

Culture, Characterization, and *In Vitro* Toxicity of Arsenic in Mouse Primary Uroepithelial Cells

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ABSTRACT

The purpose of this study was to develop a novel *in vitro* mouse uroepithelial cell culture to improve cancer risk assessment for arsenic, including use of genomic data for understanding the mode of action and cellular exposures for potential quantitative assessment and innovative dose-response assessment. Female mice 56-60 days old (C57BL/6J) were used to acquire primary bladder epithelial cells. The bladder was surgically excised and rinsed with defined keratinocyte-SFM medium (37°C). Epithelial cells were isolated by making a longitudinal incision to expose the interior cells and then gently scraping the interior to release the cells. Bladder cells from 50 mice were pooled. Cells were centrifuged and washed with keratinocyte-SFM to remove debris. Total cell yield was approximately 0.5 x 10⁶ cells per bladder and viability was >80%. Prior to seeding, RT-PCR in the cell pellet confirmed a high expression of Keratin-10, a marker of epithelial cells. Cells were seeded at approximately 0.4 x 10⁶ per well into collagen-coated, 24-well plate inserts. After acclimation of cells through growth and maintenance phases, cells were treated for 24 hr with 0, 0.1, and 0.5 μM arsenite, plus combination arsenite (As) and dimethylarsinic acid (DMA) exposures at (1.6 μM As + 0.7 μM DMA), (3 μM As + 3 μM DMA), and (5 μM As + 10 μM DMA). Cell viability analysis showed a concentration-dependent decrease in ATP relative to controls from approximately 80% at (1.6 μM As + 0.7 μM DMA) to less than 40% at (5 μM As + 10 μM DMA). This cell model may be useful in assessing arsenic toxicity and improving risk assessment models.

INTRODUCTION

Arsenic is a naturally occurring element found in soil, water, air, and food. Human exposure to arsenic occurs primarily from drinking water or occupational exposure. The USEPA has determined that inorganic arsenic is carcinogenic to humans and is associated with an increased risk of skin, lung, and bladder cancer. Arsenite metabolism occurs primarily in the liver via methylation, resulting in two organic metabolites, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), that are excreted via the bladder in the urine. These organic species are thought to be considerably less toxic than their inorganic parent, although recent evidence suggests they are show biological activity and may also be potentially carcinogenic. Therefore, development of a mouse uroepithelial model would be useful for performing innovative dose-response assessments and quantitative measurements. Use of the mouse uroepithelial model, coupled with genomic results, toxicity data, and arsenic speciation analysis, may help improve the risk assessment of arsenic for producing cancer.

METHODS

Bladder Cell Isolation, Culture, and Maintenance

Each mouse bladder was clamped (inflow and outflow). The bladder was removed and placed in a vial containing warm (37°C) Keratinocyte-SFM medium (K-SFM, supplemented with 25 mg Bovine Pituitary Extract, 5 μg mouse EGF, and 30 ng/mL Cholera Toxin, plus 1X Antibiotic/Antimycotic solution)

A longitudinal cut was made using a small pair of scissors and the bladder was rinsed with SFM medium. Bladder was transferred to a new dish containing fresh Keratinocyte-SFM medium. Using a small pointed spatula and moving away from the forceps, the cells from the interior of the bladder wall were scraped off into the fresh Keratinocyte-SFM medium.

Cells were pooled into a 50mL sterile centrifuge tubes, kept warm at 37°C, and then pelleted by centrifugation (1200 RPM for 5 min; ~200 x g). Medium was decanted off and 5 mL fresh Keratinocyte-SFM was added to resuspend cells.

Cell suspensions were filtered through a nylon filter (BD Falcon Cell Strainer #352360; 100 μm) and rinsed with an additional 5 mL medium. Viability was checked using Trypan Blue exclusion dye. Cells were counted for live (no dye) / dead (blue dye).

Cells were seeded at 250,000 cells/well in 24-well BD BioCoat plates (3 μm collagen IV-coated inserts; BD # 354545). Incubated cells at 37°C, 5% CO₂.

Test Compound and Dosing Solution Preparation

Combination exposures of sodium arsenite (As) and dimethylarsinic acid (DMA) were performed.

Test compound stocks (10 mM) were used to prepare dosing solutions of: (0.1 μM As), (0.5 μM As), (1.6 μM As + 0.7 μM DMA), (3 μM As + 3 μM DMA), and (5 μM As + 10 μM DMA) in Keratinocyte-SFM medium.

Adenosine Triphosphate (ATP) Toxicity Assay

Each well insert was processed individually. Dosing medium was removed from well and the mesh insert was cut out using a scalpel and dissecting blade (#10). Each insert was transferred to a cryotube containing 100 μL ATP cell lysis buffer. Tubes were mixed for 10 minutes to lyse cells and then transferred to 80°C storage freezer.

Cellular Adenosine triphosphate (ATP) was determined using a luciferase-based assay (ATP-Lite; Perkin-Elmer). The luminescent assay is based on production of light, resulting from reaction of the ATP with luciferase and D-Luciferin. The light emission is proportional to the amount of ATP present in the sample. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

Gene Expression and RT-PCR

RNA extraction was performed using Trizol/chloroform method. Expression of mRNA levels was determined by RT-PCR (Roche Lightcycler 1.5). Confirmation of Keratin 10, arsenic (III) methyltransferase, and PPAR-γ were used as markers of epithelial cell cultures.

RESULTS

FIGURE 1:
Mouse Bladder Epithelial Cell Isolation

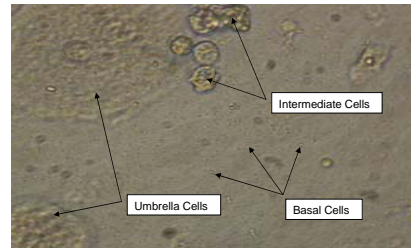


FIG. 1: Isolation of cell populations in the mouse bladder. The large polygonal (hexagonal) cells are the superficial umbrella cells that line the luminal surface of the bladder and are fully differentiated. The rounded cells are the intermediate and basal cells. The smallest cells are the undifferentiated basal cells which represent the pool of cells that may be able to regenerate and grow in culture.

FIGURE 2:
RT-PCR in Mouse Bladder Cells:
Keratin 10, PPAR-γ, and Arsenic (III) Methyltransferase (As(III)mt)

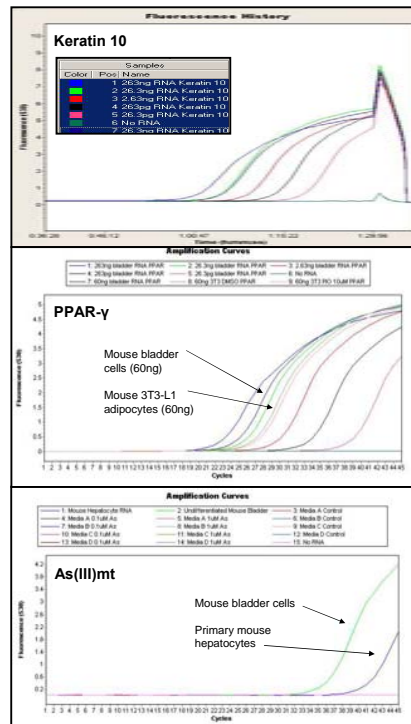


FIG. 2A:

FIG. 2B:

FIG. 2C:

FIG. 2A: Keratin 10 was detected from all tested amounts of total RNA (263ng – 26.3pg) from undifferentiated mouse bladder cells.

FIG. 2B: PPAR-γ is expressed in undifferentiated mouse bladder cells at a level higher than in mouse 3T3-L1 adipocytes.

FIG. 2C: As(III)mt was detected using RNA from undifferentiated mouse bladder cells and from untreated mouse primary hepatocytes. Approximately 20 ng of total input RNA was required in order to detect the As(III)mt transcript. Keratin 10 and PPAR-γ were able to be detected from approximately 20 pg of input RNA, and thus the As(III)methyltransferase appears to be expressed at low levels.

FIGURE 3:
Comparison of Mouse Bladder and Human Sarcoma Results after 24 and 48 Hour Exposure to Arsenite

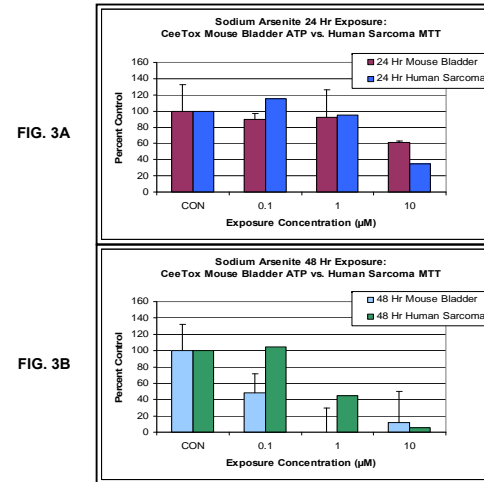


FIG. 3A:

FIG. 3B:

FIG. 3A: Following a 24 hour exposure to arsenite, mouse bladder cells were harvested for ATP analysis. Results were compared to MTT results of human sarcoma cells exposed to arsenite. Results between the two studies show similar dose-response profiles (Komissarova et al., 2005).

FIG. 3B: Following 48 hour exposure to arsenite, mouse bladder cells were harvested for ATP analysis. Results show increased toxicity at 48 hour and good concordance between the two studies (Komissarova et al., 2005).

FIGURE 4:
Combination Exposure of Arsenite + DMA:
24 Hour Exposure ATP Results

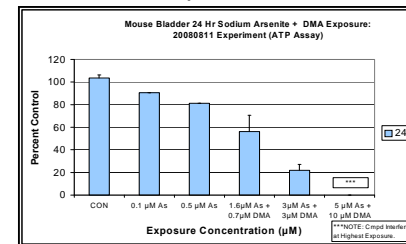


FIG. 4: Exposure to arsenite (As) and dimethylarsinic acid (DMA) was performed as a combination dosing regimen. Results show a dose-responsive decrease in the ATP toxicity marker with increasing concentrations of arsenite and DMA.

TABLE 1:
Arsenic Speciation in Dosing Solutions and Cell Supernatant

TREATMENT	Arsenic (μM)	DMA (μM)	MMA (μM)
Dosing Solution, Blank	0.01	0.01	0.00
Dosing Solution, Control	0.01	0.01	0.00
Dosing Solution, 0.1 μM Arsenite	0.10	0.01	0.00
Dosing Solution, 0.5 μM Arsenite	0.49	0.02	0.01
Dosing Solution, 1.6 μM Arsenite + 0.7 μM DMA	1.55	0.56	0.05
Dosing Solution, 3 μM Arsenite + 3 μM DMA	2.98	2.47	0.10
Dosing Solution, 5 μM Arsenite + 10 μM DMA	5.73	10.75	0.61
Cell Supernatant, Blank	0.01	0.01	0.00
Cell Supernatant, Control	0.01	0.01	0.00
Cell Supernatant, 0.1 μM Arsenite	0.11	0.01	0.00
Cell Supernatant, 0.5 μM Arsenite	0.52	0.02	0.01
Cell Supernatant, 1.6 μM Arsenite + 0.7 μM DMA	1.95	0.73	0.05
Cell Supernatant, 3 μM Arsenite + 3 μM DMA	3.19	2.80	0.20
Cell Supernatant, 5 μM Arsenite + 10 μM DMA	5.51	8.35	0.52

TABLE 1: Following a 24 hour exposure of mouse bladder epithelial cells to arsenite and DMA (Figure 4), cell supernatant was collected and analyzed for arsenic, DMA, and MMA. There were no substantial differences between the dosing solutions and the respective cell supernatants, indicating the mouse cells did not metabolize a measurable amount of the arsenic.

SUMMARY

The objective of the present study was to develop a novel *in vitro* mouse uroepithelial cell culture system to assist in addressing mode of action for bladder carcinogens, including eventual use of genomic data for understanding dose-response.

Mouse bladder uroepithelial cells in culture remain largely undifferentiated.

The mouse bladder culture expressed the gene markers Keratin 10, PPAR-γ, and Arsenic (III) methyltransferase, with Arsenic (III) methyltransferase expressed to a much lower degree than the other two.

Arsenic exposure produced a dose-responsive decrease in the ATP toxicity marker with increasing exposure concentrations.

Dose response curves of arsenic exposures are similar to those of other cell types and comparable to other published studies in the scientific literature.

No substantial differences were observed between the dosing solutions and the respective cell supernatants, indicating the mouse cells did not metabolize a measurable amount of the arsenic.

CONCLUSIONS

Development and utilization of a mouse bladder uroepithelial model may be useful for assessing mode of action for bladder carcinogens, including arsenic.

Optimization of culture conditions to improve viability and differentiation would be of great value. This may include:

- Optimization of culture medium components and growth conditions.
- Improvement of cell plating and adherence through use of various adhesion factors.
- Stimulation of growth and differentiation of cells in culture.

Genomics data would be valuable for investigating gene expression profiles and comparison across species.

The use of a mouse uroepithelial model, when coupled with toxicity results and arsenic speciation analysis, may help refine arsenic cancer risk assessment estimates.

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FIGURE 5:
Sodium Arsenite, DMA, and MMA Structures

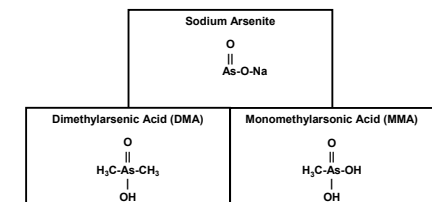


FIG. 5: Structures of Sodium Arsenite (As), DMA, and MMA.