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Reactive oxygen species regulate properties of transformation in UROtsa cells exposed to monomethylarsonous acid by modulating MAPK signaling

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Abstract

UROtsa cells exposed to 50 nM monomethylarsonous acid [MMA(III)] for 52 wk (MSC52) achieved hyperproliferation, anchorage independent growth, and enhanced tumorigenicity. MMA(III) has been shown to induce reactive oxygen species (ROS), which can lead to activation of signaling cascades causing stress-related proliferation of cells and even cellular transformation. Previous research established the acute activation of MAPK signaling cascade by ROS produced by MMA(III) as well as chronic up regulation of COX-2 and EGFR in MSC52 cells. To determine if ROS played a role in the chronic pathway perturbations by acting as secondary messengers, activation of Ras was determined in UROtsa cells [exposed to MMA(III) for 0–52 wk] and found to be increased through 52 wk most dramatically after 20 wk of exposure. Ras has been shown to cause an increase in $O_2^{\cdot-}$ and be activated by increases in $O_2^{\cdot-}$, making ROS important to study in the transformation process. COX-2 upregulation in MSC52 cells was confirmed by real time RT-PCR. By utilizing both antioxidants or specific COX inhibitors, it was shown that COX-2 upregulation was dependent on ROS, specifically, $O_2^{\cdot-}$. In addition, because previous research established the importance of MAPK activation in phenotypic changes associated with transformation in MSC52 cells, it was hypothesized that ROS play a role in maintaining phenotypic characteristics of the malignant transformation of MSC52 cells. Several studies have demonstrated that cancer cells have lowered superoxide dismutase (MnSOD) activity and protein levels. Increasing levels of MnSOD have been shown to suppress the malignant phenotype of cells. SOD was added to MSC52 cells resulting in slower proliferation rates (doubling time = 42 h vs 31 h). ROS scavengers of $\cdot OH$ also slowed proliferation rates of MSC52 cells. To further substantiate the importance of ROS in these properties of transformation in MSC52 cells, anchorage independent growth was assessed after the addition of antioxidants, both enzymatic and non-enzymatic. Scavengers of $\cdot OH$, and $O_2^{\cdot-}$ blocked the colony formation of MSC52 cells. These data support the role for the involvement of ROS in properties of transformation of UROtsa cells exposed to MMA(III).

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Keywords

Arsenic carcinogenesis; MMA(III); UROtsa cells

Introduction

Although high levels of arsenic have long been associated with an increased risk of cancer, the mechanisms of induction of carcinogenesis remain unclear. Arsenic is an atypical carcinogen because it is classified as neither an initiator nor promoter under the categories of carcinogenic agents (Huang et al., 2004). A large problem in determining the mechanism of induction of bladder carcinogenesis is the fact that there are limited animal models to utilize. Animal models for arsenical-induced cancer have been developed, but unfortunately, none specific to bladder cancer with As(III) or MMA(III) exposure alone. There is one model of DMA(V)- induced bladder cancer in rats, but these studies were performed at high concentrations (100 ppm) and 2 years of exposure (Cohen et al., 2007; IARC 2004; EPA-SAB 2007).

To study the induction of arsenical-induced carcinogenesis in the human bladder, an immortalized, non-tumorigenic human urothelial cell line, UROtsa, has been established as an *in vitro* model to study the molecular mechanisms behind arsenical-induced carcinogenicity of the bladder, a primary target of arsenicals (Sens et al., 2004).

Following exposure to either 1 μ M As(III) or 50 nM MMA(III) for 52 weeks, UROtsa cells gained the phenotypic characteristics of hyperproliferation, colony growth in soft agar, and tumors when heterotransplanted into nude mice (URO-ASSC cells and MSC52 cells) (Sens et al., 2004; Bredfeldt et al., 2006). These cells were used as a model to investigate the mechanism behind the transformation. MSC [12, 24, 52 wk exposures to 50 nM MMA(III)] cells, showed permanent alterations in MAPK signaling. Both cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR or ERBB1) expression increased in a time-dependent fashion. These changes in expression correlate with phenotypic alterations and the development of malignancy. Elevated ERBB2 and COX-2 were seen after acute exposure to MMA(III), suggesting that the short-term perturbations noted in this pathway can lead to long-term changes after chronic exposure to MMA(III) (Figure 1) (Eblin et al., 2007).

Although the generation of oxidative stress is not widely accepted as a significant contributor to the mode of action of all arsenicals, previous research has established the importance of reactive oxygen species (ROS) in the increased MAPK signaling, specifically the upregulation of COX-2, after short-term exposure to arsenicals (Figure 1) (Jung et al., 2003; Drobna et al., 2003; Benbrahim-Tallaa et al., 2005; Cooper et al., 2007; Ramos et al., 2006; Eblin et al., 2008). In addition, low-level MMA(III) exposure has been linked to the generation of ROS (Nesnow et al., 2002; Eblin et al., 2006; Wang et al., 2007). ROS are regarded as having carcinogenic potential, so it is plausible that the increased ROS seen after acute arsenical exposure can lead to the long-term perturbations seen in the MAPK signaling after chronic MMA(III) exposure.

ROS are associated with multiple cellular functions, in particular for these studies, cellular proliferation. In addition, MAPK upregulation seen in MSC52 cells is linked with increases in cellular proliferation. Several studies suggest that increased ROS are involved in carcinogenesis: a) some growth factors such as EGF, have been shown to increase ROS production in cells for regulating cell migration and proliferation; b) the use of natural antioxidants can inhibit cancer cell proliferation and tumor growth; and c) from both the literature and previous studies in this laboratory, ROS induce MAPK, NF- κ B, and AP-1 which are all associated with cancer development (Xia et al., 2007; Eblin et al., 2007). A plausible

role for ROS which leads to the development of MMA(III) related cancers would be in the form of increased cellular signaling due to the ROS acting as secondary messengers in the MAPK signaling cascade. This increased signaling could trigger an increase in stress-related proliferation driving rapid growth and genomic instability as arsenicals have been shown to induce intracellular proliferative signals and also to over-ride cell cycle checkpoints (Rossman, 2003).

Some long term alterations seen in human bladder cancer in the MAPK pathway include the activation of Ras and increases in COX-2 protein, making them important proteins to study in terms of MMA(III) induced hyperproliferation following long-term exposure (Simeonova et al., 2002; Luster et al., 2004; Eblin et al., 2007). Various forms of Ras have been shown to cause an increase in $O_2^{\cdot-}$ and be activated by increases in $O_2^{\cdot-}$, making ROS important to study in the transformation process (Kowluru et al., 2007; Lin et al., 2006; Heo et al., 2006; Heo et al., 2005; Seru et al., 2004). In addition, H-Ras has been directly linked with increased proliferation of cells. In a neuroblastoma cell line stably transfected with H-Ras, Seru and colleagues (2004) detected an increase in $O_2^{\cdot-}$, which could be ameliorated by the addition of 4-(2-aminoethyl) benzenesulfonyl fluoride, a specific inhibitor of the membrane superoxide generating system NADPH oxidase. These effects depended on the MAPK/ERK1/2 pathway, as the specific MEK inhibitor, PD98059, prevented H-Ras-mediated increase in ROS and specifically $O_2^{\cdot-}$. COX-2 protein was shown to be upregulated in the MC52 cells, but the link between the upregulation and increased ROS has not been investigated (Eblin et al., 2007).

Results of many studies indicate that $O_2^{\cdot-}$ is the primary ROS produced by As(III), and its formation leads to the production of H_2O_2 , 1O_2 , and $\cdot OH$ and that SOD mimetics have been shown to be effective against ROS-mediated diseases, such as inflammation, ischemia-reperfusion injury, cancer, and the aging process (Buetler et al., 2004; Shi et al., 2004). Several studies have demonstrated that cancer cells have lowered MnSOD activity when compared to their normal counterparts, independent of the underlying cause (Buetler et al., 2004). Studies suggest that increased levels of MnSOD protein suppress the malignant phenotype of cells, as shown by decreased growth rate, lower colony formation in soft agar, and less tumor formation in nude mice as compared to parental non-malignant cells (Yang et al., 2002).

This study examines the role of ROS on cell signaling in the MAPK pathway, hyperproliferation and anchorage independent growth, two phenotypic hallmarks of tumorigenesis, in UROtsa cells chronically exposed to 50 nM MMA(III). It is hypothesized that ROS play a role as a signaling molecule in the MAPK cascade, activating both Ras and COX-2 proteins, leading to constitutive activation in MMA(III) exposed cells, resulting in increased proliferation and anchorage independent growth. As MMA(III) has been shown to increase the formation of ROS in UROtsa cells after acute exposures, it was important to investigate the role of ROS in the transformation process and subsequent altered growth patterns of chronically exposed UROtsa cells.

Methods

Chemicals

Sodium arsenite, protease inhibitor cocktail, peg-superoxide dismutase, peg-catalase, and potassium iodide were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic, and 1X trypsin-EDTA (0.25%) were acquired from Gibco Invitrogen Corporation (Carlsbad, CA). Diiodomethylarsine (MMA(III) iodide, CH_3AsI_2) was prepared by the Synthetic Chemistry Facility Core (Southwest Environmental Health Sciences Center, Tucson, AZ) using the method of Millar et al., (1960). Water used in studies was distilled and de-ionized. Ras Assay Reagent consisting of Raf-1 RBD and agarose, Anti-Ras clone RAS10, Mg^{2+} Lysis/Wash

Buffer, GTP γ S (10 mM), and GDP (100 mM) were all purchased as part of the Ras Activation Assay Kit (Upstate, Temecula, CA).

Cells

UROtsa cells and URO-ASSC cells were a generous gift from Drs. Donald and Maryann Sens (University of North Dakota). URO-MSC cells were created in our laboratory according to Bredfeldt et al., (2006). Cell culture conditions were derived from those previously described by Bredfeldt et al., (2004).

Intracellular O₂^{•-} staining

UROtsa or MSC52 cells were seeded at 1×10^6 cells per bioptechs dish overnight in DMEM plus 10% FBS media. The cells were then stained with 5 μ M di-hydroethidium (DHE) (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 min at 37°C, then washed with 1x PBS. Images were captured with an upright Zeiss LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) with a 40x “dipping” lens. DHE was excited at 480 nm and emission was collected with a 567 nm long pass filter.

Ras activity assay

Ras activity was determined using a Ras Activation Assay Kit (Upstate, Temecula, CA) according to package instructions. Briefly, confluent URO-MSC cells were lysed in 0.5 ml of the diluted Mg²⁺ Lysis/Wash Buffer containing protease inhibitor cocktail (Sigma, St. Louis, MO). Genomic DNA was sheared and centrifuged (5 min, $14,000 \times g$, 4°C). Supernatant fractions were snap frozen in liquid nitrogen and stored at -80°C for future use. Protein concentration (BCA assay) was adjusted so that each 0.5 ml aliquot contained 1 mg/ml protein. Treatment samples and the positive and negative control samples were then generated following manufacturer’s protocol. Samples were separated via SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Piscataway, NJ). Immunoblotting for activated Ras was achieved with anti-Ras, clone RAS10, and an HRP conjugated goat anti-mouse polyclonal antibody (BD Biosciences, San Diego, CA). Densitometry was performed using Quantity One (Biorad, Hercules, CA).

Isolation of Nucleic Acids

UROtsa cells were plated 6×10^5 cells per well in 6-well plates and grown according to Eblin et al., (2008) in serum-containing media. Nucleic acids were isolated as previously described (Oshiro et al., 2005). Total RNA was isolated from all cells using the RNeasy Mini kit (Qiagen, Valencia, CA). All samples were quantified using absorbance at 260 nm on the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Real-Time RT-PCR

Total RNA (250 ng) was converted to cDNA (Applied Biosystems, Foster City, CA). Converted cDNA was added to Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific Taqman Primer/Probe (Applied Biosystems, Foster City, CA) and was then subjected to Real-Time PCR analysis using the ABI 7500 Real-Time detection system (Applied Biosystems, Foster City, CA). Results were calculated using the Delta Ct method normalizing to GAPDH expression for each sample. ABI Assay ID number are available upon request.

Effect of anti-oxidants on URO-MSC52 cell increased COX-2 protein

Cells were treated with anti-oxidants, KI (5 mM), or SOD (100 units/ml) or with COX inhibitors NS398 (10 μ M) or indomethacin (10 μ M) every 2 days. Cells were subcultured every 4 days. After 14 days of incubation, protein was isolated and western blot analysis performed.

Western Blot analysis

Western blot analysis was adapted from Eblin et al., (2006b). Confluent URO-MSC cells were scraped into RIPA (Radio-Immunoprecipitation Assay) Buffer with protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined by the BCA assay (Sigma, St. Louis, MO). Thirty micrograms of each sample was separated via SDS-PAGE with Mini-Protean II (BioRad, Hercules, CA) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Inc/GE Healthcare, Piscataway, NJ). Immunoblotting for proteins of interest (SOD1, SOD2, Catalase, COX-2, and GAPDH) was achieved with monoclonal, HRP-conjugated secondary antibodies (Bioscience Resource Project, Saco, ME; Cayman Chemical, Ann Arbor, MI; Calbiochem, La Jolla, CA) and fresh enhanced chemiluminescent (ECL) solution (Pierce, Rockford, IL). Data shown representative of (N=3) experiments

Cell growth kinetics following exposure to anti-oxidants

Growth kinetics were adapted from Bredfeldt et al., (2006). Growth curves for UROtsa and URO-MSC52 after 24 h, 48 h, and 96 h of exposure to SOD, catalase, or KI were obtained via trypan blue exclusion assay. Cells were plated in 6-well plates at a density of 2×10^5 cells per well. Cells were removed from the plates via trypsin and counted. Growth curves were generated based on increases in cell population per 24 h periods for a total time of 96 h. These growth curves were then used to calculate doubling time (N=3).

ATP viability assay

Cellular ATP levels in UROtsa or MSC52 cells following anti-oxidant exposure were determined using an ATP Cell Viability Assay Kit (BioVision Research, Mountain View, CA) according to company protocol. UROtsa or MSC52 cells were seeded at 1×10^6 cells per well in 6-well plates (Falcon) and grown according to Eblin et al., (2008) in serum-containing media. To test the viability of cells exposed to cellular antioxidants, ATP levels were determined for UROtsa and MSC52 cells after 24 h, 48 h, and 96 h of exposure to SOD (100 units/ml), catalase (200 units/ml), or KI (5 mM). Levels of ATP were analyzed using a curvet-based TD-20/20 Luminometer (Turner Biosystems, Sunnyvale, CA).

Caspase-3 activity assay

Activation of caspase-3 was assessed in UROtsa or MSC52 cells following anti-oxidant exposure through utilization of a caspase-3 fluorescence assay kit (Cayman Chemical, Ann Arbor, MI) according to protocol. Cells were seeded in a 96-well plate at 5×10^4 cells/well in 100 μ l of culture medium. To analyze the induction of apoptosis in cells exposed to cellular antioxidants, caspase-3 levels were determined for UROtsa and MSC52 cells after 24 h, 48 h, and 96 h exposures to SOD (100 units/ml), catalase (200 units/ml), or KI (5 mM). Fluorescent intensity was analyzed using a fluorescent 96-well plate reader (Molecular Devices, Sunnyvale, CA) at an excitation of 485 nm and an emission of 535 nm.

Effect of anti-oxidants and inhibitors of ROS generation on MSC52 cell anchorage-independent growth

Protocol adapted from Eblin et al., (2007). Anchorage-independent growth was detected by colony formation in soft agar. For colony formation in soft agar, cells were removed from culture flask with trypsin and suspended in culture medium supplemented with 0.3% agar. The agar

enriched with cells was overlaid onto 0.6% agar medium in a 24-well plate with a density of 1×10^4 cells per well. Cells were treated with anti-oxidants, KI (5 μ M), SOD (100 units/ml), or catalase (200 units/ml), or inhibitors manumycin A, *N*-vanillylnonanamide, or allopurinol every 2 days. After 14 days of incubation, colonies were manually counted with an Olympus CK2 microscope (Olympus America, Inc. Melville, NY). Data represents colonies formed in single plane of agar.

Statistics

Graphs were generated in Microsoft Office Excel (Microsoft Corp., Redmond, WA). Data from the trypan blue proliferation assay, densitometry, and real-time RT-PCR are expressed as the average of three experiments. These data are represented as the mean $\pm$ SEM. Densitometry was performed using Quantity One Analysis (Bio Rad, Hercules, CA). Statistical significance was determined using the Student t-test for comparison of samples and statistical significance was marked by either an asterisk (*) or a cross ($\dagger$). Statistical significance was determined if $p < 0.05$.

Results

MSC52 cells have higher inherent levels of $O_2^{\cdot-}$

After transformation of UROtsa with MMA(III), MSC52 cells are no longer exposed to MMA(III). Previous studies have established that cancer cells have increased endogenous ROS, so it is necessary to determine if endogenous ROS production has been increased in MSC52 cells, and if so, how that increase affects MAPK signaling, as they have been shown to be linked in acute studies (Xia et al., 2007). Similar to data in the literature, MSC52 cells have increased endogenous ROS production when compared to normal non-transformed UROtsa, suggesting that the some cellular processes are altered to cause the increased ROS (Figure 2).

Ras Activation following chronic exposure of UROtsa to 50 nM MMA(III)

Increases in Ras protein activation have been linked to increased ROS production (Yang et al., 2002), so Ras activation was investigated throughout the time course of 50 nM MMA(III) treatment (Figure 3). Ras protein increased throughout the transformation, with significant increases between 16–28 wk and peak activity occurring at 20 wk. The activation and subsequent decrease of Ras protein is indicative of cellular changes associated with carcinogenesis, specifically occurring when cancer changes from a non-invasive to a more aggressive phenotype (Zhang et al., 2001). The peak in Ras protein activation occurs just before the MMA(III)-exposed UROtsa acquire the phenotypic property of anchorage independent growth. Specifically of interest to the rest of the study is that $O_2^{\cdot-}$ has been shown to be increased following Ras activation (Kowluru et al., 2007; Seru et al., 2004). In addition, Shi et al., (2004) showed that arsenical exposure could also increase $O_2^{\cdot-}$.

COX-2 induction in transformed UROtsa

In further support of the importance of MAPK signaling in the MSC cells when coupled with the activation of Ras, steady state COX-2 mRNA was shown to be increased in MSC cells (Figure 4a). These data are in accordance with previous studies demonstrating COX-2 protein induction following MMA(III) exposure in MSC-52 cells (Eblin et al., 2007). Interestingly, URO-ASSC cells, UROtsa cells transformed with 1 μ M As(III) for 52 wk, had a statistically significant decrease in COX-2 protein suggesting that MAPK signaling plays a more important role following MMA(III) exposure in UROtsa cells. Due to this difference between URO-ASSC and MSC52 and the activation of MAPK signaling, the remainder of the study focused solely on MSC52 cells and their altered properties.

COX-2 induction can be decreased by ROS scavengers

To determine if there was a link between the changes seen in MAPK signaling after chronic exposure to increased ROS, the antioxidants SOD and KI were used to decrease ROS for a period of two wk in MSC52 cells. These antioxidants were investigated following acute exposures to both As(III) and MMA(III) and were shown to decrease the formation of ROS within UROtsa cells as visualized by confocal microscopy (Eblin et al., 2008). In addition, the COX-1 inhibitor, indomethacin, and the COX-2 inhibitor NS398 were used for two wk as controls for the decrease in COX-2 protein. After the incubation of MSC52 cells for two wk in the presence of these compounds, a clear decrease in COX-2 protein can be seen following NS398 and SOD treatment (Figure 4b). A decrease was also seen following KI treatment, similar to the decrease seen with indomethacin, the COX-1 inhibitor, suggesting that $\text{OH}^\cdot$ is not necessarily as important in COX-2 induction as $\text{O}_2^{\cdot-}$. These data suggest that ROS-induced MAPK signaling causes an upregulation of the MAPK signaling cascade that leads to the induction of COX-2 protein, which can result in the increased proliferation and anchorage independent growth of MSC52 cells. As previous work has shown that anchorage independent growth was dependent on COX-2, it was next important to investigate the importance of ROS on this phenotype (Eblin et al., 2007).

ROS scavengers slow proliferation rates of MSC52 cells

As increased ROS occurs after 50 nM MMA(III) exposure and Ras, a protein important in cellular proliferation pathways, is activated during the transformation process of UROtsa cells chronically exposed to 50 nM MMA(III), it was important to investigate the effect of the inhibition of ROS on the increased proliferation rates of the transformed MSC52 cells (Figure 5A,B). The transformed cells were exposed to peg-catalase, peg-SOD, and potassium iodide (KI), a scavenger of $\text{OH}^\cdot$ and proliferation rates were assessed. ROS inhibitors of $\text{OH}^\cdot$ and $\text{O}_2^{\cdot-}$, KI and SOD respectively, significantly slowed the proliferation rates of the MSC52 cells which supports a role of ROS in the increased proliferation rates.

ROS inhibitors do not lead to increased apoptosis or cell death

To ensure that the slowed proliferation rates were due strictly to effects caused by the antioxidant enzymes used, both cell viability and apoptosis were measured in MSC52 cells exposed to CAT, SOD, or KI for up to 96 h (Figure 5 C & D). There was no significant increase in apoptosis, as measured by Caspase-3 activation, seen over the 96 h exposure period to the antioxidants confirming the decrease in cell growth and subsequent hyperproliferation of the MSC52 cells, in the absence of cell death. In addition, there was no decrease in ATP viability when comparing treated cells with control MSC52 cells, further indicating the absence of cell death and validating the decrease in cell proliferation. The addition of catalase to MSC52 cells significantly increased growth after 24 h. The MSC52 cells with catalase maintained higher amounts of ATP than passage matched MSC52 cells without catalase throughout the course of the experiment (Figure 5D).

ROS inhibitors decrease anchorage independent growth of transformed UROtsa

ROS inhibitors, KI and SOD, both decreased the anchorage independent growth associated with UROtsa cell transformation after MMA(III) exposure (Figure 6). MSC52 cells showed a diminished colony formation in soft agar after a two-wk growth period. These data show that colony formation in soft agar following MMA(III) exposure is partially due to a downstream effect of ROS generation and establish the importance of ROS in the phenotypic alterations of MSC52 cells.

Anchorage independent growth of UROtsa is dependent on ROS generation by NADPH oxidase, MAPK signaling, and mitochondrial oxidase

Because increases in ROS are likely responsible for COX-2 upregulation and phenotypic alterations in MSC52 cells, it was necessary to determine where this ROS was being generated in the cells as these cells are no longer exposed to MMA(III). The changes being measured are due to permanent biological alterations in the cells and not the presence of the chemical species, MMA(III). A variety of compounds were selected which inhibited various properties in the cells related to ROS generation: allopurinol, an NADPH oxidase inhibitor, manumycin A, which inhibits ras activation, and *N*-vanillylnonanamide, an inhibitor of xanthine oxidase. Non-cytotoxic concentrations were determined in UROtsa and MSC52 cells (data not shown), and the cells were treated with the compounds for a period of two weeks and their growth in soft agar was evaluated. The compounds that blocked growth in soft agar were manumycin A, and allopurinol, suggesting that these are the complexes or proteins responsible for the increase in ROS generation (Figure 7).

Discussion

Chronic exposure of UROtsa cells to 50 nM MMA(III) results in both anchorage independent growth and hyperproliferation, phenotypes associated with malignant transformation. Previous investigations ascertained that acute exposure to MMA(III) resulted in increased ROS as evidenced by DCFDA fluorescence, as well as altered signaling resulting from this generation of ROS (Nesnow et al., 2002; Eblin et al., 2006; Wang et al., 2007; Drobna et al., 2005; Eblin et al., 2007). It is therefore important to study if these acute changes result in permanent alterations after chronic exposure to low-level MMA(III) (Figure 1). Previous research has also established the importance of the MAPK pathway in the anchorage independent growth of MSC52 cells, as well as chronic upregulation of both EGFR and COX-2 through the transformation process of UROtsa into MSC52 cells (Eblin et al., 2006).

The current study establishes the importance of ROS generation by low-level MMA(III) in the upregulated MAPK signaling seen following transformation, and the subsequent hyperproliferation and anchorage independent growth of MSC52 cells. To investigate the importance of ROS in MMA(III)-associated transformation, it was important to determine if there was increased endogenous ROS in the MSC52 cells without the presence of MMA(III). It was shown that there were increased ROS, supporting the necessity to look at processes in the cells that can result in this secondary ROS generation related to signaling alterations previously characterized. The MAPK pathway has been shown to be constitutively activated by MMA(III), so it was important to examine chronic changes in this pathway, such as Ras activation. The Ras protein acts through a positive feedback mechanism whereby it not only produces ROS, but is also activated as a result of ROS (Seru et al., 2004). Activated Ras protein was significantly increased as soon as 8 wk following chronic exposure to 50 nM MMA(III). This increase remained statistical from 16–28 wk, with a large peak at 20 wk of exposure (Figure 3). The activation of Ras is not only important in terms of ROS secondary generation, but is also important in terms of the transformation of cells. Overexpression of various forms of Ras, in particular, H- or V-ras occurs in more than 50% of human urothelial carcinomas, and is thought to result from upstream receptor tyrosine kinase activation (Zhang et al., 2001). In this study, only Ras activation was studied, not specific upregulation. Ras activation is important in the non-invasive pathway of urothelial tumorigenesis or the initial transition of normal cells to metastatic cells. This increased activation of Ras occurring at approximately 20 wk, followed by the MSC cells gaining characteristics of a carcinogenic phenotype further supports this idea. A Ras inhibitor was used to treat the MSC52 cells and was shown to inhibit anchorage independent growth, suggesting that Ras and the MAPK pathway are important modulators of MSC52 cells anchorage independent growth.

Following the determination that Ras protein is activated by chronic exposure to low-level MMA(III), it was important to determine what role ROS played in the upregulation of the MAPK signaling pathway and the subsequent hyperproliferation of MSC52 cells. Previous research has linked the induction of COX-2 with ROS generation after short-term exposure to 50 nM MMA(III). The MAPK pathway was shown to be induced throughout the transformation process with both EGFR and COX-2 mRNA and protein showing the highest increase after 52 wk exposure to 50 nM MMA(III) (Eblin et al., 2007; Figure 4). This pathway is important to study in terms of proliferation and anchorage independent growth as it has been linked to enhanced proliferation rates in cancer cells, as well as both proteins showing induction in human bladder tumors (Wadwa et al., 2005; Eschwege et al., 2003; Nguyen et al., 1994). An interesting finding was that Ras activation peaked before the gain in the phenotypic property of anchorage independent growth. COX-2, on the other hand, has been shown to be an important protein required for sustaining anchorage independent growth, therefore the fact that COX-2 peaks later may be necessary to continue the transformation process.

Support for the importance of ROS in the induction of COX-2 protein and the increased MAPK signaling seen in MSC52 cells comes from the experiments that show the increased COX-2 is dependent on the continual production of ROS. The ROS antagonist SOD decreased COX-2 protein in a manner similar to the COX-2 specific inhibitor, NS398 following treatment of the MSC52 cells for 2 wk (Figure 4). KI also decreased COX-2 protein, but in a manner similar to the COX-1 inhibitor, indomethacin. This suggests that MAPK signaling in MSC52 cells is more reliant on the generation of $O_2^{\cdot-}$ than $OH^{\cdot}$ as a signaling molecule. This is important as it establishes the importance of ROS in the induction of MAPK signaling seen in these cells.

As MAPK signaling was shown to be dependent on ROS and MAPK since inhibitors of these decreased anchorage independent growth (Eblin et al., 2007), it was important to determine if ROS inhibitors would effect the characteristics of hyperproliferation of MSC52 cells and also their ability to form colonies in soft agar. KI and SOD both slowed proliferation rates, causing the doubling time to slow to closer to that of the control UROtsa. The ROS antagonists caused a decrease in the colony formation of MSC52 cells in soft agar, which is an important indicator of cell tumorigenicity. Anchorage independent growth and hyperproliferation are important markers of carcinogenic potential so any compounds that can cause changes in these phenomena are important to investigate.

Because the MSC52 cells are no longer exposed to MMA(III), and there is still increased endogenous ROS, it was important to determine what cellular components could play a role in the maintenance of this increase. Using growth in soft agar as an endpoint, MSC52 cells were treated with various inhibitors of secondary ROS generation. Due to the fact that direct inhibition of ROS with antioxidants caused a decrease in growth in soft agar, and manumycin A and allopurinol also blocked growth in soft agar, it was determined that activated Ras and NADPH oxidase could play a role in producing secondary ROS. These data on the importance of the activation of Ras in anchorage independent growth support previous research that established the importance of MAPK signaling in the growth in soft agar as inhibition of COX-1, COX-2, PI3K, and src all led to decreased colony formation (Eblin et al., 2007). In addition, the dependence on NADPH oxidase supports the idea that a secondary system is generating the ROS produced by MMA(III), not the compound itself.

Chronic exposure to 50 nM MMA(III) results in altered phenotype of UROtsa cells that lead to increased proliferation of the cells and anchorage independent growth. In addition, chronically upregulated MAPK signaling can be detected in an increasing amount following 12, 24, and 52 wk of exposure to MMA(III). All of these changes can be decreased by the subsequent treatment of the MSC52 cells with the ROS antagonists, SOD and KI, which block $O_2^{\cdot-}$ and $OH^{\cdot}$, respectively. These data support the importance of ROS in the phenotypic

properties of UROtsa cells exposed to 50 nM MMA(III) for 52 wk. As malignant transformation of cells is not a reversible phenomenon, it is important to consider why ROS inhibitors are having these effects on MSC52 cells. We hypothesize that increased ROS are being used as signaling molecules in pathways related to proliferation and anchorage independent growth, such as MAPK. These data suggest that ROS are involved in the maintenance of phenotypic changes of MSC52 cells related to the upregulation of COX-2. Future work needs to address the relevance of these finding to in vivo research as recent studies in animals have shown that antioxidants cannot inhibit the effects of arsenic nor block carcinogenesis (Cohen et al., 2007; Waalkes et al., 2007). An important note though, is that the levels of arsenicals used in UROtsa cells in these studies are much lower than those used in the animal studies with antioxidants. Antioxidants were used in these studies, not to block arsenical induced carcinogenesis, but to determine the role ROS play in altered signaling. Future research with both arsenicals and anti-oxidants can clarify the role of antioxidants in blocking the transformation of UROtsa cells exposed long-term to arsenicals. Further research is important to perform as establishing the role of ROS in the generation of carcinogenesis following chronic MMA(III) exposure is critical to determine both markers of the transformation process as well as potential therapeutic opportunities.

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Figure 1.
Summary of changes seen in UROtsa cells following both acute and chronic treatment with 50 nM MMA(III) that are associated with increased ROS.

Figure 2.
DHE fluorescence detecting the presence of increased ROS in MSC52 cells. A) Normal UROtsa have minimal background fluorescence. B) MSC52 cells have increased $O_2^{\cdot-}$ when compared to control UROtsa. The images are representative of n=3.

Figure 3.

Effect of chronic 50 nM MMA(III) treatment on Ras activation in UROtsa cells. UROtsa cells were treated with 50 nM MMA(III) for 0–52 wk and immunoprecipitation performed for activated Ras (21 kDa). Densitometry analysis of western blots for activated-Ras shows time-dependent increase with exposure to MMA(III) until 5 mo and then a subsequent decrease in Ras. Data shown is average $\pm$ SEM ($n \geq 3$). GTP is the positive control. GDP is the negative control. (*) marks statistical significant increase when compared to GDP negative control ($P < 0.005$). These samples are taken from frozen cells that were isolated throughout the course of transformation with MMA(III).

Figure 4.

A) Quantitative real-time RT-PCR expression of COX-2 mRNA after treatment with 50 nM MMA(III) for 52 wk. COX-2 expression is relative to GAPDH and was normalized to control UROtsa. Data shown is average $\pm$ SEM ($n \geq 3$). Asterisks (*) denote statistically significant changes ($p < 0.05$) from control UROtsa. B) COX-2 protein expression in MSC52 cells treated for 2 wk with ROS antagonists or COX inhibitors. A decrease in COX-2 protein occurs following 2-wk treatment with either KI, SOD, or NS398. Shown is representative western blot of $n=3$.

Figure 5. Effect of ROS antagonists on proliferation rates of UROtsa cells exposed to MMA(III) for 52 wk

A) Normal untreated UROtsa cells have no change in proliferation following exposure to ROS antagonists. B) MSC52 cells have an increased doubling time following exposure to both KI and SOD when compared to untreated MSC52 cells. C) There is no significant increase in caspase-3 activity following addition of ROS antagonists to MSC52 cells when compared to untreated MSC52 cells.

D) There is no significant decrease in viability as measured through levels of ATP following the addition of ROS antagonists to MSC52 cells when compared to untreated MSC52 cells. Data shown is average $\pm$ SEM ($n \geq 3$). (*) marks statistical significance.

Figure 6.

Anchorage independent growth of MSC52 cells is decreased following treatment with ROS antagonists. UROtsa and MSC52 were grown in soft agar in the presence of ROS antagonists for 2 wk. Data shown is average $\pm$ SEM ($n \geq 3$). (*) marks statistically significant decrease in colony formation ($p < 0.05$); (†) marks statistically significant decrease in colony formation ($p < 0.10$).

Figure 7.

Anchorage independent growth of MSC52 cells is decreased following treatment with inhibitors of secondary ROS generation. Colonies of MSC52 cells were grown in the presence of non-cytotoxic concentrations of inhibitors for 2 wk. Data shown is average $\pm$ SEM ($n \geq 3$). (*) marks statistically significant decrease in colony formation ($p < 0.05$).