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Regulation of transgelin and GST-pi proteins in the tissues of hamsters exposed to sodium arsenite.

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ABSTRACT

Hamsters were exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days. Equal amounts of proteins from urinary bladder or liver extracts of control and arsenic-treated hamsters were labeled with Cy3 and Cy5 dyes, respectively. After differential in gel electrophoresis and analysis by the DeCyder software,several protein spots were found to be down-regulated and several were up regulated. Our experiments indicated that in the bladder tissues of hamsters exposed to arsenite, transgelin was down-regulated and GST-pi was up-regulated. The loss of transgelin expression has been reported to be an important early event in tumor progression and a diagnostic marker for cancer development [29-32]. Down-regulation of transgelin expression may be associated with the carcinogenicity of inorganic arsenic in the urinary bladder. In the liver of arsenite-treated hamsters, ornithine aminotransferase was up-regulated, and senescence marker protein 30 and fatty acid binding protein were down-regulated. The volume ratio changes of these proteins in the bladder and liver of hamsters exposed to arsenite were significantly different than that of control hamsters.

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Introduction

Chronic exposure to inorganic arsenic can cause cancer of the skin, lungs, urinary bladder, kidneys, and liver [1-6]. The molecular mechanisms of the carcinogenicity and toxicity of inorganic arsenic are not well understood [7-9). Humans chronically exposed to inorganic arsenic excrete MMA(V), DMA(V) and the more toxic +3 oxidation state arsenic biotransformants MMA(III) and DMA (III) in their urine [10, 11], which are carcinogen [12]· After injection of mice with sodium arsenate, the highest concentrations of the very toxic MMA(III) and DMA(III) were in the kidneys and urinary bladder tissue, respectively, as shown by experiments of Chowdhury et al [13].

Many mechanisms of arsenic toxicity and carcinogenicity have been suggested [1, 7, 14] including chromosome abnormalities [15], oxidative stress [16, 17], altered growth factors [18], cell proliferation [19], altered DNA repair [20], altered DNA methylation patterns [21], inhibition of several key enzymes [22], gene amplification [23] etc. Some of these mechanisms result in alterations in protein expression.

Methods for analyzing multiple proteins have advanced greatly in the last several years. In particularly, mass spectrometry (MS) and tandem MS (MS/MS) are used to analyze peptides following protein isolation using two-dimensional (2-D) gel electrophoresis and proteolytic digestion [24]. In the present study, Differential In Gel Electrophoresis (DIGE) coupled with Mass Spectrometry (MS) has been used to study some of the proteomic changes in the urinary bladder and liver of hamsters exposed to sodium arsenite in their drinking water. Our results indicated that transgelin was down-regulated and GST-pi was up-regulated in the bladder tissues. In the liver tissues ornithine aminotransferase was up-regulated, and senescence marker protein 30, and fatty acid binding protein were down-regulated.

1. Materials and Methods

1.1. Chemicals

Tris, Urea, IPG strips, IPG buffer, CHAPS, Dry Strip Cover Fluid, Bind Silane, lodoacetamide, Cy3 and Cy5 were from GE Healthcare (formally known as Amersham Biosciences, Uppsala, Sweden). Thiourea, glycerol, SDS, DTT, and APS

were from Sigma-Aldrich (St. Louis, MO, USA). Glycine was from USB (Cleveland, OH, USA). Acrylamide Bis 40% was from Bio-Rad (Hercules, CA, USA). All other chemicals and biochemicals used were of analytical grade. All solutions were made with Milli-Q water.

1.2. Animals

Male hamsters (Golden Syrian), 4 weeks of age, were purchased from Harlan Sprague Dawley, USA. Upon arrival, hamsters were acclimated in the University of Arizona animal care facility for at least 1 week and maintained in an environmentally controlled animal facility operating on a 12-h dark/12-h light cycle and at 22-24°C. They were provided with Teklad (Indianapolis, IN) 4% Mouse/Rat Diet # 7001 and water, ad libitum, throughout the acclimation and experimentation periods.

1.3. Sample preparation and labelling

Hamsters were exposed to sodium arsenite (173 mg) in drinking water for 6 days and the control hamsters were given tap water. On the 6th day hamsters were decapitated rapidly by guillotine. Urinary bladder tissues and liver were removed, blotted on tissue papers (Kimtech Science, Precision Wipes), and weighed. Hamster urinary bladder or liver tissues were homogenized in lysis buffer (30mMTris, 2M thiourea, 7M urea, and 4% w/w CHAPS adjusted to pH 8.5 with dilute HCl), at 4°C using a glass homogenizer and a Teflon coated steel pestle; transferred to a 5 ml acid-washed polypropylene tube, placed on ice and sonicated 3 times for 15 seconds. The sonicate was centrifuged at 12,000 rpm for 10 minutes at 4°C. Small aliquots of the supernatants were stored at -80°C until use (generally within one week). Protein concentration was determined by the method of Bradford [25] using bovine serum albumin as a standard. Fifty micrograms of lysate protein was labeled with 400 pmol of Cy3 Dye (for control homogenate sample) and Cy5 Dye (for arsenic-treated urinary bladder or liver homogenate sample). The samples containing proteins and dyes were incubated for 30 min on ice in the dark. To stop the labeling reaction, 1uL of 10 mM lysine was added followed by incubation for 10 min on ice in the dark. To each of the appropriate dye-labeled protein samples, an additional 200 ug of urinary bladderor liver unlabeled protein from control hamster sample or arsenic-treated hamster sample was added to the appropriate sample. Differentially labeled samples were combined into a single Microfuge tube (total protein 500 ug); protein was mixed with an equal volume of 2x sample buffer [2M thiourea, 7M urea, pH 3-10 pharmalyte for isoelectric focusing 2% (v/v), DTT 2% (w/v), CHAPS 4% (w/v)]; and was incubated on ice in the dark for 10 min. The combined samples containing 500 ug of total protein were mixed with rehydration buffer [CHAPS 4% (*w/v*), 8M urea, 13mM DTT, IPG buffer (3-10) 1% (v/v) and trace amount of bromophenol blue]. The 450 ul sample containing rehydration buffer was slowly pipetted into the slot of the Immobiline DryStrip Reswelling Tray and any large bubbles were removed. The IPG strip (linear pH 3-10, 24 cm) was placed (gel side down) into the slot, covered with drystrip cover fluid (Fig. 1), and the lid of the Reswelling Tray was closed. The Immobilline DryStrip was allowed to rehydrate at room temperature for 24 hours.

Figure 1

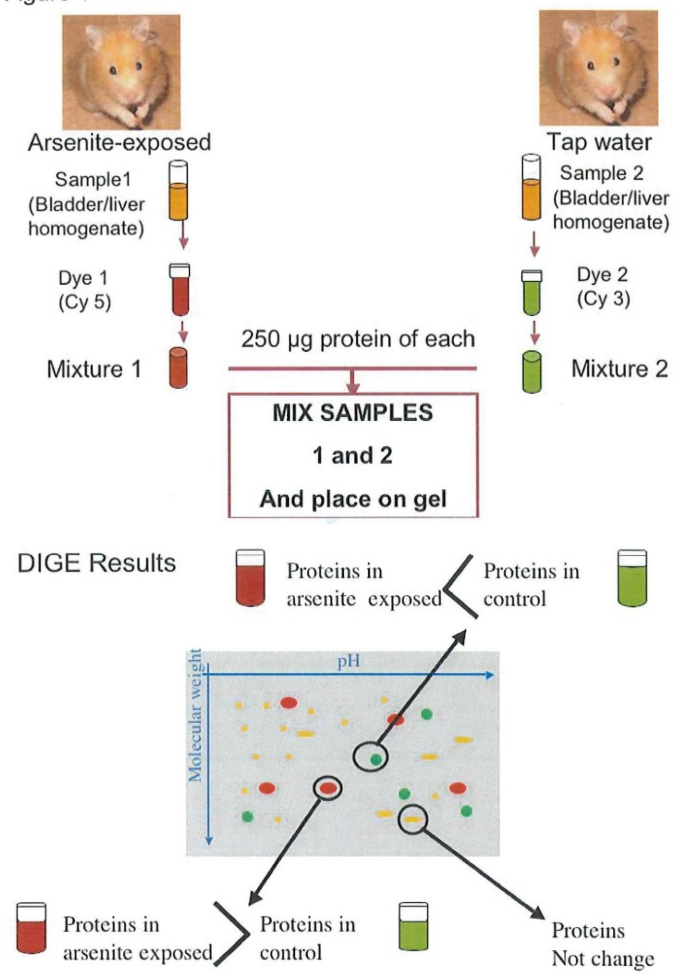

Figure 1. Flow sheet for Differential In-Gel Electrophoresis (DIGE) experiments [26].

2.4 Two-dimensional gel electrophoresis

2.4.1. First dimension Isoelectric focusing (IEF)

The labeled sample was loaded using the cup loading method on universal strip holder. IEF was then carried out on EttanIPGphor II using multistep protocol (6 hr @ 500 V, 6 hr @ 1000 V, 8 hr @ 8000 V). The focused IPG strip was equilibrated in two steps (reduction and alkylation) by equilibrating the strip for 10 min first in 10 ml of 50mM Tris (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.5% (w/v) DTT, followed by another 10 min in 10 ml of 50mM Tris (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 4.5% (w/v) iodoacetamide to prepare it for the second dimension electrophoresis.

2.4.2. Second dimension SDS-PAGE

The equilibrated IPG strip was used for protein separation by 2D-gel electrophoresis (DIGE). The strip was sealed at the top of the acrylamide gel for the second dimension (vertical) (12.5% polyacrylamide gel, 20x25 cm x 1.5 mm) with 0.5% (w/v) agarose in SDS running buffer [25 mMTris, 192 mM Glycine, and 0.1% (w/v) SDS]. Electrophoresis was performed in an Ettan DALT six electrophoresis unit (Amersham Biosciences) at 1.5 watts per gel, until the tracking dye reached the anodic end of the gel.

2.4.3. Image analysis and post-staining

The gel then was imaged directly between glass plates on the Typhoon 9410 variable mode imager (Sunnyvale, CA, USA) using optimal excitation/emission wavelength for each DIGE fluor: Cy3 (532/580 nm) and Cy5 (633/670 nm). The DIGE images were previewed and checked with Image Quant software (GE Healthcare) where all the two separate gel images could be viewed as a single gel image. DeCyde v.5.02 was used to analyze the DIGE images as described in the Ettan DIGE User Manual (GE Healthcare). The appropriate up-/down regulated spots were filtered based on an average volume ratio of ± over 1.2 fold. After image acquisition, the gel was fixed overnight in a solution containing 40% ethanol and 10% acetic acid. The fixed gel was stained with Sypro Ruby (BioRad) according to the manufacturer protocol (Bio-Rad Labs., 2000 Alfred Nobel Drive, Hercules, CA 94547).

2.5. Identification of proteins by MS

2.5.1. Protein spot picking and digestion

Sypro Ruby stained gels were imaged using an Investigator ProPic and HT Analyzer software, both from Genomic Solutions (Ann Arbor, MI). Protein spots of interest that matched those imaged using the DIGE Cy3/Cy5 labels were picked robotically, digested using trypsin as described previously [24] and saved for mass spectrometry identification.

2.5.2. Liquid chromatography (LC)- MS/MS analysis

LC-MS/MS analyses were carried out using a 3D quadrupole ion trap mass spectrometer (Thermo Finnigan LCQ DECA XP PLUS; Thermo Finnigan, San Jose, CA) equipped with a Michrom Paradigm MS4 HPLC (Michrom Biosources, Auburn, CA) and a nanospray source, or with a linear quadrupole ion trap mass spectrometer (Thermo Finnigan LTQ), also equipped with a Michrom MS4 HPLC and a nanospray source. Peptides were eluted from a 15 cm pulled tip capillary column (100 um I.D. x 360 um O.D.; 3-5 um tip opening) packed with 7 cm Vydac C18 (Vydac, Hesperia, CA) material (5 µm, 300 Å pore size), using a gradient of 0-65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% triflouroacetic acid) over a 60 min period at a flow rate of 350 nL/min. The ESI positive mode spray voltage was set at 1.6 kV, and the capillary temperature was set at 200°C. Dependent data scanning was performed by the Xcalibur v 1.3 software on the LCQ DECA XP+ or v 1.4 on the LTQ [27], with a default charge of 2, an isolation width of 1.5 amu, an activation amplitude of 35%, activation time of 50 msec, and a minimal signal of 10,000 ion counts (100 ion counts on the LTQ). Global dependent data settings were as follows: reject mass width of 1.5 amu, dynamic exclusion enabled, exclusion mass width of 1.5 amu, repeat count of 1, repeat duration of a min, and exclusion duration of 5 min. Scan event series were included one full scan with mass range of 350-2000 Da, followed by 3 dependent MS/MS scans of the most intense ion.

2.5.3. Database searching

Tandem MS spectra of peptides were analyzed with Turbo SEQUEST, version 3.1 (Thermo Finnigan), a program that allows the correlation of experimental tandem MS data with theoretical spectra generated from known protein sequences. All spectra were searched against the latest version of the non redundant protein database from the National Center for Biotechnology Information (NCBI 2006; at that time, the database contained 3,783,042 entries).

2.6. Statistical analysis

The means and standard error were calculated. The Student's t-test was used to analyze the significance of the difference between the control and arsenite exposed hamsters. P values less than 0.05 were considered significant. The reproducibility was confirmed in separate experiments.

3. Results

3.1. Analysis of proteins expression

After DIGE (Fig. 1), the gel was scanned by a Typhoon Scanner and the relative amount of protein from sample 1 (treated hamster) as compared to sample 2 (control hamster) was determined (Figs. 2, 3). A green spot indicates that the amount of protein from sodium arsenite-treated hamster sample was less than that of the control sample. A red spot indicates that the amount of protein from the sodium arsenite-treated hamster sample was greater than that of the control sample. A yellow spot indicates sodium arsenite-treated hamster and control hamster each had the same amount of that protein.

Several protein spots were up-regulated (red) or down-regulated (green) in the urinary bladder samples of hamsters exposed to sodium arsenite (173 mg As/L) for 6 days as compared with the urinary bladder of controls (Fig. 2).

In the case of liver, several protein spots were also over-expressed (red) or under-expressed (green) for hamsters exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days (Fig. 3).

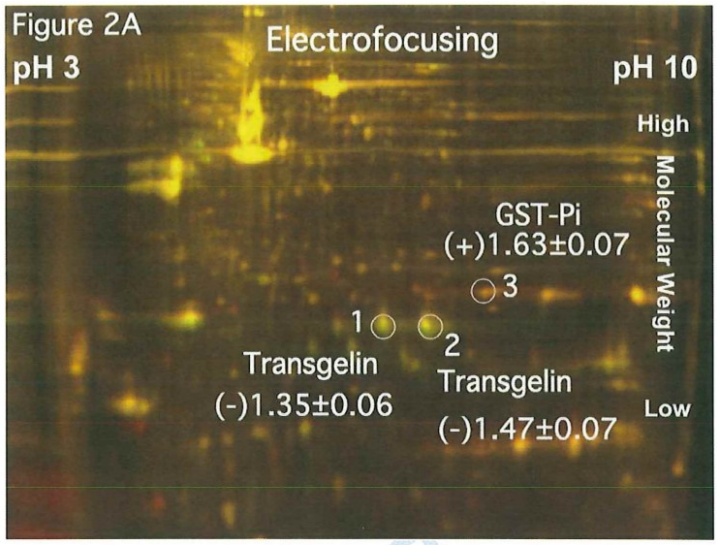

Figure 2. A Typhoon image of a DIGE gel of urinary bladder tissue samples labeled with CyDye DIGE Fluors. Before adding to the gel, the bladder sample of control hamster was labeled with Cy3 minimal dye, and the bladder sample of sodium arsenite-treated hamster was labeled with Cy5 minimal dye. The control hamsters were given tap water.

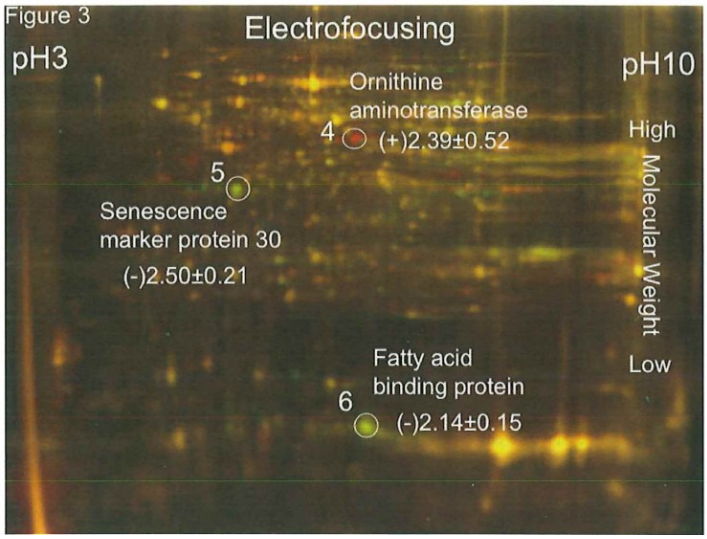

Figure 3. A Typhoon image of a DIGE gel of liver tissue samples pre-labeled with CyDye DIGE Fluors. The liver sample of control hamster was pre-labeled with Cy3 minimal dye, and the liver sample of sodium arsenite treated hamster was pre-labeled with Cy5 minimal dye. The control hamsters were given tap *w*ater.

The urinary bladder samples were collected from the first and second experiments in which hamsters were exposed to sodium arsenite (173 mg A*s*/L) in drinking water for 6 days and the controls were given tap water. The urinary bladder samples from the 1st and 2nd experiments were run 5 times in DIGE gels on different days. The protein expression is shown in Figure 2 and Table 1.

Table 1. Urinary bladder proteins that are down-regulated or up-regulated in hamsters exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days. Proteins in the DIGE gels were identified by using LC-MS/MS. Values (fold change) are the mean ± SE; (n=5).

Spot No.	Accession no[a]	Identification Protein	Mol. Wet. (kDa)	No. of Peptides	Percent Coverage	Fold Change (n=5)	p value (n=5)
1	CAA929	Transgelin	22.58	7	27.9%	(-)1.35±0.06	p<0.001
2	CAA929	Transgelin	22.58	7	28.4%	(-)1.47±0.07	p<0.001
3	Q60550	GST-class pi	23.43	13	61.0%	(+)1.63±0.07	p<0.001

[a]Accession number from NCBI (2006)

The liver samples from the 1st and 2nd experiments were also run 3 times in DIGE gels on different days. The proteins expression were shown in Figure 3 and Table 2. The volume ratio changed of the protein spots in the urinary bladder and liver of hamsters exposed to arsenite were significantly differences than that of the control hamsters (Table 1 and 2).

Table 2. Liver proteins that are down-regulated or up-regulated in hamsters exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days. Proteins in the DIGE gels were identified by using LC-MS/MS. Values (fold change) are the mean ± SE; (n=4).

Spot No.	Accession no[a]	Identification Protein	Mol. Wet. (kDa)	No. of Peptides	Percent Coverage	Fold Change (n=4)	p value (n=4)
4	AAH08119	OAT	48.36	9	23.2%	(+)2.39±0.52	p<0.05
5	BAC76714	SMP 30	33.25	12	50.2%	(-)2.50±0.21	p<0.001
6	AAV33399	L-FABP	10.89	4	48.0%	(-)2.14±0.15	p<0.001

Note: "Accession number from NCBI (2006); OAT, ornithine aminotransferase; SMP 30, senescence marker protein 30; L-FABP, liver fatty acid binding protein.

3.2. Protein spots identified by LC-MS/MS

3.2.1. Bladder

The spots of interest were removed from the gel, digested, and their identities were determined by LC-MS/MS (Fig. 2 and Table 1). The spots 1, 2, & 3 from the gel were analyzed and were repeated for the confirmation of the results (experiments; 173 mg As/L). The proteins for the spots 1, 2, and 3 were identified as transgelin, transgelin, and glutathione S-transferase-Pi, respectively (Fig. 2).

3.2.2. Liver

We also identified some of the proteins in the liver samples of hamsters exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days (Fig. 3). The spots 4, 5, & 6 from the gels were analyzed and were repeated for the confirmation of the results. The proteins for the spots 4, 5, and 6 were identified as ornithine aminotransferase, senescence marker protein 30, and fatty acid binding protein, respectively (Fig. 3).

4. Discussion

The identification and functional assignment of proteins is helpful for understanding the molecular events involved in disease. We exposed hamsters to sodium arsenite in drinking water. Controls were given tap water. DIGE coupled with LC-MS/MS was then used to study the proteomic change in arsenite-exposed hamsters. After electrophoresis DeCyder software indicated that several protein spots were down-regulated (green) and several were up-regulated (red).

Our overall results as to changes and functions of the proteins we have studied are summarized in Table 3.

Table 3. The functions of down-regulated and up-regulated proteins in the urinary bladder and liver tissues of hamsters exposed to Na arsenite.

Spot #	Expression	Protein	Function
Urinary Bladder			An actin cross-linking protein that is sensitive to transformation and shape-changes of the cells. However, its function is not clear. The loss of transgelin expression has been found in prostate cancer cells [30], in human colonic neoplasms [31], in the urinary bladders of rats having bladder outlet obstruction [32], in human breast and colon tumors [33].
1	Down	Transgeline	
2	Down	Transgeline	
3	Up	GST-pi	It participates in antioxidant defenses for protection against oxidative stress [37]. Over expression of GST-Pi has been found in the tissues of colon and esophagel cancer [39], in human stomach carcinomas [40], in human lung tumor [42], breast cancer [43].
Liver			
4	Up	OAT	OAT has a role in regulating mitotic cell division and it is required for proper spindle assembly in human cancer cell [49].
5	Down	SMP 30	SMP 30 expressed mostly in the liver. By stimulating membrane calcium-pump activity it protects cells against various injuries [50].
6	Down	L-FABP	High levels of saturated, branched chain fatty acids are deleterious to cells and resulting in lipid accumulation and cytotoxicity. FABP expression has protected the cells against branched chain saturated fatty acid [55].

Note: OAT, ornithine aminotransferase; SMP 30, senescence marker protein 30; L-FABP,liver fatty acid binding protein.

4.1. Bladder

In the case of the urinary bladder tissue of hamsters exposed to sodium arsenite (173 mg As/L) in drinking water for 6 days, transgelin was down-regulated and GST-pi was up-regulated. This is the first evidence that transgelin is down-regulated in the bladders of animals exposed to sodium arsenite.

Transgelin, which is identical to SM22 or WS3-10, is an actin cross linking/gelling protein found in fibroblasts and smooth muscle [28, 29]. It has been suggested that the loss of transgelin expression may be an important early event in tumor progression and a diagnostic marker for cancer development [30-33]. It may function as a tumor suppressor via inhibition of ARA54 (co-regulator of androgen receptor)-enhanced AR (androgen receptor) function. Loss of transgelin and its suppressor function in prostate cancer might contribute to the progression of prostate cancer [30]. Down-regulation of transgelin occurs in the urinary bladders of rats having bladder outlet obstruction [32]. Ras-dependent and Ras-independent mechanisms can cause the down regulation of transgelin in human breast and colon carcinoma cell lines and patient-derived tumor samples [33]. Transgelin plays a role in contractility, possibly by affecting the actin content of filaments [34]. In our experiments loss of transgelin expression may be associated or preliminary to bladder cancer due to arsenic exposure. Arsenite is a carcinogen [1].

In our experiments, LC-MS/MS analysis showed that two spots (1 and 2) represent transgelin (Fig. 2 and Table 1). In human colonic neoplasms there is a loss of transgelin expression and the appearance of transgelin isoforms (31).

GST-pi protein was up-regulated in the bladders of the hamsters exposed to sodium arsenite. GSTs are a large family of multi-functional enzymes involved in the phase II detoxification of foreign compounds [35]. The most abundant GSTs are the classes alpha, mu, and pi classes [36]. They participate in protection against oxidative stress [37]. GST-omega has arsenic reductase activity [38].

Over-expression of GST-pi has been found in colon cancer tissues [39]. Strong expression of GST-pi also has been found in gastric cancer [40], malignant melanoma [41], lung cancer [42], breast cancer [43] and a range of other human tumors [44]. GST-pi has been up-regulated in transitional cell carcinoma of human urinary bladder [45]. Up-regulation of glutathione – related genes and enzyme activities has been found in cultured human cells by sub lethal concentration of inorganic arsenic [46].

There is evidence that arsenic induces DNA damage via the production of ROS (reactive oxygen species) [47]. GST-pi may be over-expressed in the urinary bladder to protect cells against arsenic-induced oxidative stress.

4.2. Liver

In the livers of hamsters exposed to sodium arsenite, ornithine amino transferase was over-expressed, senescence marker protein 30 was under-expressed, and fatty acid binding protein was under-expressed. Ornithine amino transferase has been found in the mitochondria of many different mammalian tissues, especially liver, kidney, and small intestine [48]. Ornithine amino transferase knockdown in human cervical carcinoma and osteosarcoma cells by RNA interference blocks cell division and causes cell death [49]. It has been suggested that ornithine amino transferase has a role in regulating mitotic cell division and it is required for proper spindle assembly in human cancer cells [49].

Senescence marker protein-30 (SMP-30) is a unique enzyme that hydrolyzes diisopropyl phosphorofluoridate. SMP-30, which is expressed mostly in the liver, protects cells against various injuries by stimulating membrane calcium-pump activity [50]. SMP-30 acts to protect cells from apoptosis [51]. In addition it protects the liver from toxic agents [52]. The livers of SMP-30 knockout mice accumulate phosphatidylethanolamine, cardiolipin, phosphatidyl-choline, phosphatidylserine, and sphingomyelin [53].

Liver fatty acid binding protein (L-FABP) also was down-regulated. Decreased liver fatty acid-binding capacity and altered liver lipid distribution has been reported in mice lacking the L-FABP gene [54]. High levels of saturated, branched-chain fatty acids are deleterious to cells and animals, resulting in lipid accumulation and cytotoxicity. The expression of fatty acid binding proteins (including L-FABP) protected cells against branched-chain saturated fatty acid toxicity [55].

Limitations: we preferred to study the pronounced spots seen in DIGE gels. Other spots were visible but not as pronounced. Because of limited funds, we did not identify these others protein spots.

In conclusion, urinary bladders of hamsters exposed to sodium arsenite had a decrease in the expression of transgelin and an increase in the expression of GST-pi protein. Under-expression of transgelin has been found in various cancer systems and may be associated with arsenic carcinogenicity [30-33). Inorganic arsenic exposure has resulted in bladder cancer as has been reported in the past [1]. Over-expression of GST-pi may protect cells against oxidative stress caused by arsenite. In the liver OAT was up regulated and SMP-30 and FABP were down regulated. These proteomic results may be of help to investigators studying arsenic carcinogenicity.

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