 2007; Schaub *et al*., 2004). Urine was mixed with denaturing buffer (8M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50mM Tris, pH9), vortexed, and the pH adjusted to 4.5, followed by dilution with an equal volume of CM10 binding buffer (100mM ammonium acetate, pH 4.5). Arrays were equilibrated with binding buffer and 190 μl of sample was added to each spot, incubated 1 h, then washed with binding buffer and deionized water. Sinapinic acid, prepared in 50% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA), was added twice to each spot. Arrays were analyzed in a 2- to 10-kDa mass-to-charge ratio (*m*/*z*) range by the ProteinChip Reader (Series PBS II, Bio-Rad, Hercules, CA). A negative control consisting of buffers with no sample and an internal control consisting of a repeated sample (Nevada study) or pooled samples (Chile study; pooled due to limited sample quantity) were included on one out of every three arrays to monitor reproducibility. All samples were run in duplicate and the coefficient of variance (CV) was calculated for each detected peak with a pooled CV calculated based on the following equation:

$$\sqrt{\frac{\sum_{i=1}^{n} CV^2}{n}}$$

where CV represents the CV of an individual peak and n represents the number of peaks detected. Duplicates with a pooled CV greater than 30% were repeated.

Statistical Analysis

Clusters were generated by Biomarker Wizard software (Ciphergen Biosystems, Fremont, CA) from 2 to 10 kDa with the following settings: first pass signal-to-noise ratio (S/N) 5, second pass S/N 2, minimum peak threshold of 25%, and a cluster mass window of 0.3%. Statistical analysis was performed in Biomarker Wizard using the Mann-Whitney U test to generate p values. The Grubbs' test was used to determine the presence of an outlier for the 4.37 kDa peak. Pearson correlation coefficients were used to determine correlations between peak intensities and other parameters including micronuclei frequency, other peak intensities, and urinary As species. Gene expression analysis was performed with a Student t-test followed by the Student-Newman-Keuls multiple comparison test with p values < 0.05 considered statistically significant and results reported from the Newman-Keuls test.

Protein Purification and Identification

Urine was centrifuged (5000 × g) and supernatants precipitated with 50% acetone followed by centrifugation (12,000 × g). Protein precipitates were dried in a Speed Vac, reconstituted with sample buffer (Bio-Rad, Hercules, CA), run on a 16.5% Tris-tricine gel (Bio-Rad) and stained according to manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The 6.5 kDa marker, an unstained band and a band near the 6.5 kDa marker were cut from the gel and protein was extracted from a portion of each band. For protein extraction, pieces of each gel band were incubated with 50% methanol/10% acetic acid followed by ACN, and incubated overnight with 50% formic acid/25% ACN/15% isopropanol. Supernatant was analyzed on an NP20 array (Bio-Rad).

The remaining gel band pieces chosen for identification were washed with 100mM ammonium bicarbonate (NH_4HCO_3) and incubated with 100mM NH_4HCO_3 and dithiothreitol. One hundred mM iodoacetamide was added and the sample was washed with 50% ACN/50% 100mM NH_4HCO_3. ACN was added and the sample was dried in a Speed Vac and incubated with 0.02 μg/μl modified trypsin (Promega, Madison, WI) in 25mM NH_4HCO_3 followed by addition of 25mM NH_4HCO_3 and overnight incubation at 37°C. Additional peptides were extracted twice with 60% ACN/0.1% formic acid and added to the supernatant. These tryptic peptides were submitted for peptide identification.

Mass Spectrometry

For disulfide bond and mass analysis, a CM10 array was analyzed using a ProteinChip Tandem MS Interface as a front-end SELDI ion source for the Applied Biosystem/MDS Sciex QStar Hybrid LC/MS/MS System (Models QStar XL, Foster City, CA). For protein identification, a nano LC column, made of 10 cm Polaris c18 5-μm packing material (Varian, Palo Alto, CA) in a 100-μm inner diameter glass capillary with an emitter tip, was coupled to an ESI source mounted on a Thermo-Finnigan Decca XP Plus mass spectrometer (Thermo Electron, Waltham, MA). A linear gradient from 0 to 45% at 200 nl/min was used to elute peptides. Buffer A was 5% ACN and 0.02% heptaflurobutyric acid (HBFA); buffer B was 80% ACN and 0.02% HBFA.

The program SEQUEST was used to identify peptides and proteins from the complete human database (Yates *et al*., 1995) with a search using a peptide ion tolerance of 1.5 atomic mass unit and a fragment ion tolerance of 1.0. Data were reported with Xcorr cutoffs of 1.8 for $a + 1$ charge state, 2.5 for $a + 2$, and 3.5 for $a + 3$.

HBD-1 Immunoassay

Rabbit anti-HBD-1 antibody was donated by Dr. Tomas Ganz (University of California, Los Angeles, CA). PS10 arrays were prepared as described previously (Diamond *et al*., 2001). Briefly, PBS was added to the arrays followed by incubation with Protein G (Calbiochem, San Diego, CA). Arrays were blocked with 1M tris pH8, washed with PBS containing 0.5% Triton X-100 (PBS-Triton X), and anti-HBD-1 antibody (0.5 mg/ml) was applied. Following a series of washes, 150 μl of urine from either the Nevada or Chile study was diluted with 50 μl of PBS-Triton X and added to the array followed by additional washes with PBS-Triton X and DI water. After drying, 1 μl of alpha-cyano hydroxycinnamic acid prepared with 50% ACN, 0.5% TFA was added twice.

RESULTS

Protein Array Analysis of Urine from the Nevada Study Reveals Differentially Expressed Proteins in High versus Low Exposure Groups

General characteristics and As exposure levels of subjects included in the SELDI-TOF MS urinary analyses are presented

in Table 1. No significant difference was noted between ages of the high and low exposure groups. Using urinary As levels as a measure of exposure, exposed subjects from Nevada and Chile had approximately 20- and 12-fold higher levels than those in the low exposure groups, respectively. Following CM10 analysis of 31 individual urine samples from the Nevada study (17 females, 14 males), one sample (female, high exposure) was excluded because of poor reproducibility (CV between duplicates > 30% following three independent SELDI-TOF MS runs). Analysis of the remaining 30 samples revealed a significant decrease in peak intensity of two polypeptides in the high versus low exposure groups (results summarized in Table 2; a list of all detected peaks is provided as supplementary data). Two polypeptides at 2.21 ($p = 0.014$) and 4.37 kDa ($p = 0.017$) displayed a 33 and 24% reduction in mean peak intensities, respectively. Upon stratification by sex, both polypeptides remained significantly decreased among high versus low exposed men. Specifically, there were 53 and 36% reductions in the mean peak intensities of the 2.21 and 4.37 kDa polypeptides (Fig. 1), respectively. An additional polypeptide detected at 2.81 kDa was not different in the high versus low exposure groups in the unrestricted analysis, but was significantly decreased ($p = 0.012$) by 50% in the men with high As exposure. There were no significant peaks detected between the female high and low exposure groups (Table 2) and removal of current smokers (Table 1; 4 females high exposure, 4 females low exposure) did not alter these results (data not shown). An inverse correlation was observed between the 4.37 kDa peak intensity and the micronuclei frequency observed among men ($r = -0.71$, $p \leq 0.008$; Fig. 2A) but not women ($r = -0.26$, $p \leq 0.35$; Fig. 2B) in the original cross-sectional micronucleus study (Warner *et al.*, 1994). In addition, we noted that the 4.37 kDa peak intensity was inversely correlated with total urinary As, iAs, MMA, and DMA levels in males but not females (male results summarized in Table 3).

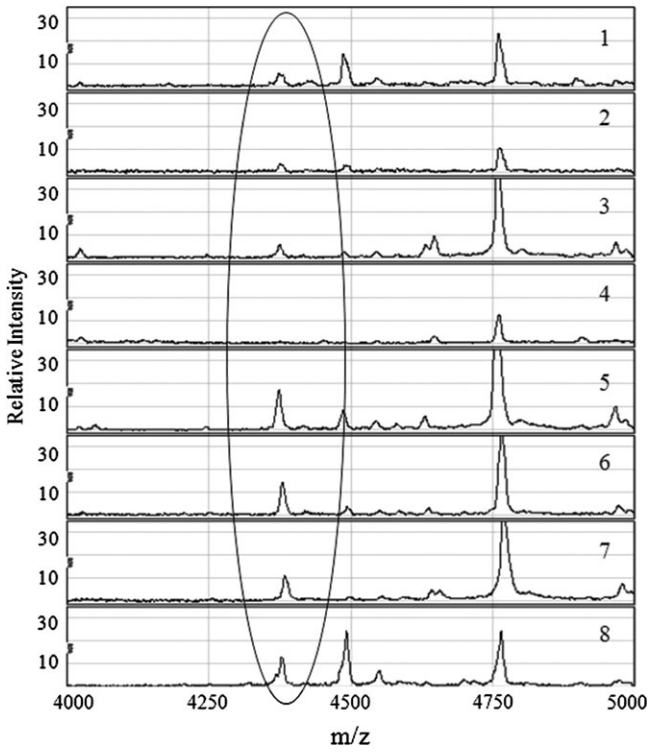

FIG. 1. Differential expression of the 4.37 kDa peptide in male urine from the Nevada study. Representative spectra from CM10 (weak cation exchange) array analysis of male urine with high (1–4) and low (5–8) As exposure levels. The circled peak represents the differentially expressed 4.37 kDa peptide.

TABLE 2
SELDI-TOF MS Analysis

Peak (kDa)	*p* Value	High exposure M.I. (SD)	Low exposure M.I. (SD)
Nevada			
Males and females combined			
2.21	0.014	4.46 (2.26)	6.67 (3.68)
4.37	0.017	7.98 (5.81)	10.56 (5.00)
Males			
2.21	0.001	3.87 (2.55)	8.22 (2.92)
4.37	0.018	6.52 (3.57)	10.21 (3.84)
2.81	0.012	5.22 (3.66)	10.58 (6.89)
Females			
N/A	N/A	N/A	N/A
Chile			
Males and females combined			
N/A	N/A	N/A	N/A
Males			
4.37	0.017	12.03 (8.59)	21.09 (10.26)
4.62	0.029	2.73 (2.25)	4.94 (2.97)
4.76	0.027	35.81 (20.82)	52.92 (17.08)
4.96	0.018	4.67 (3.30)	7.52 (2.33)
Females			
7.69	0.031	0.93 (0.85)	1.90 (1.09)
8.07	0.021	1.36 (1.23)	3.83 (2.93)

Note. Table indicates results of final analysis, excluding samples with poor reproducibility or outliers. M.I., mean intensity; SD, standard deviation; N/A, not available (no significant peaks).

Confirmation of Differentially Expressed Proteins in an As Exposed Population from Chile

CM10 analysis of 60 urine samples (26 females, 34 males) from the Chile study resulted in the exclusion of two male samples due to poor reproducibility (one high exposure, one low exposure). As noted in the Nevada study, there were no significant differences in ages between the high and low exposure groups. There were no significantly different peaks when males and females were analyzed together (results summarized in Table 2; a list of all detected peaks is provided as supplementary data). However, as observed in the Nevada study, stratification by sex led to the detection of a greater number of significantly different peaks in the male analysis

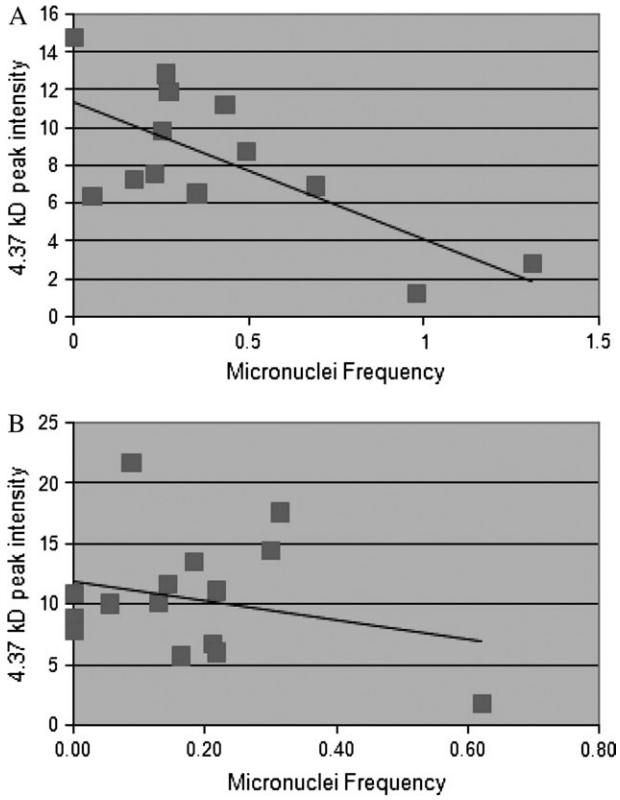

FIG. 2. Correlation between the 4.37 kDa peak intensities and micronuclei frequency in the Nevada study. At 4.37 kDa peak intensities from SELDI-TOF MS analysis of males (A) and females (B) are plotted against micronuclei frequency.

than the female analysis. Analysis of the female samples led to the detection of two polypeptide peaks significantly decreased in females with high versus low As exposure (7.69 and 8.07 kDa; Table 2). Analysis of the male samples led to the detection of four polypeptide peaks significantly reduced in males with high As versus low exposure (4.37, 4.62, 4.76, and 4.96 kDa) (Fig. 3). Of particular interest was the 4.37 kDa polypeptide ($p = 0.039$) which displayed a 32% reduction in the males with high exposure. Upon removal of one sample (high exposure), which was an outlier for this peak ($p < 0.01$), a 43% reduction was observed with $p = 0.017$ in males with high versus low exposure. Removal of both current and former smokers (Table 1) did not alter these results (data not shown). The similarity in mass and percent reduction between this and the decreased 4.37 kDa polypeptide in the Nevada study suggested that they were the same protein. Additionally, as observed in the Nevada samples, this polypeptide was not associated with exposure in females. Consistent with the Nevada study, the 4.37 kDa peak intensities were inversely correlated with urinary DMA levels in males in the Chile study. Interestingly, in the Chile study, the 4.76 kDa peak intensities were also inversely correlated with urinary DMA levels in males (Table 3). Results were not adjusted for multiple comparisons in either study.

TABLE 3
Correlation between 4.37 and 4.76 kDa Peak Intensities and Urinary As Species

		Total As	iAs	MMA	DMA
Nevada					
4.37 kDa	*r*	−0.62	−0.7	−0.65	−0.64
	p Value	0.02	0.01	0.02	0.02
4.76 kDa	*r*	−0.3	−0.38	−0.25	−0.27
	p Value	0.29	0.21	0.42	0.37
Chile					
4.37 kDa	*r*	−0.29	−0.08	−0.04	−0.37
	p Value	0.14	0.69	0.86	0.05
4.76 kDa	*r*	−0.3	−0.12	−0.11	−0.4
	p Value	0.11	0.54	0.57	0.03

Note. Analyses shown are for male subjects included in final analyses excluding samples with poor reproducibility or outliers.

Protein Purification and Identification

The observed decrease of a 4.37 kDa polypeptide in males with high As exposure in both the Nevada and Chile studies led us to pursue this polypeptide for identification. Reduced and nonreduced urine were analyzed on a CM10 array using a ProteinChip Tandem MS Interface as a front-end SELDI ion source for the Applied Biosystem/MDS Sciex QStar Hybrid LC/MS/MS System (Models QStar XL) to determine the

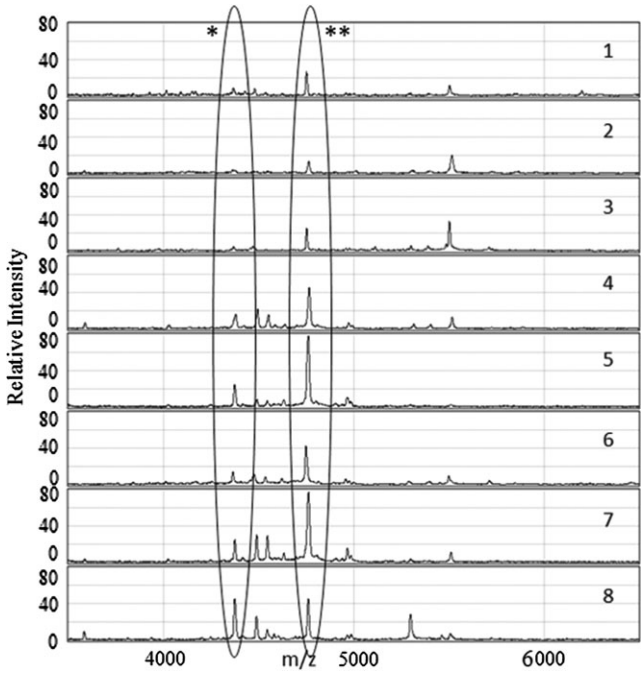

FIG. 3. Differential expression of the 4.37 and 4.76 kDa protein peaks in male urine from the Chile study. Representative spectra from CM10 array analysis of male urine with high (1–4) and low (5–8) As exposure levels. The circled peak (*) represents the differentially expressed 4.37 kDa peptide and the circled peak (**) represents the differentially expressed 4.76 kDa peptide.

presence of disulfide bonds and to obtain more accurate masses. This analysis showed a 6 Da shift in both the 4.37 and 4.76 kDa polypeptides upon reduction (data not shown), indicating the presence of three disulfide bonds, and the monoisotopic masses were determined to be 4363.88 and 4748.03 Da for these polypeptides, respectively.

For peptide sequence analysis, urine polypeptides were precipitated with acetone and separated on a tris-tricine gel. A distinct band near the 6.5 kDa marker was detected and no bands were observed below 4 kDa. Extraction of the polypeptides from the gel band near the 6.5 kDa marker and analysis on an NP20 array showed a series of peptides in the 4 kDa region (data not shown). Tryptic digestion and identification of this gel band led to a list of candidate proteins with the highest sequence coverage for a 23 amino acid tryptic peptide consistent with the sequence for HBD-1 (Swiss-Prot number P60022). This peptide had a 33.8% sequence coverage of the 68 amino acid precursor protein and a 52.2% sequence coverage of the processed 44 amino acid polypeptide reported to be the predominant form in male urine (Valore *et al.*, 1998). HBD-1 also was the only protein containing three disulfide bonds among the candidate proteins, providing additional evidence for its identity.

HBD-1 is synthesized as a 68 amino acid precursor and exists in the urine in multiple forms ranging from 36 to 44 amino acids that differ by N-terminal processing (Valore *et al.*, 1998; Zucht *et al.*, 1998). The amino acid sequence of the unprocessed peptide is MRTSYLLLFT LCLLLSEMAS GGNFLTGLGH RSDHYNCVSS GGQCLYSACP IFTKIQGTCY RGKAKCCK with the tryptic peptide identified corresponding to amino acids 32–54. Previous studies of urinary HBD-1 have identified the two most abundant variants as peptides corresponding to amino acids 29–68 and 25–68 (Valore *et al.*, 1998) with calculated monoisotopic masses of 4362.99 and 4747.23 Da, respectively. The reduction experiment utilizing the ProteinChip Interface provided more accurate monoisotopic masses for the decreased 4.37- and 4.76 kDa proteins as 4363.88 and 4748.03 Da, respectively, indicating that these proteins likely correspond to the 40 and 44 amino acid variants. In addition, a correlation was noted between the 4.37 and 4.76 kDa peak intensities in males in both the Nevada and Chile study ($r = 0.617$, $p < 0.02$; $r = 0.6$, $p < 0.01$, respectively) providing added evidence of a relationship between these two protein peaks. Further, greater relative expression of the 4.76 versus 4.37 kDa peak in our SELDI-TOF MS spectra is consistent with the report that the 44 amino acid peptide is the predominant form in male urine.

Immunoassay Confirms the Identity of HBD-1 Peptides

Analysis of urine with a SELDI-TOF MS immunoassay confirmed the 4.37 and 4.76 kDa peptides as HBD-1 (Fig. 4). An additional peak was present in some but not all urine samples, at 4.49 kDa that most likely represents an additional HBD-1 peptide corresponding to the amino acids 28–68.

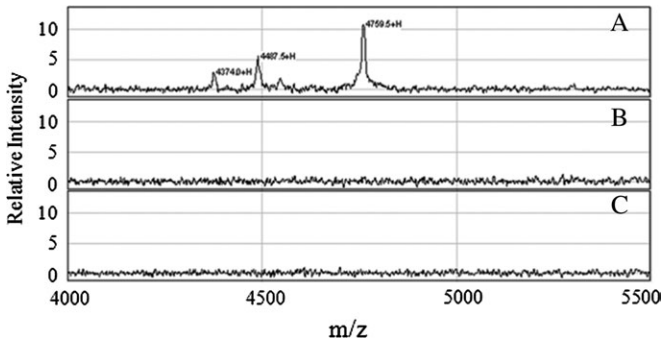

FIG. 4. Immunoassay confirmation of HBD-1. PS10 arrays were incubated with anti-HBD-1 antibody followed by urine. (A) Antibody with a low exposure urine sample, (B) antibody with no urine, (C) no antibody with urine. These spectra are representative of analysis of urine from the Nevada and Chile studies.

As Treatment Decreases HBD-1 Gene Expression in HeLa and 293T Cell Lines

To further validate our findings, we examined the effect of As exposure on *HBD1* gene expression in two human cell lines. HeLa cells, a cervical cancer cell line, and the human embryonic kidney cell line, 293T, were chosen because both the cervix and kidney have been shown to have abundant *HBD1* expression (Pazgier *et al.*, 2006; Valore *et al.*, 1998; Zucht *et al.*, 1998) and the kidney is a target organ of As exposure. First, we examined the effect of iAs^{III} in the form of $NaAsO_2$ on both cell lines (Figs. 5A and 5B gray bars). HeLa and 293T cells exhibited dose-dependent decreases in *HBD1* gene expression after treatment with iAs^{III} for 48 h with significant decreases at 3 and 10μM (225 and 749 μg/l $NaAsO_2$, respectively; Fig. 5A, gray bars) in Hela cells and a significant decrease at 3μM (Fig. 4B, gray bars) in 293T cells. The 10μM dose was toxic to 293T cells, but not to HeLa cells.

Many of the toxic effects of As are thought to be mediated through its methylated metabolite MMA^{III}. To determine the effect of MMA^{III} on *HBD1* expression, HeLa cells were exposed to up to 3.0μM $MAs^{III}O$ which resulted in > 90% loss of viable cells (data not shown). *HBD1* mRNA expression was significantly decreased following treatment at nontoxic doses of 0.1, 0.3, or 1μM $MAs^{III}O$ (11, 32, and 106 μg/l, respectively; Fig. 5A, black bars). 293T cells treated with $MAs^{III}O$ demonstrated a more gradual decrease in *HBD1* mRNA levels than HeLa cells with a significant decrease being observed at 1.0μM $MAs^{III}O$ (Fig. 5B, black bars). This difference in susceptibility to $MAs^{III}O$ was not due to differences in overall expression of *HBD1* as the cycle threshold values were similar for the two cell lines (data not shown).

DISCUSSION

In an initial study comparing the urinary proteome of individuals exposed to high and low levels of As in drinking

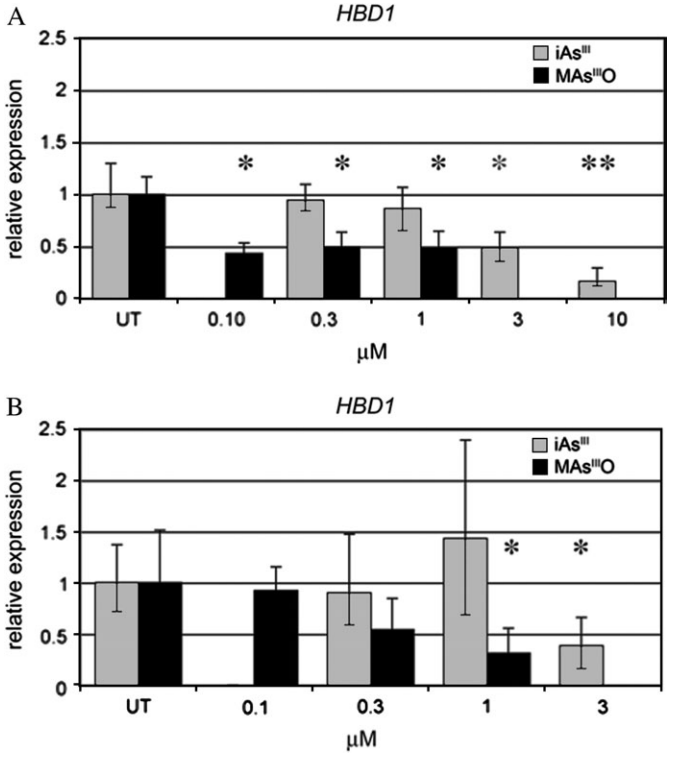

FIG. 5. Suppression of *HBD1* mRNA expression by iAs^{III} and $MAs^{III}O$ in HeLa cells (A) and 293T cells (B). *HBD1* mRNA expression following 48 h treatment with iAs^{III} as $NaAsO_2$ (gray bars) or $MAs^{III}O$ (black bars) is shown. Relative quantitation of mRNA levels are presented as values normalized to the respective untreated samples. $*p \leq 0.05$, $**p \leq 0.01$.

water in Nevada, we found inverse associations among the peak intensity of a 4.37 kDa HBD-1 peptide and urinary As levels and bladder cell micronuclei frequency (as a measure of genotoxicity) in men. These findings were confirmed in a larger, independent population from Chile with a similar range of high and low urinary arsenic levels. Additionally, in the Chile study, we observed an inverse association between levels of a 4.76 kDa HBD-1 peptide and urinary As, indicating that As exposure may lead to an overall decrease in HBD-1 rather than affecting the processing of a specific HBD-1 peptide. Further evidence provided by *in vitro* studies indicated that both iAs^{III} and MMA^{III} exposure directly lead to decreased *HBD-1* mRNA expression.

MS/MS analysis of the extracted polypeptides in the 4 kDa range led to a list of candidate proteins, of which HBD-1 had the highest sequence coverage. Use of a ProteinChip Tandem MS Interface and sample reduction indicated the presence of three disulfide bonds in both the 4.37 and 4.76 kDa peaks, which are characteristic of defensins (Pazgier *et al*., 2006). An HBD-1 specific SELDI-TOF MS-based immunoassay was used to confirm the identity of both peaks as HBD-1. Finally, correlation between the 4.37 and 4.76 kDa peak intensities and a similar reduction in both the 4.37 and 4.76 kDa peaks together with previous reports of multiple HBD-1 forms present in the urine (Valore *et al*., 1998; Zucht *et al*., 1998) provide additional evidence for their identity as HBD-1 peptides.

To determine if the observed reduction in urinary HBD-1 occurs at the mRNA level, HeLa and 293T cell lines were treated with $NaAsO_2$ or $MAs^{III}O$ followed by *HBD1* gene expression analysis. Both cell lines demonstrated a decrease in *HBD1* mRNA following As treatment. However, $MAs^{III}O$ exerted a greater reduction in *HBD1* expression compared with $NaAsO_2$, which is consistent with reports that MMA^{III} is the most toxic As species *in vitro* (Mass *et al*., 2001; Petrick *et al*., 2000, 2001; Styblo *et al*., 2000). Studies in humans have demonstrated a positive association between the proportion of urinary MMA and risk for As-induced cancers (Chen *et al*., 2003a, b; Steinmaus *et al*., 2006) providing additional evidence that monomethylated As is more toxic than the dimethyl or inorganic species. Although extrapolation between cell line treatments and human exposures is difficult, the doses used in these *in vitro* experiments fall within the range that the bladder would encounter based on the urinary levels of the populations analyzed in this study. Interestingly, an inverse correlation was observed between the 4.37 kDa peak intensities and urinary levels of As and its metabolites in males in the Nevada study, whereas the 4.37 and 4.76 kDa peaks were inversely associated only with urinary DMA levels in males from the Chile study. The reasons for these differences are unclear and may be due to genetic differences in metabolism between the two populations. Therefore, although the *in vitro* analysis indicates that MMA^{III} is more potent at reducing *HBD1* gene expression, there are differences between the association of urinary HBD-1 peptides and urinary MMA metabolites between these two populations. Furthermore, HeLa cells exhibited decreased *HBD1* gene expression at lower As concentrations than 293T cells. Reasons for the difference in sensitivity between the two cell lines remain to be determined.

HBD-1 is a cationic antimicrobial peptide constitutively expressed in multiple tissues including epithelial cells of the respiratory tract and urogenital system (Pazgier *et al*., 2006; Valore *et al*., 1998; Zucht *et al*., 1998). Although most studies have focused on the antimicrobial activity of HBD-1 (Valore *et al*., 1998), there is evidence of cytotoxic and chemotactic activities suggesting that HBD-1 may have additional biological effects (Yang *et al*., 1999; Zucht *et al*., 1998). Analysis of renal epithelial neoplasm subtypes identified HBD-1 as one of the markers for conventional renal cell carcinoma (RCC), with decreased *HBD-1* expression both at the mRNA and protein levels (Young *et al*., 2001, 2003). Similarly, a larger study of *HBD-1* mRNA and protein expression in tissue from conventional RCC and prostate cancer patients found loss of HBD-1 protein expression in 90% of RCC and 82% of prostate cancers with either complete protein loss or weak staining. In a subset of samples, this protein loss also correlated with mRNA loss (Donald *et al*., 2003). Another study suggested that *HBD-1* may be a tumor suppressor gene for urological cancers (Sun *et al*., 2006). These studies together indicate that HBD-1

may play a role in carcinogenesis and may be a potential biomarker of RCC.

It is plausible that decreased HBD-1 levels due to As exposure play a role in the development of associated urogenital and/or kidney cancers. However, this study does not address this or which specific tissue(s) may be affected by As. Urinary HBD-1 peptides originate primarily from the kidney (Valore *et al*., 1998), which is the major organ of excretion and plays a minor role in metabolism of As, thus providing a biological interaction by which As may affect urinary HBD-1 levels. Reasons why our findings were observed only in males are unclear. However, this observation, along with the inverse correlation between the 4.37 kDa peak intensities and micronuclei frequency in males from the Nevada study, is consistent with our previous report of higher bladder cell micronuclei frequency in males with high versus low exposure from this study (Warner *et al*., 1994). Several studies have reported differences in As metabolism between sexes (Bates *et al*., 1992) and some studies have suggested that men could be more susceptible to the toxic effects of As (Guha Mazumder *et al*., 1998; Moore *et al*., 1996; Ohtsuka *et al*., 2004; Watanabe *et al*., 2001). This suggests that sex hormonal status may influence As toxicity and may explain the sex differential in As-associated HBD-1 protein expression levels identified in the present study. These differences may also explain the inverse correlation observed between HBD-1 levels (as a measure of the 4.37 kDa peak intensities) and micronuclei prevalence in men and not women.

Although sample collection and storage conditions may interfere with reproducibility, great care was taken to ensure that collection and storage conditions were identical among samples. Although there are no studies on the effect of various storage conditions on HBD-1 peptide levels, identical handling of all samples and strict adherence to a maximum of four freeze/thaw cycles (Papale *et al*., 2007; Schaub *et al*., 2004) indicate that sample handling should not influence the results reported here. Variations in urine pH can affect protein peak detection (data not shown). Thus, although there was no significant difference in urinary pH levels between high and low exposure (data not shown), all samples were adjusted to the same pH prior to protein array preparation. A difference in total protein content may also affect these results. However, no difference in total protein content between exposure groups or sexes was observed (Bradford protein quantitation of a subset of samples; data not shown). This is also reflected in the SELDI-TOF MS spectra by peaks that did not change intensities across samples. Although smoking status may also influence results, both studies had few smokers (Table 1) and removal of such samples did not affect results.

Major strengths of this study are the novel findings involving a widespread environmental contaminant, the replication of findings in two independent populations and an independent *in vitro* validation to confirm the relationship between As exposure and decreased *HBD-1*, inclusion of frequency-matched subjects to reduce potential sources of variability and confounding, and the standardization of preanalytical variables specific to sample preparation (i.e. urine pH, number of freeze-thaw cycles) to reduce variation. This study identified reduced levels of HBD-1 in men with high versus low As exposure in two independent populations, and provided *in vitro* evidence that this effect is directly related to As exposure and that it occurs at the gene expression level. These findings provide novel evidence that HBD-1 plays a role in As-associated toxicity and may be a biomarker of early response to As exposure. Results from these studies also shed light on a new mechanism through which As may increase risk of urological malignancies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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