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Impairment of antioxidant defenses as a contributor to arsenite-induced cell transformation

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Abstract Arsenite (As) causes transformation of human osteogenic sarcoma cells (HOS) when applied continuously at low doses (0.1–0.5 μM) during 8-weeks of exposure. However, the mechanisms by which As transforms human cells are not known. We investigated whether alterations occurred in gene expression and protein levels of antioxidant defense proteins, such as superoxide dismutase 1 (SOD1) and ferritin. In comparison to control HOS cells, 0.1 μM As induced greater cell proliferation and decreased anti-oxidant defenses. The tumor suppressor protein p53 was also decreased at both mRNA and protein levels. Further, pig3 (p53-induced-gene 3), a homolog of NQO1 (NADPH quinone oxidoreductase 1), was also down-regulated after 8 weeks of As challenge. The treatment of HOS cells with dicumarol, a NQO1 inhibitor, caused a dose-dependent decline in p53 protein levels, proving the effect of an antioxidant enzyme on p53 expression and, potentially, downstream processes. Caffeic acid phenethyl ester, an antioxidant, prevented the As-induced decreases in SOD1, p53, and ferritin mRNA and protein levels. SOD1, p53 and ferritin levels were inversely related to As-induced cell proliferation. Cumulatively, these results strongly suggest that impairment in antioxidant defenses contributes to As-induced human cell transformation and that the p53 pathway is involved in the process.

Keywords Arsenite · CAPE · Ferritin · NQO1 · p53 · SOD1

Abbreviations

As	Arsenite
CAPE	Caffeic acid phenethyl ester
HOS	Human osteogenic sarcoma
NQO1	NADPH quinone oxidoreductase 1
SOD1	Superoxide dismutase 1
pig3	p53-induced gene 3
AsT-HOS	Arsenite-transformed HOS cells
As-8w-HOS	HOS cells treated with arsenite for 8 weeks

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Introduction

Arsenic is a metalloid element, which forms a number of poisonous compounds. It has been widely used in industrial, agriculture, and medical areas (http://www.inchem.org/documents/ehc/ehc/ehc224.htm). It is present in the soil, ground water, food, and airborne particles as the result of both natural and human activities, with arsenic levels in industrial emissions being about three times higher than the levels in natural sources (Galanis et al. 2009; Rossman 2003). The U.S. Environmental Protection Agency has placed arsenic at the top of its Superfund contamination list (EPA 1996). Arsenic's human carcinogenicity has been well documented (Galanis et al. 2009; Huang et al. 2004; Kitchin 2001; Rossman 2003; Salnikow and Zhitkovich 2008). However, the molecular mechanisms by which arsenic induces human cancer remain elusive.

One classic pathway that could contribute to carcinogenesis is the ability of a carcinogen to induce mutagenesis. Although arsenic has been reported to mediate mutagenesis via oxidative damage (Chang et al. 2010; Galanis et al. 2009; Kessel et al. 2002; Liu et al. 2001), it is not a strong mutagen since mutagenesis in mammalian cells has been shown to occur primarily at highly toxic concentrations of arsenite and several of its metabolites (Klein et al. 2007). Notably, As has been shown to induce delayed mutagenesis in HOS cells, as well as malignant cell transformation, when administered at extremely low doses (0.025–0.1 μM) over an extended exposure duration of 30 cell passages (Mure et al. 2003).Caffeic acid phenethyl ester (CAPE), a natural component present in propolis made by honeybees, has the ability to inhibit As-induced HOS cell transformation as we previously reported (Yang et al. 2005). CAPE has been well known for its anti-inflammatory, antioxidant, and anti-tumor properties. The mechanism by which CAPE inhibits As-induced HOS cell transformation is not yet known.

The current studies were designed to investigate alterations in antioxidant defense pathways involving ferrritin and other antioxidant protective proteins that could be involved in arsenite-induced human cell transformation. Using the previously developed model of HOS cell transformation induced by a chronic low-dose exposure to arsenite and its modulation by CAPE (Yang et al. 2005), we found that As causes significant decreases in SOD1, ferritin, pig3 (p53-induced gene 3, a NQO1 homolog), and p53 gene expression and/or protein levels, and we discuss these effects as possible contributors to arsenic-induced carcinogenesis.

Materials and methods

Materials

Sodium arsenite ($NaAsO_2$, As), monoclonal mouse-anti-β-tubulin antibodies (Ab), minimum essential medium alpha (α-MEM), MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], dicumarol, and CAPE (caffeic acid phenethyl ester) were obtained from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Ferritin standard and anti-ferritin Ab were purchased from Research Diagnostics, Inc. (Flanders, NJ). Anti-SOD1 Ab was obtained from Santa Cruz (Santa Cruz, CA). Anti-p53 and peroxidase-conjugated anti-mouse Abs were from Cell Signaling (Beverly, MA). Radioimmunoprecipitation assay (RIPA) lysis buffer was from Upstate Inc. (Lake Placid, NY). Complete proteinase inhibitor cocktail was from Roche Molecular Biochemicals (Indianapolis, IN). Dicumarol was dissolved in 0.4 N NaOH stock solutions just before use and kept in the dark.

Cell culture and treatment with arsenite

HOS cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in complete α-MEM supplemented with 10 % FBS, 100 μg/ml penicillin/streptomycin, and incubated for 8 weeks with: (1) 0.1 μM As, (2) 0.1 μM As plus 0.5 μM CAPE, (3) 0.5 μM CAPE alone, and (4) no exposures (control HOS). Sodium arsenite (As) was dissolved in sterile HPLC grade water, while CAPE was dissolved in sterile DMSO. When cells were treated with CAPE, the final DMSO concentration was $\leq$ 0.01 %. The same DMSO amount was used to treat control cells.

After 8-week incubation, exposed and control cells were screened by a soft agar assay for loss of anchorage-dependence (ability to form colonies in

soft agar) as a measure of transformation (Mure et al. 2003). Only HOS cells treated with $NaAsO_2$ alone were capable of forming large clones in soft agar (Yang et al. 2005); some of these clones were isolated from soft agar and propagated in complete medium. After re-cloning in soft agar, these cells were propagated again and designated AsT-HOS (As-transformed HOS). In parallel experiments, cells derived from the 8-week exposures to As ± CAPE were cultured in arsenite-free α-MEM with 5 % FBS for at least two additional weeks after the removal of As and/or CAPE, then various endpoints were assessed. Cells exposed to As for 8 weeks but not screened in soft agar were designated arsenite-treated HOS cells (As-8w-HOS) cells. Control HOS cells were kept under the same conditions for 8 weeks but in the absence of As or CAPE.

Morphology and growth saturation density determination

Passage-matched parental HOS and As-8w-HOS were seeded in 90 mm Petri dishes (3×10^6/dish). Four days later, the cells were trypsinized and counted using a cell counter (Beckman Particle Counter, Beckman-Coulter Inc, Fullerton CA). Cell morphology was evaluated under phase contrast microscope.

Growth potential of four types of cells

Growth potential was determined in microplates using the MTT assay (Alley et al. 1988). Cells derived from the four 8-week exposure groups (untreated control, As alone, As + CAPE, CAPE alone), as well as AsT-HOS cells were seeded, each in two separate 96-well plates (1×10^3 cells/well in 100 µl α-MEM with 5 % FBS), incubated for 24 h, and washed once with PBS to eliminate the non-attached cells. One plate per exposure group was subjected to the MTT assay after overnight attachment as the initial growth control. After 72 h, the other plates were subjected to the MTT assay to measure the 72-h growth. Growth potential was expressed as the A_{570} (absorbance) value at the 72-h point divided by the A_{570} of the initial growth control. For the MTT assay, cell culture medium was changed to phenol red-free α-MEM (100 µl) before adding MTT. Ten microliter MTT (5 mg/ml in PBS) were added to each well for 4-h incubation at 37 °C. After removal of media, the resulting formazan crystals were dissolved in 200 µl DMSO. Absorbance was determined at 570 nm (A_{570}) using a microplate reader (Molecular Devices, Sunnyvale, CA) and the background absorbance measured at 690 nm was subtracted.

Determination of sod, pig3, p53, and ferritin mRNA expression by gene arrays and RT-PCR

The passage-matched parental HOS and As-8w-HOS cells in α-MEM containing 5 % FBS were cultured to about 80 % confluence in 60 mm culture dishes, washed with PBS, lysed with TRIzol, and RNA was extracted according to the manufacturer's instructions (Invitrogen Life Technologies, Inc., Carlsbad, CA).

RNA (5 µg) from each As ± CAPE-treated cell population was used for gene array assays according to the protocol provided by the manufacturer (SuperArray Biosciences Corp., Frederick, MD). Gene expression was detected by the alkaline phosphatase substrate CDP-Star, which generated chemiluminescent signals detected by exposure to X-ray film.

RT-PCR reverse transcription of total RNA was carried out according to he instructions provided by SuperScript® III First-Strand Synthesis System kit (Invitrogen Life Technologies, Inc., Carlsbad, CA). The resultant cDNA was amplified by PCR to determine expression of the H-chain and L-chain of ferritin, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, using the following gene-specific primers:

H-Ferritin: 5′-CGC CAG AAC TAC CAC CAG GAC-3′ (sense) and

5′-GGA AGT CAC CCC ACG GCT ATG-3′ (antisense) (Yang et al. 2001);

L-Ferritin: 5′-TTC CTC TCC GCT TGC AAC CT-3′ (sense) and

5′-CAC TCA TCT TCA GCT GGC TTC T-3′ (antisense) (Ghio et al. 1997);

GAPDH: 5′-CGG AGT CAA CGC ATT TGG TCG TAT-3′ (sense) and

5′-AGC CTT CTC CAT GGT TGG TGA AGA C-3′ (antisense).

PCR was carried out in a Whatman Biometra Thermal Cycler (Labrepco. Inc, Horsham PA) (Yang et al. 2001). After initial denaturation at 94 °C for 5 min, PCR was performed as follows: denaturation at 94 °C for 0.5 min, annealing at 55 °C for 1 min, and extension at 72 °C for 10 min. After separation of

PCR products on 1 % agarose gel, they were visualized by SYBR Gold Nucleic Acid Gel Stain (Molecular Probes Inc. Eugene, OR).

Detection of SOD1 and p53 proteins by Western blotting and ferritin by ELISA

Cells were lysed in RIPA buffer containing a protease inhibitor cocktail and the lysates were centrifuged at 15,000×g for 15 min. Thirty microgram of the extracted proteins were fractionated on a 12 % SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane. SOD1 and p53 were detected using specific antibodies (1:1,000 dilution), with β-tubulin serving as an internal control (Ab: 1:3,000 dilution). Specific banding was visualized using peroxidase-conjugated secondary antibodies with Western Lightning Plus Chemiluminescence Reagent (Perkin-Elmer, Waltham, MA) as the substrate. Cytoplasmic and nuclear proteins were separated using a commercial kit (Panomics Inc, Fremont, CA) and ferritin present in cell lysates, cytoplasm, and nuclei were detected by ELISA. The results were normalized to b-tublin in each sample (Zhang and Huang 2002).

The effect of NQO1 inhibitor dicumarol on p53 expression

The protein levels of p53 were measured while inhibiting the NQO1 activity by dicumarol. Parental HOS cells were seeded in 6-well plates at 2×10^6 cells/well in 2-ml complete medium. After cell attachment, they were washed two times with warm PBS, then treated with freshly prepared dicumarol (dissolved in 0.4 N NaOH) at the final concentrations of 200, 400, or 600 μM, which had no effects on the pH of the media. The same amount of NaOH was added to untreated cells as a vehicle control. After 4-h treatment at 37 °C, cells were washed once with cold PBS, scraped into 1 ml PBS, and short-term cell viability was determined by the trypan blue exclusion assay. Cell suspensions were centrifuged, RIPA lysis buffer was used to extract proteins from cell pellets, and p53 protein levels were detected by Western blotting.

Statistical analysis

To assure reproducibility, the gene array experiments were performed independently twice. All other graphed data represent the means of three independent experiments. The experimental differences were determined by two-tailed Student's t test, with $p \leq 0.05$ taken as a significant difference in all cases using SPSS.

Results

Morphological differences between parental HOS cells and As-8w-HOS, and the effects of CAPE

After 8-week exposure of HOS cells to arsenite and/or CAPE, all four treatment groups were grown in α-MEM with 5 % FBS for more than 2 weeks. Viewing the cells under the phase contrast microscope, it became evident that the control HOS cell populations consist of mixed fibroblast- and epithelial-like cells, with some cells containing multiple-nuclei. These control cells exhibited flatter morphology and lower saturation density when compared to As-8w-HOS cells. As-8w-HOS cells lost the polarity characteristic of control HOS cells, and also exhibited fibroblast morphology with loss of contact growth inhibition and more compacted growth. After 4 days growth, the number of harvested As-8w-HOS cells was 1.6 times higher than that of control HOS cells (not shown).

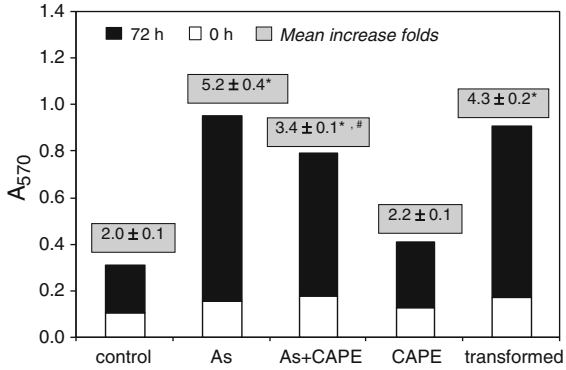

Fig. 1 Cell proliferation. Cells derived from the four 8-week exposure HOS cell groups (untreated control, As alone, As + CAPE, CAPE alone) were analyzed for cell growth using a 72-h MTT assay, as described in "Materials and methods". The experiments were performed twice, cells from each group in seven wells each. Data derived from all wells were used for statistical evaluation ($n = 14$). * Indicates significant ($p < 0.01$) differences versus control; # indicates significant ($p < 0.01$) difference versus As-treated group

Inhibition of arsenite-induced proliferation by CAPE

As shown in Fig. 1, As-8w-HOS (As-treated) and AsT-HOS (As-transformed) cells exhibit significantly higher proliferation potential than the parental HOS cells ($p < 0.01$). Incubation of HOS cells with CAPE did not change their proliferation capacity when compared with passage-matched HOS cells. However, when HOS cells were concomitantly treated with arsenite and CAPE, the proliferation was significantly ($p < 0.05$) lower than that of HOS cells treated with arsenite alone, which indicates that CAPE possesses the ability to suppress arsenite-mediated increases in HOS cells proliferation.

sod1, pig3, ferritin, and p53 gene and protein expression in control HOS and As-8w-HOS cells

RNA samples isolated from the parental HOS and As-8w-HOS cells were analyzed for gene expression using the human signal transduction Pathway Finder Q series array and Stress and Toxicity gene G series array (both from SuperArray) for *pig3, sod1, and p53*, and by RT-PCR for ferritin. Figure 2a, b, c shows that mRNA levels of *p53*, *sod1*, and *pig3* in As-8w-HOS cells are significantly lower than those in the matched control HOS cells. Similarly, RT-PCR results showed (Fig. 2c) that both H and L chains of ferritin mRNA, especially the heavy chain, are decreased in As-8w-HOS cells (lane 3) in comparison to control HOS cells (lane 2) (90 and 50 % decrease, respectively). In parental HOS cells, the expression of H chain was higher than that of L chain, whereas, in the As-8w-HOS cells, the relationship was reversed due to the dramatically decreased H chain levels (Fig. 2c). Thus, As not only modulated ferritin's gene expression, but it also affected the composition of its subunits. ELISA and Western blotting showed that, similar to the gene expression results, protein levels of ferritin, SOD1 and p53 also were decreased in AsT-HOS and As-8w-HOS cells in comparison to the control HOS cells ($p < 0.05$, Figs. 3, 4).

Figure 4 illustrates differences in the distribution of ferritin between cytoplasm and nucleus among the four different groups of treated HOS cells. Relative amounts of ferritin in cytoplasm are much higher than those in nuclei, which suggests that cytoplasmic ferritin is more vulnerable to chronic As exposure than that in the nucleus. Notably, although ferritin levels were virtually abolished by arsenite in the cytoplasm of AsT-HOS and As-8w-HOS cells, there were still 50 % of the levels present in the nuclear fractions of control HOS cells in both AsT-HOS and As-exposed HOS cells.

CAPE counteracts arsenite-mediated decline in SOD1, p53, and ferritin protein levels

The concomitant exposure of HOS cells to As and CAPE ameliorated arsenite-induced decreases in p53 and ferritin levels, but it did not reverse the As-induced decline in SOD1 (Fig. 3). Treatment of HOS cells with CAPE alone did not significantly change the production of these three proteins (Figs. 3, 4). Although CAPE by itself did not show any effect on either cytoplamic or nuclear ferritin distribution, it prevented the As-induced decrease of nuclear ferritin to a much greater extent than that in cytoplasm, with its levels being around 80 % (nuclear) and 15 % (cytoplasmic) of HOS cells treated only with CAPE.

The relationships between cell proliferation and p53, ferritin or SOD1 expression

The control levels of proliferation and expression of p53, ferritin and SOD1 were assumed to be 1, while changes mediated by As and/or CAPE treatments were expressed as fold-change above or below these levels. Figure 5 shows that, in the differently treated groups, cell proliferation inversely correlates with p53, ferritin, and SOD1 protein levels.

Inhibition of NQO1 causes a decrease in p53 levels in HOS cells

Figure 6 shows that dicumarol, an NQO1 inhibitor, decreases p53 levels in the parental HOS cells. In comparison to the vehicle control, a 4-h treatment with 400 or 600 μM dicumarol caused 25 and 40 % decreases in p53, respectively. These results suggest that arsenite decreases p53 levels in HOS cells, at least in part, through inhibiting pig3, a NQO1 homolog.

Discussion

Although oxidative stress has been shown to be important in arsenic exposure mediated carcinogenesis

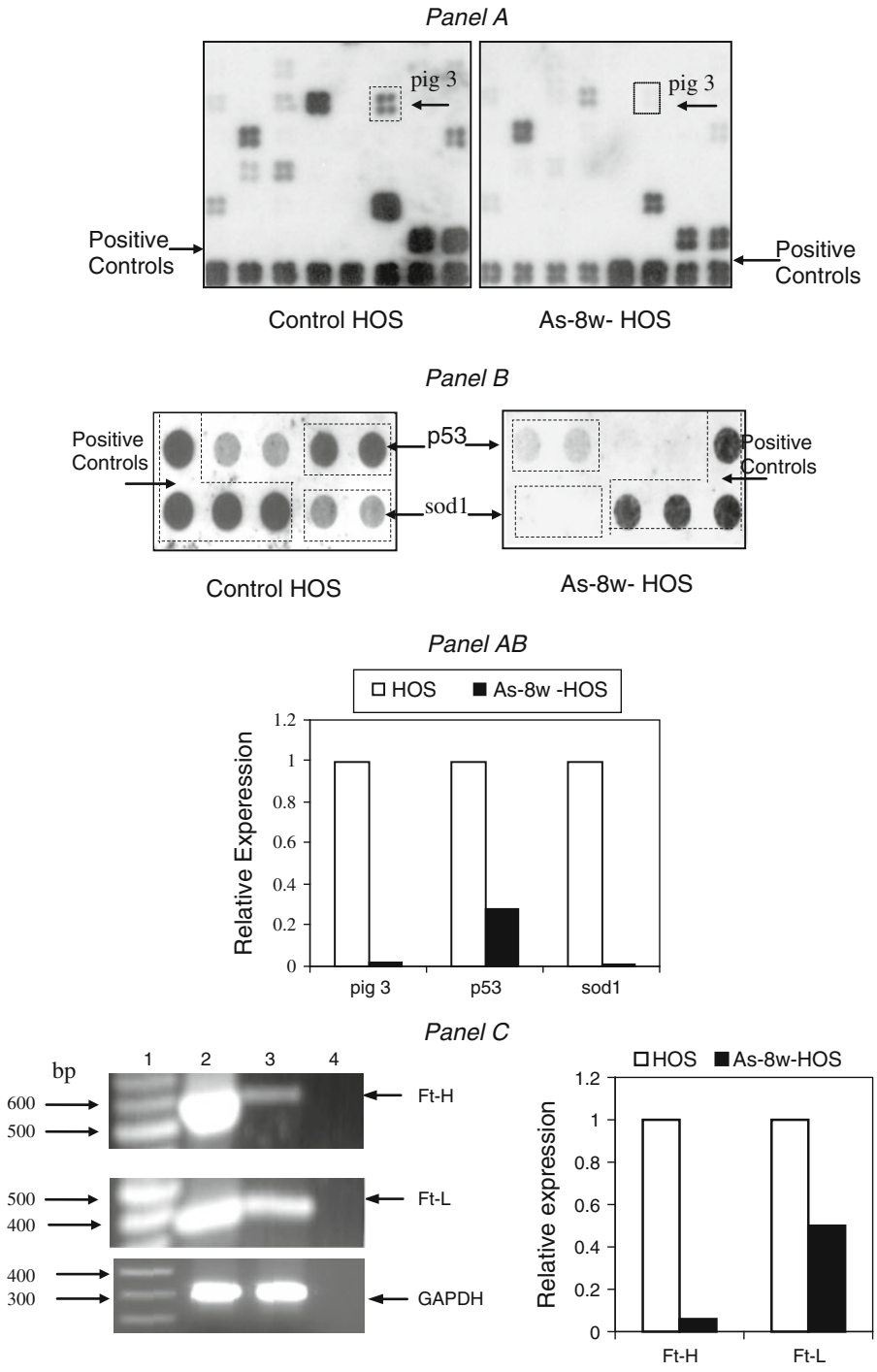

Fig. 2 Expression of p53, pig3, sod1, and ferritin genes in control HOS and As-8w-HOS cells. **a** mRNA expression of pig3 (NQO1-like) and p53 using the SuperArray Q gene array series, as described in "Materials and methods". **b** Illustrates the expression of sod1 and p53 using the SuperArray G series. **ab** Represents quantitation of the gene array signal densities normalized with housekeeping gene GAPDH. **c** Represents the analysis of RT-PCR products of ferritin H chain (Ft-H) and ferritin L chain (Ft-L). GAPDH was utilized as an internal control. Markers (*Lane 1*), Control HOS cells (*Lane 2*), As-8w-HOS cells (*Lane 3*). To control for the possibility of genomic DNA contamination, PCR was also performed using template samples that had not been reverse-transcribed (*Lane 4*). All assays were performed at least twice. Histograms (**c**) represent quantitative differences in As-treated cells when compared to array and PCR controls

(Chang et al. 2010; Galanis et al. 2009; Huang et al. 2004; Kitchin 2001), there remains a need for more firmly establishing the mechanisms of its involvement. Although HOS cells are tumor cells, they do not form large clones in soft agar, which is associated with a potential of tumor growth in animals. As-mediated growth of HOS cells in soft agar indicates additional genetic and/or epigenetic changes in cells that increase

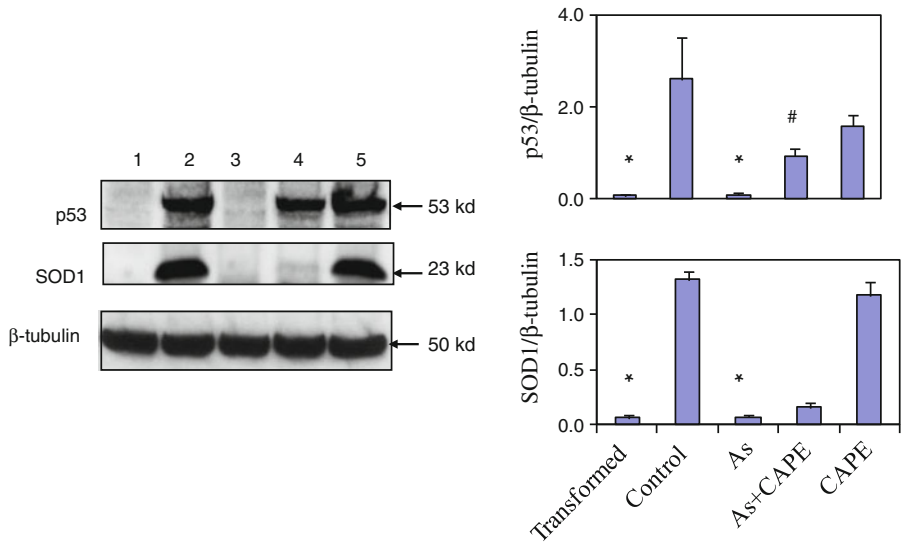

Fig. 3 Western blotting assessment of p53 and SOD1 levels in different exposure cell types. Control HOS cells were cultured and passaged continuously for 8 weeks in the absence of arsenite and CAPE (HOS controls) or in the presence of 0.1 μM arsenite ± 0.5 μM CAPE. To eliminate studying acute-phase effects of and to determine stable long-term changes induced by arsenic, AsT-HOS cells (*Lane 1*), control HOS cells (*lane 2*), 8-week As-treated HOS cells (*lane 3*), HOS cells exposed to As + CAPE (*lane 4*), and CAPE-treated HOS cells (*lane 5*) were cultured in As-free medium for 2 weeks post-treatment prior to the analysis. A representative blot is shown with similar results obtained in four separate experiments. The results were analyzed by the UN-SCAN-IT software version 4.1 (Silk Scientific Corp) and normalized to *β*-tubulin by calculating ratios of p53/*β*-tubulin and SOD1/*β*-tubulin. * Indicates a significant ($p < 0.05$) difference versus control HOS cells, while #indicates a significant difference ($p < 0.05$) vs. As-8w-HOS cells (As)

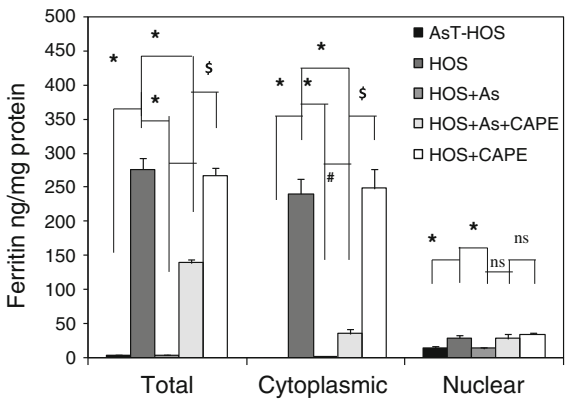

Fig. 4 ELISA detection of ferritin. Control HOS cells were cultured and passaged continuously for 8 weeks in the absence of As and CAPE (controls) or in the presence of 0.1 μM arsenite ± 0.5 μM CAPE. Total cell lysate, cytoplasmic and nuclear protein fractions were used for ferritin quantitation by ELISA. The results were normalized to protein utilized for ELISA. * Indicates a significant ($p < 0.05$) difference versus control HOS cells, # $p < 0.05$ difference versus As-8w-HOS cells (As), $ $p < 0.05$ difference versus HOS + CAPE. ns Indicates a non-significant difference. Data shown are representative of four independent experiments

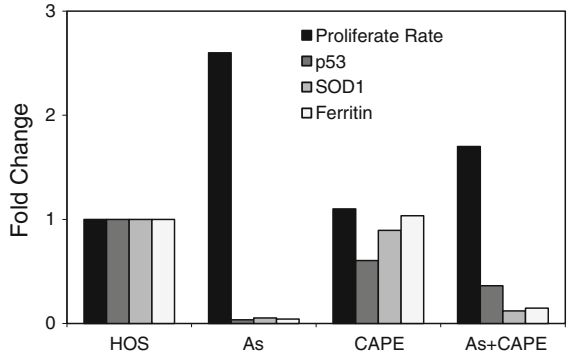

Fig. 5 Relationships between proliferation rates and p53, SOD1, or ferritin levels. A relationship between the proliferation capacity (Fig. 2), and p53 (Fig. 4), SOD1 (Fig. 4), or ferritin (Fig. 5) was determined. The expression of the above endpoint in control HOS is set as 1, while the effects of the different treatments are expressed as a fold-change

their malignant potential. Our previous results showed that chronic exposures to low As doses induce malignant HOS cell transformation. Concomitantly, increases in bioavailable cellular iron and decreases in both inflammatory cytokines and antioxidant enzymes

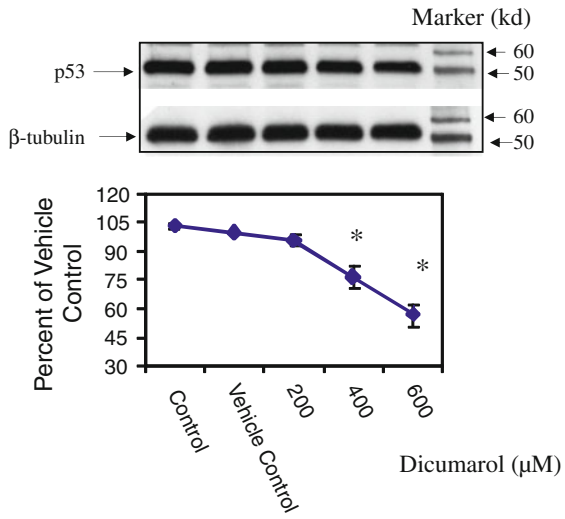

Fig. 6 Inhibition of p53 expression in HOS cells by dicumarol. As described in "Materials and methods", parental HOS cells were exposed to 200, 400, or 600 μM dicumarol and extracted proteins analyzed for p53 by Western blotting. The trypan blue exclusion assay showed that the cell viability was above 95 % in all treatment groups. * Indicates a significant ($p < 0.05$) difference versus vehicle-control group. The results are from three independents experiments

levels were observed, thus, indicating that these factors could be involved in the cell transformation process (Wu et al. 2006; Yang et al. 2005).

Iron is usually thought to be a pro-oxidant hazard because of its participation in the Haber–Weiss reaction (Koppenol 2001). Ferritin, consisting of 24 subunits, is the major intracellular iron storage protein in most types of cells. It is considered an important anti-oxidant cytoprotectant (Erdmann et al. 2005). Its subunits consist of both the light (L) and the heavy (H) chain types with molecular weight of 19 and 21 kD, respectively. The ferroxidase activity of the H chain allows ferritin to store iron and, thus, protect cells from cyclical reduction/oxidation reactions that tend to propagate and amplify oxidative damage (Levi et al. 1988). Our data show that As-8w-HOS and AsT-HOS cells have reduced ability to produce ferritin, which was evident at the transcriptional level because of the decreased expression of both H- and L-chain mRNAs (Fig. 2, panel C). The variability in ferritin subunit composition could affect the rates of iron uptake and release in different tissues (Proulx-Curry and Chasteen 1995) and, consequently, could further increase the content of bioavailable iron within the cells. In fact, we previously showed that arsenite-treated HOS cells contain much higher "free" iron levels (bound to low molecular weight cellular chelators, such as citrate) than the parental HOS cells do (Wu et al. 2006). The As-induced decline in ferritin and concomitant increase in iron levels could create oxidative stress and consequent cellular vulnerability and/or DNA damage. It was previously reported that arsenic increases iron release from ferritin in vitro (Ahmad et al. 2000). Such iron release could occur if SOD1 expression is greatly decreased by arsenite and, consequently, superoxide anion radical levels would be increased and cause the reduction of Fe^{3+} to Fe^{2+} in the ferritin core, thus allowing the release of iron in the bioactive form. Another possibility or contributing factor is that during arsenite-induced cell transformation processes, arsenite induces oxidative stress by decreasing ferritin gene expression and/or directly attacking ferritin molecules. This could lead to a loss in iron-binding capacity and a more facile release of iron, which would further contribute to ROS production and oxidative damage. In most cell types, ferritin is located in cytoplasm, but it has also been observed in the nuclei of rat hepatocytes after a single subcutaneous dose instillation of iron-dextran (Smith et al. 1990), in normal chicken corneal epithelial, and human erythromyeloblastoid leukemia cell lines (Cai et al. 1997; Pountney et al. 1999), as well as in rodent neurons during the development and after hypoxic ischemic insult (Thompson et al. 2002). Here, we presented evidence that ferritin also is present in HOS cell nuclei and that its levels are affected by submicromolar arsenic exposure. It was reported that nuclear ferritin has the ability to protect DNA from certain types of damage, such as that induced by UV(Cai et al. 1998), but the exact mechanism by which nuclear ferritin damage is involved in arsenic's carcinogenesis needs to be further investigated (Wu et al. 2006).

In addition to the effects on ferritin and iron levels, 0.1 μM arsenite transformed HOS cells also exhibit a decline in the expression of various other genes relevant to oxidative stress (Yang et al. 2005), including the antioxidant enzymes pig3 (NQO1 homolog) and SOD1 (Fig. 2). The alterations in *sod1* gene expression were paralleled by similar changes in protein levels. SOD and NQO1 are enzymes known to combat oxidative stress.

Our data showing arsenite's effects on pig3, SOD1, and ferritin appear to contradict some other published results showing that arsenic elevates these anti-

oxidant proteins (Lee and Ho 1995; Lemarie et al. 2008; Pi et al. 2003). The difference between those studies and our results is likely due to important differences in arsenic doses used and experimental design. Our study here employed a long-term (8-week) chronic exposure to low submicromolar doses of arsenite (0.1 or 0.5 μM), while the other published studies often utilized short-term (12–16 h) treatments with 100-fold higher arsenic doses (12 μM) in different cell models. We have previously shown that short-term exposures to arsenite stimulate antioxidant defense genes while long-term exposures lead to their suppression (Wu et al. 2006). Our experiments here were designed to better mimic human chronic long-term, low-dose exposure to arsenic.

Under conditions of chronic oxidative stress induced by arsenite, anti-oxidant enzymes and proteins are consumed and, if not replaced, can lead to a gradual loss of cellular anti-oxidant defenses resulting in further ROS accumulation and ongoing oxidative damage to cellular macromolecules, including DNA. Our preliminary data (not shown) show that As-8w-HOS cells have higher 8-oxo-dG levels in their DNA than untreated HOS cells ($3.04/10^6$dG vs. $1.96/10^6$dG). However, in the arsenic-transformed epithelial cells model, it was found that ROS generation decreased, which was responsible for cell growth and colony formation by activation of NF-κB pathway (Chang et al. 2010).

Another important clue into the mechanisms of As-induced carcinogenic responses is our finding that tumor suppressor protein 53 (p53) is greatly decreased in As-8w-HOS and AsT-HOS cells. In human bronchial epithelial cells, the expression of p53 rendered the cells refractory to transformation by arsenite, whereas the counterpart p53 knockdown cells could be transformed by arsenite(Chang et al. 2010). Since p53 is the guardian of the genome, lowered p53 protein content and altered function could allow gene mutations to accumulate at a faster rate in organisms exposed to arsenite, eventually leading to a carcinogenic response. It was reported that 0.1 μM As decreases p53 protein levels by up-regulating the mdm2 pathway (Hamadeh et al. 1999). Our present data also indicate that decreased p53 is involved in As-induced cell transformation (Fig. 3), but not necessarily through mdm2 pathway, since there was no significant difference in mdm2 gene expression in our gene arrays under the same conditions that

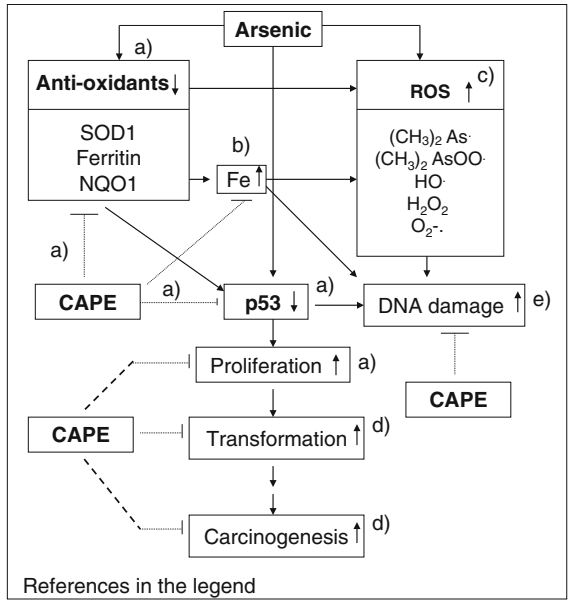

Fig. 7 Scheme of the oxidative damage pathway in arsenic-induced HOS cell transformation and effects of CAPE. According to the accumulated evidence, arsenic can be metabolized by methylation to $(CH_3)_2As\cdot$, $(CH_3)_2AsOO\cdot$, hydroxyl, and superoxide radicals under conditions of decreased anti-oxidant defenses that include SOD1, NQO1 and ferritin. The diminishing levels of ferritin cause free iron accumulation in cells, while decreased SOD1 allows for $O_2^{-\cdot}$ accumulation, which reduces both cellular iron and that stored in ferritin from Fe^{3+} to Fe^{2+}. Iron that is released from ferritin becomes bioavailable for redox activity and this bioavailable Fe catalyzes hydroxyl radicals' production (Higgy et al. 1997; Russo and Russo 2001). Free radicals are known to cause oxidative DNA damage as well as oxidation of other cellular macromolecules. Further, arsenic decreases p53 levels either directly or through a pathway sensitive to declining NQO1. A decrease in p53 protein content suppresses apoptosis and, at the same time, increases proliferation of cells with damaged DNA. Some of these As-induced processes (*solid line*) were shown here to be counteracted by CAPE (*dotted lines*). The results of this study are denoted in the scheme as (a), whereas other elements of this scheme have been previously published in citations (b) through (e) as follows: (b) reference (Asher et al. 2002a); (c) references (Chang et al. 2010; Galanis et al. 2009; Liu et al. 2001); (d) reference (Yang et al. 2005); (e) references (Kessel et al. 2002; Mure et al. 2003)

significantly down-regulated p53 (data not shown). Degradation of p53 can be mediated by ubiquitin-independent or ubiquitin-dependent pathways (Asher et al. 2002a, b); mdm2 belongs to the latter. The ubiquitin-independent pathway is regulated by NQO1, which can interact with and stabilize p53 (Anwar et al. 2003; Asher et al. 2001; Asher et al. 2002a, b). To prove the involvement of the NQO1 pathway in As-

induced p53 decline, HOS cells were treated with dicoumarol, a NQO1 inhibitor, which caused a dose-dependent decrease ($p < 0.05$) in p53 protein levels (Fig. 7). Based on these data, we concluded that, in our model, arsenite decreases p53 at least in part, via the NQO1 pathway. These results suggest that the observed decline in the antioxidant enzyme pig3, a NQO1 homolog, causes the decrease in p53 which, in turn, could lead to the proliferation of cells with damaged DNA and a decrease of apoptotic cell death.

The HOS cell model employed herein clearly shows that the impairment of anti-oxidant defenses and p53 regulation are both involved in the process of arsenite-induced cell transformation. This conclusion is strengthened by the finding that a concomitant treatment of HOS cells with As and CAPE, an anti-oxidant, suppresses As-induced HOS cells' growth in soft agar (Yang et al. 2005) and reduces their proliferation capacity (Fig. 1). Concomitantly-administered CAPE also prevented As-induced reduction in p53 and ferritin levels and allowed As-treated HOS cells to retain 70 % of the p53 and 15 % of ferritin levels present in the parental HOS treated only with CAPE (Fig. 3, panels a and b). These results, considered together with the negative correlation with cell proliferation potential, suggest that p53 serves as a pivotal link in As-induced malignant HOS cell transformation (Fig. 4), as determined by soft-agar growth of large clones.

The schematic in Fig. 7 illustrates a potential oxidative stress-mediated mechanism operative in arsenite-induced carcinogenesis, showing CAPE-mediated inhibition at various stages of the process. Inorganic arsenic is metabolized by methylation into pentavalent methylated species, such as dimethyl arsenic acid (DMA), which can be further metabolized into trivalent dimethylarsine. The latter compound can react with mono-molecular oxygen forming the $(CH_3)_2As^{\bullet}$ and superoxide anion radicals. $(CH_3)_2As^{\bullet}$ radical can acquire another molecule of oxygen to form $(CH_3)_2AsOO^{\bullet}$ and hydroxyl radicals (Kitchin 2001). This illustrates As-induced conditions of long-term oxidative stress, with anti-oxidant defenses diminished, coupled with severe decreases in ferritin resulting in a greater accumulation of bioavailable iron, which facilitates hydroxyl radicals' production. Further, arsenite decreases p53 levels, at least in part due to As-mediated decline in NQO1 (pig3), allowing cells to continue replicating on a damaged DNA template, losing normally strict-regulation of the cell cycle. In conclusion, oxidative stress and higher cell proliferation capacity are strong forces in the carcinogenic process and appear to be operational, as shown here, in arsenite-induced human cell transformation.

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