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Interleukin-8 (IL-8) over-production and autocrine cell activation are key factors in monomethylarsonous acid [MMA(III)]-induced malignant transformation of urothelial cells

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Abstract

The association between chronic human exposure to arsenicals and bladder cancer development is well recognized; however, the underlying molecular mechanisms have not been fully determined. We propose that inflammatory responses can play a pathogenic role in arsenic-related bladder carcinogenesis. In previous studies, it was demonstrated that chronic exposure to 50 nM monomethylarsenous acid [MMA(III)] leads to malignant transformation of an immortalized model of urothelial cells (UROtsa), with only 3 mo of exposure necessary to trigger the transformation-related changes. In the three-month window of exposure, the cells over-expressed pro-inflammatory cytokines (IL-1 β , IL-6 and IL-8), consistent with the sustained activation of NF κ B and AP1/c-jun, ERK2, and STAT3. IL-8 was over-expressed within hours after exposure to MMA(III), and sustained over-expression was observed during chronic exposure. In this study, we profiled IL-8 expression in UROtsa cells exposed to 50 nM MMA(III) for 1 to 5 mo. IL-8 expression was increased mainly in cells after 3 mo MMA(III) exposure, and its production was also found increased in tumors derived from these cells after heterotransplantation in SCID mice. UROtsa cells do express both receptors, CXCR1 and CXCR2, suggesting that autocrine cell activation could be important in cell transformation. Supporting this observation and consistent with IL-8 over-expression, CXCR1 internalization was significantly increased after three months of exposure to MMA(III). The expression of MMP-9, cyclin D1, bcl-2, and VEGF was significantly increased in cells exposed to MMA(III) for 3 mo, but these mitogen-activated kinases were significantly decreased after IL-8 gene silencing, together with a decrease in cell proliferation rate and in anchorage-independent colony formation. These results suggest a relevant role of IL-8 in MMA(III)-induced UROtsa cell transformation.

Keywords

bladder cancer; arsenic; monomethylarsonous acid; IL-8; CXCL8; CXCR1; inflammation

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Introduction

Bladder cancer is the second most common cancer of the urinary tract in the world. Among the malignant tumors, bladder tumor is the seventh in frequency, representing 3.2% of all cancers (Arroyo et al., 2004; Barton et al., 2005). It is mainly diagnosed in white males living in urban areas with a frequency double that seen in Hispanic, Asian or African-descendent males (Surveillance, Epidemiology, and End Results (SEER) Program, 1996–2008). In 2010, the number of anticipated new cases was 70,530 with almost 20 % of the cases ending in death.

Although risk factors for bladder cancer development include cigarette smoking (Silverman et al., 1992; Boffeta, 2008) and occupational exposure to aromatic amines (Baan et al., 2008), over the last 13 years chronic exposure to arsenic (As) have been recognized as a relevant risk factor for exposed populations worldwide. In fact, new studies have shown a decreased risk of bladder cancer after a reduction of arsenic intake from drinking water in Taiwan and Argentina (Su et al., 2011; Pou et al., 2010).

The proposed molecular mechanisms of As-induced carcinogenesis are numerous, but the inflammatory response as a pathogenic factor has gained attention since there is evidence showing that *in vitro* the exposure to arsenicals induces the production or activation of inflammatory mediators (Yamamoto et al., 2008; Fry et al., 2007; Lemarie et al., 2006; Simeonova et al., 2003).

Our earlier studies reported that chronic exposure of an immortalized urothelial cell line (UROtsa) to concentrations as low as 50 nM of the highly reactive trivalent arsenic metabolite, monomethylarsonous acid [(MMA(III))] results in malignant transformation after 52 weeks. Subsequent investigations found that the critical period in which the continuous exposure to MMA(III) induces irreversible changes in these cells is around 12 weeks (3 mo) exposure (Wnek et al., 2010).

UROtsa cells from different stages of MMA(III)-induced malignant transformation were profiled for inflammatory cytokine production to determine the role of inflammation in malignant transformation of these cells. In that study different pro-inflammatory as well as anti-inflammatory cytokines were released in the cell culture media as soon as 12 h of exposure (Escudero-Lourdes et al. 2010). The over-expressed cytokines included IL-1 α and β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, and IL-15 along with a decreased expression of IL-23. Among all these over-expressed cytokines, interleukin-8 (IL-8) is one of the most important pro-inflammatory cytokines associated with tumor progression and invasion (Xie, 2001; Lee et al. 2003; Brat et al., 2005; Waugh and Wilson, 2008). The over-expression of this cytokine was more prominent after 3 mo (12 weeks) of exposure to MMA(III), which was also consistent with the sustained over-activation of mitogen activated kinases (MAPK, ERK 2, and p38, as well as NF κ B and AP1/c-Jun).

IL-8, also known as CXCL8, is classified as a CXC chemokine. The CXC chemokines specifically induce the migration of neutrophils and are potent promoters of angiogenesis. Expression of IL-8 has been shown to be regulated by different stimuli including inflammatory cytokines (e.g., TNF- α , IL-1 α), chemical and environmental stresses such as exposure to chemotherapy agents, oxidative stress, hypoxia, and steroid hormones (Xie, 2001). IL-8 activates cells by binding to either CXCR1 or CXCR2 surface receptors. Interestingly, the silencing of CXCR1 gene expression in prostate cancer cells leads to a cell cycle arrest and a decrease in cyclin D1 and in extracellular signal-regulated kinases 1/2 (ERK 1/2) levels, while the transfection of CXCR1 and CXCR2 enhances human melanoma tumorigenesis, as well as subsequent growth and invasion (Singh et al., 2009b).

Evidence has suggested a crucial role of IL-8 in bladder cancer development. In both the human bladder cancer model and the orthotopic bladder cancer model, the production of high levels of different growth factors, including IL-8, VEGF and basic fibroblast growth factor (bFGF), has been demonstrated (Chikazawa et al., 2008; Black and Dinney, 2007). The transfection of the non-tumorigenic cell line 233 J-P, expressing the IL-8 gene in immune-compromised mice, results in tumors that develop spontaneous metastases. Silencing of the IL-8 gene with anti-sense probe in the 233 J-P cells diminished the tumorigenicity and metastatic potential of the cell line (Black and Dinney, 2007). Chikazawa et al., (2008), demonstrating that high IL-8 and bFGF expression correlates with an important increase in tumorigenicity and metastases in urothelial cells clones selected from the highly tumorigenic and metastatic human cell line 253J B-V after orthotopic implantation in mice.

Although these findings support the notion that IL-8 plays an important role in bladder cancer, there have been no definitive investigations regarding the role of this cytokine in the pathogenesis of arsenic-induced bladder cancer. In this investigation, the role of IL-8 in the MMA(III)-induced malignant transformation of UROtsa cells was examined by determining the expression of IL-8 and its receptors CXCR1/CXCR2 in different phases of the transformation process. In addition, the effect of IL-8 on UROtsa cells through autocrine activation was indirectly determined by the silencing of the IL-8 gene using interference RNA. Finally the impact of IL-8 silencing on proliferation, anchorage-independent growth, and tumor formation in immune-deficient mice was examined.

Methods

Cells

UROtsa cells, which were previously shown to be able to methylate inorganic *arsenic* (Bredfeldt et al, 2004), were generously provided by Drs. Mary Ann and Donald Sens (University of North Dakota). This cell line was immortalized by transfection with SV40 large T antigen (Rossi et al., 2001). Cell culture conditions were those previously described by Bredfeldt et al. (2006). Stock cells cultures were grown on 100-cm² tissue culture flasks using Dulbecco's modified eagle medium (DMEM) enriched with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37° C in 5% CO₂. Cells were allowed to reach 85–90% confluence before the experiments were conducted. Normal non-exposed UROtsa cells as well as passage-matched UROtsa cells chronically exposed to 50 nM MMA(III) for 1, 2, 3 and 5 mo, every other day, were used for all the studies. In some experiments, 12 mo-exposed cells were used as positive control or end point for the transformation process. To keep the cells passage-matched, the cells were prepared as previously described (Escudero-Lourdes et al., 2010). Human lymphoblastoid cell line (LCL 18564) was used as control for CXCR1 and CXCR2 determination by flow cytometry.

Enzyme-linked immunoassay (ELISA)

Before the assays were performed, passage-matched UROtsa cells that were chronically exposed to 50 nM MMA (III) for 1, 2, 3 and 5 mo were grown for 24 h without the toxicant and then harvested. Cell suspensions were seeded in 12-well plates in triplicate and incubated for 24 h to settle. The medium was removed and fresh serum-free DMEM was added 24 h before the supernatants were recovered. IL-8 released from the cells into the cell culture media was detected using a specific IL-8 commercial ELISA system, according to the manufacturer's instructions (Pierce, Rockford, IL). The sensitivity of the assay enables the detection of cytokine levels as low as 3 pg/ml. Absolute levels of IL-8 secreted by cells were determined using the assay standards provided in the assay kit. Data are presented as mean ± SEM of three separate experiments.

Gene expression

The RNA from 80–90% confluent cell cultures was isolated using the TRIzol® (Invitrogen, Paisley, UK) method, according to the supplier's protocol. Total RNA was quantified spectrophotometrically and stored at –80°C until use. Total RNA (500 ng) was reverse transcribed to cDNA using the manufacturer's instructions (Applied Biosystems, Foster City, CA). Converted cDNA (10 ng) was then subjected to real-time PCR analysis to determine the levels of GAPDH, IL-8, MMP-9, cyclin D1, bcl-2, and VEGF mRNA in each cell line using human gene-specific Taqman Primer/Probe technology (Applied Biosystems) and by performing PCR in the ABI 7500 real-time detection system (Applied Biosystems, Foster City, CA). Fold changes for each gene expression were calculated using the Delta Ct method normalizing to GAPDH expression for each sample. In another experiment, nonexposed UROtsa cells were exposed to 10 ng/ml recombinant IL-8 (SIGMA) in order to compare the expression profile of the above mentioned genes with that from 3 mo MMA(III)-exposed cells.

Flow cytometry for CXCR1 and CXCR2 expression—Unexposed UROtsa cells and cells exposed to 50 nM of MMA(III) for 1 to 5 mo were grown under normal conditions until 85–90% confluence. The cultures were then washed once with ice-cold phosphate buffered saline (PBS), and trypsinized for 7 min on ice. Cells were then washed in cold PBS containing 0.2% fetal bovine serum (FBS) and stained for 30 min with antibodies to either CXCR1 or CXCR2. After washing and fixing with 2% paraformaldehyde, direct immunofluorescence was performed on either saponin-permeabilized or nonpermeabilized UROtsa cells by exposing cells to saturating amounts of fluorescein- or phycoerythrin (PE)-conjugated mouse antibodies to human CXCR1 and CXCR2 (Santa Cruz Biotechnology). After staining, cells were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with an air-cooled 15 mW argon ion laser tuned to 488 nm. The emission fluorescence of FITC conjugated to anti-CXCR1 IgG was detected and recorded using a 530/30 bandpass filter in the FL1 channel. The PE-conjugated anti-CXCR2 IgG was detected in the FL2 channel using a 585/42 bandpass filter. List mode data files consisting of 10,000 events gated on FSC (forward scatter) versus SSC (side scatter) were acquired and analyzed using CellQuest PRO software (BD Biosciences, San Jose, CA) at a rate of 200–400 events per second. Appropriate electronic compensation was adjusted by acquiring cell populations stained with each fluorophore individually, as well as an unstained control.

Proliferation assay—To assay proliferation rates, a suspension of 1×10^5 cells in high-glucose Dulbecco's modified Eagle Medium (DMEM) was prepared from 80–90% confluent cell cultures. A volume of 100 μ l of this suspension was seeded in a 96-well plate. The cells were allowed to settle for 24 h. The proliferation rate was evaluated using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI) at 24 and 72 h.

Colony formation in soft agar—The ability of cells to form colonies in soft agar was performed as previously described (Bredfeldt et al., 2006). Briefly, a base layer of 0.6% Noble agar (Becton, Dickinson, and Company, Sparks, MD) in culture media was poured into 24-well plates and allowed to solidify. After solidification, enough cells to have a final cell concentration of 10–15,000/well were resuspended in 500 μ l of 0.3% Noble agar and placed on top of the base layer. Serum-enriched DMEM (500 μ l) was added on top of the agar in each well. Fresh medium was added to each well every 48 h. Cells were incubated under normal culture conditions up to 3 weeks, and the colonies were then counted using a microscope at 10 $\times$ magnification. A minimum of three independent experiments were performed.

Lentiviral infection—Lentivirus delivered plasmids (pLK0.1 puro) generating small hairpin RNA (shRNA) against IL-8 mRNA were obtained from Sigma-Aldrich (Sigma, St. Louis, MO). Transduction of these cell lines was performed following the manufacturer's protocol. Briefly, 3 mo-exposed cells were seeded in a 6-well tissue culture dish and allowed to attach for 24 h. After 24 h, cells were transduced with lentiviral particles after medium containing 6 µg/ml hexadimethrine bromide (Sigma, St. Louis, MO) was added.

After 24 h, the medium containing the lentiviral particles was removed and replaced with fresh medium. After another 24 h, the medium was replaced with medium containing 2 µg/ml puromycin. Medium containing puromycin was subsequently replaced every two days. Cells were maintained in selection medium throughout all experimental procedures.

Non target vector particles (non targeting control, NTC) were used as controls for each cell line. To determine that the IL-8 gene expression level in transduced cells, real-time PCR using specific Taq man probes, as well as ELISA assay, was performed in each cell line as described above.

For better understanding, cells exposed to MMA(III) for 3 mo without transduced shRNA are designated in this work as 3 mo CTL; cells transduced with shRNA for IL-8 gene as 3 mo IL8(−), and cells transduced with lentivirus carrying non targeting-shRNA as 3 mo NTC.

Tumorigenicity in severe combined immunodeficiency (SCID) mice—Ten million 3 mo CTL cells, 3 mo IL8 (−) or 3 mo NTC were implanted subcutaneously on the dorsal flank of 6–8-week-old male SCID mice (10 mice per group). The tumor growth of each mouse was monitored over time by measuring tumor volumes with a caliper. At necropsy, tumors were harvested from each animal after approximately 59 days, and 1/2 of the tumor was placed in 10% neutral buffered formalin for 24 h and then transferred to 70% EtOH. The tissues were taken to the Tissue Acquisition and Cellular/Molecular Analysis Shared Service at the Arizona Cancer Center for analysis. All procedures were performed in accordance with approved protocols of the University of Arizona Institutional Animal Care and Use Committee.

Histological analysis of xenograft tumors—Tumor samples were paraffin-embedded, sectioned, stained with hematoxylin and eosin (H&E), and analyzed by light microscopy by Dr. Raymond B. Nagle (University of Arizona, Department of Pathology). Samples of each experimental group were chosen to assay for IL-8, VEGF, and VEGFR2, as well as for the proliferation marker Ki67, by immunohistochemical analysis.

Statistical analysis—All values are expressed as mean ± SD. Differences between groups were determined using two-way ANOVA and Bonferroni's post test. A p value equal to or less than 0.05 was considered statistically significant.

Results

IL-8 is over-expressed in UROtsa cells exposed chronically to MMA(III)

In our previous work using a 16-cytokine array, we found that different pro-inflammatory cytokines were over-produced in UROtsa cells after acute and chronic exposure to 50 nM MMA(III) (Escudero-Lourdes et al., 2010). Among the key cytokines of interest was IL-8, due to its known role in tumorigenesis. Consistent with this finding, in this study the IL-8 mRNA level was three times over-expressed by cells exposed to MMA(III) for 3 or more mo (Figure 1). IL-8 secreted in the medium showed a 4.8- and 3.6-fold increase for cells exposed for 3 mo and 12 mo, respectively, compared to unexposed UROtsa cells (Figure 2).

Five month-exposed cells did not show significant changes compared to control in this experiment.

IL-8 is expressed in tumors derived from hetero-transplanted UROtsa cells

The over-expression of IL-8 correlates with tumor development, disease progression, and recurrence in different type of cancers. The over-expression of IL-8 in the bladder has been found mainly in carcinoma *in situ* and invasive tumors compared to non-invasive carcinomas (Izawa et al., 2001). Our group previously showed that cells exposed to MMA(III) for 3 mo or more are able to produce highly proliferative squamous-type tumors when they are subcutaneously injected into severe combined immunodeficiency (SCID) mice, which established this as a critical phase for irreversible MMA(III)-induced malignant transformation of UROtsa cells (Wnek et al., 2010). To determine the presence and distribution of IL-8 in the xenograft tumors and its association with tumor progression, specific immunohistochemical analysis for IL-8 was performed on tumors from cells exposed to MMA(III) for 3 mo.

The immunohistochemical analysis of tumor tissues showed that tumors derived after the heterotransplantation of unexposed UROtsa cells in SCID mice, which form discreet-size tumors (4–6 mm diameter), showed a weak signal for IL-8 production (Figure 3). In these tumors, IL-8 was mainly produced by the basal layer cells, and its localization inside the cells was mainly perinuclear. In contrast, tumors derived from cells exposed to MMA(III) for 3 mo, which were larger than the tumors formed by normal UROtsa cells (10–15 mm diameter in average), showed a higher IL-8 production with an intra-cytoplasmic localization. In these tumors, IL-8 production was found to be more prominent not only in the basal layer cells but also in 50- to 100- μ m diameter spots within the superficial layer (differentiated cells), with an intra-cytoplasmic distribution.

The change in IL-8 production and distribution within the tumor was consistent with a more proliferative and, as a consequence, more necrotic tumor (as demonstrated by the proliferation marker Ki67), which showed extensive muscle invasive areas compared to those tumors derived from unexposed UROtsa cells (Figure 3).

In humans, IL-8 produced by tumor cells can stimulate tumor and stromal cells expressing CXCR1 or CXCR1 either through an autocrine or paracrine fashion. This can lead to the expression of angiogenesis-related factors and the promotion of endothelial cell proliferation, tumor growth, and metastasis (Waugh and Wilson, 2008). However, mice do not express IL-8 or its receptors, suggesting that the IL-8 produced is able to stimulate only the tumor cells developed from the heterotransplantation of UROtsa cells. To evaluate the role of the autocrine activation by the IL-8 produced by UROtsa cells, the expression of its receptors was determined by flow cytometry.

Increased internalization of CXCR1 and CXCR2 in MMA(III)-exposed UROtsa cells suggests a sustained autocrine cell activation by secreted IL-8

The internalization of chemokine receptors is known to occur after the ligand binds to the corresponding receptor at the cell surface, and this process can dramatically reduce the expression of the receptor on the cell membrane (Neel et al., 2005). Unexposed UROtsa cells were processed to determine the constitutive expression of surface and internal IL-8 receptors by flow cytometry. Both, CXCR1 and CXCR2 were found in the surface of UROtsa cells with specific mean fluorescence intensities of 150 and 39, respectively (Figure 4). However, only 13.6 % of the cells expressed surface CXCR1 while 28 % expressed surface CXCR2 (Figure 4a). Interestingly, 99.7% of the cells expressed the internal receptor CXCR1, whereas 58 % expressed CXCR2 (Figure 4b) with a specific mean fluorescence

intensities of 855 and 31, respectively. As control, a human lymphoblastoid cell line (LCL 18564) was processed in the same way as UROtsa cells for CXCR1 and CXCR2 determination. The results for unpermeabilized and permeabilized cells were consistent with those found for the UROtsa cells, where both receptors were expressed mainly intracellularly and with CXCR1 being predominant over CXCR2 (Figure 4c).

Based on the fact that IL-8 is over-expressed in the UROtsa cells that were chronically exposed to MMA(III), the enhanced receptor internalization observed would support the hypothesis of a sustained IL-8-dependent autocrine cell over-activation. Therefore, the presence of intracellular CXCR1 and CXCR2 were determined in cells chronically exposed to 50 nM MMA(III) for 0, 1, 2, 3, 5, and 12 mo. Consistent with IL-8 over-production, the results showed that UROtsa cells exposed for 3 and more months to this arsenic metabolite have a significant enhancement in CXCR1 internalization rate (Figure 5a and b), supporting the notion that MMA(III) induces cell activation through an increase in IL-8 production.

IL-8 over-production in chronically exposed UROtsa cells is coincident with an increase in the expression of genes associated with tumor progression, cell survival, and invasion

IL-8 mediated cell activation through CXCR1 and CXCR2 induces the activation of serine/threonine kinases, protein tyrosine kinases, and RHO-GTPases, inducing the translocation of different transcription factors, which leads to the expression of proteins associated with cell proliferation, survival, and cell invasion (Waugh and Wilson, 2008). To determine whether the over-expression of IL-8 in this critical phase of MMA(III)-induced cell transformation is related to the sustained activation of critical intracellular pathways and transcription factors that leads to the expression of genes associated with tumor growth, apoptosis inhibition, and invasion, the gene expression of MMP-9, cyclin D1, bcl-2, and VEGF was determined in 3 mo-exposed cells.

The gene expression pattern elicited by cells after chronic exposure to MMA(III) was also compared to UROtsa cells grown for 24 h in the presence of 100 nM recombinant IL-8. Compared to nonexposed UROtsa cells, the cells exposed to MMA(III) for 3 mo consistently over-expressed genes coding for cyclin D1, bcl-2, and MMP-9 proteins (Figure 6). Interestingly the same gene expression signature was found for UROtsa cells [unexposed to MMA(III)] that were acutely exposed to externally added recombinant IL-8.

IL-8 gene silencing in UROtsa cells leads to a decrease in cell proliferation rate, colony formation in soft agar, and genes associated with tumor progression and invasion

Cells exposed to MMA(III) for 3 mo were shown to have malignant characteristics, including faster cell growth and the ability to grow in soft agar and form tumors in SCID mice (Wnek et al., 2010). After IL-8 gene expression was silenced in these cells, these characteristics were evaluated to determine the relevance of IL-8 expression in MMA(III)-induced cell transformation.

Through the use of shRNA lentiviral particles, 99.8% silencing of IL-8 gene expression of 3 mo IL8(–) was confirmed by real-time PCR. Specific commercial ELISA assay results showed that IL-8 protein expression was reduced in shRNA IL-8 transduced cells by around 60% compared to control cells (Figure 7). The silencing of the IL-8 gene in these cells led to a significant decrease in cell proliferation rate (an average of 50%) (Figure 8A) and also an 81% decrease in anchorage-independent cell growth as determined by colony formation in soft agar (Figure 8B). Consistent with these findings, 3 mo IL8(–) cells showed a significant decrease in the expression of the cyclin D1 gene and also of the MMP-9 and bcl-2 coding genes, compared to 3 mo CTL cells, which expressed “normal” quantities of IL-8 (Figure 9).

IL-8 silencing decreases tumor formation by MMA(III)-transformed UROtsa cells in SCID mice

To determine the role of IL-8 on MMA(III)-transformed UROtsa cells in their ability to form tumors in SCID mice, UROtsa cells exposed to MMA(III) for 3 mo that showed characteristics of transformation, such as growth in soft agar and shorter doubling time, were transduced with lentivirus carrying a shRNA for IL-8. Cells were subcutaneously injected into SCID mice and monitored for tumor growth. All mice inoculated with 3.5 mo CTL, 3 mo NTC, or 3 mo IL8(-) cells showed a palpable tumor within 60 days after cell transplantation. However, 3 mo IL8(-) cells produced tumors with an average volume of $570 \pm 73 \text{ mm}^3$, compared to the average tumor volume with 3 mo CTL or 3 mo NTC (1278 ± 106 and 915 ± 148 , respectively), which represents around of 60 % less tumor volume compared to controls (Figure 10). The expression of IL-8 in tumor tissues was confirmed by immunohistochemical staining (data not shown).

Discussion

The association between human chronic exposure to arsenicals and bladder cancer development has been repeatedly documented (Volanis et al., 2010, Huang et al., 2004, Chiou et al., 2001.), but the role of the inflammatory response as a pathogenic mechanism had not been evaluated before.

It is well known that inorganic arsenic (iAs) is metabolized through a series of reduction and methylation reactions leading to the production of methylated compounds. Monomethylarsonous acid [MMA(III)] has been proven to be highly reactive and therefore more toxic than iAs and has been used to study the toxicological effect of *in vitro* exposure to arsenicals. In a previous work using an immortalized urothelial cell line (UROtsa cells), we demonstrated that a significant inflammatory response, characterized by the over-production of cytokines, such as IL-6, IL-1b, MIF-1, and IL-8, was elicited in UROtsa cells acutely exposed to MMA(III) and that a sustained cellular production of these cytokines was observed, if the cells were continuously exposed to MMA(III) for 3 mo (Escudero-Lourdes et al., 2010). Based on the literature, all these cytokines appear to play an important role in carcinogenesis; however, IL-8 seems to be highly relevant in bladder cancer development and invasiveness (Chikazawa et al., 2008.; Black and Dinney, 2007).

Normal urothelial cells constitutively express IL-8, which is essential for cell survival (Tseng-Rogenski and Libert, 2009); however, the over-expression of this chemokine is associated with tumor growth and metastasis in urothelial cell carcinoma of the bladder (Chikazawa et al. 2008). In this work, we profiled IL-8 expression in UROtsa cells chronically exposed to MMA(III) and found that chronic exposure to 50 nM MMA(III) for 3 mo or longer showed a sustained over-expression of IL-8. This finding is consistent with a higher rate of CXCR1 internalization.

The chemokine receptors normally undergo a basal level of internalization or recycling even in the absence of ligand; however, IL-8 binding can enhance CXCR2 internalization and trafficking by up to 80% (Neel et al., 2005). This process plays a relevant role in the regulation of the inflammatory process (Zaslever et al., 2001). In our model, we found that nonexposed UROtsa cells expressed CXCR1 and CXCR2 mainly intracellularly, which is consistent with the notion that IL-8 is needed for the normal growth of urothelial cells; however, exposure to 50 nM MMA(III) for more than 3 mo increased CXCR1 internalization by up to 70%.

These receptors show a distinctive ligand binding pattern. CXCR2 is activated by multiple CXC chemokines, including growth-related oncogenes (GRO α , h, and g), neutrophil-

activating peptide, and granulocyte chemotactic protein-2 (Waugh and Wilson, 2008); however, CXCR1 is activated only in response to binding of IL-8 and granulocyte chemotactic protein-2. Due to this receptor specificity, and the fact that UROtsa cells exposed for 3 mo or longer over-express IL-8, it is likely that IL-8 is mainly responsible for the increased CXCR1 internalization in UROtsa cells chronically exposed to MMA(III).

Consistent with these findings, the expression of genes associated with cell proliferation, survival, and invasion are increased in cells exposed for 3 mo or longer to MMA(III). These include genes representative of tumor progression-related processes, such as metalloproteinase 9 (MMP-9), involved in angiogenesis and invasion, cyclin D1, which promotes transition from G1 to S phase of the cell cycle, bcl2 protein, which negatively regulates apoptosis, and vascular endothelial growth factor (VEGF), which stimulates vasculogenesis and angiogenesis.

Interestingly, the gene expression pattern found for the genes coding for MMP-9, cyclin D1, Bcl-2, and VEGF in 3 mo-exposed cells was also seen when untreated UROtsa cells were exposed to recombinant IL-8 for 24 h. This suggests that when UROtsa cells are exposed to MMA(III), IL-8 production plays an important role in the over-expression of these genes.

In vivo, the inoculation of SCID mice with 3 mo-exposed cells led to the formation of tumors of increased size and higher expression of the proliferation marker Ki67, compared to control UROtsa cells. These tumors express IL-8 at increased levels at the basal layer compared to those formed from nonexposed UROtsa cells. IL-8 production was also found in the superficial layer, but was not found in the superficial layer of tissue from nonexposed UROtsa cells. Due the fact that mice do not express IL-8 (Jeyaseelan 2005), the only source of this cytokine has to be the heterotransplanted cells.

To further determine the role of IL-8 in MMA(III)-induced urothelial cells transformation and tumor growth, IL-8 gene expression was silenced using iRNA. Stable cultures of UROtsa cells that were exposed for 3 mo to 50 nM MMA(III) depleted of IL-8 were established. The stable shRNA transfectants [3 mo IL-8(-)] that expressed significantly reduced IL-8 mRNA and protein (≤ 99 % and 60 %, respectively) or control (NTC) transfectants (3 mo NTC) were characterized. 3 mo IL8(-) cells showed a significant reduction in cell proliferation (50% in average), consistent with an substantial decrease in cyclin D1 (76.5%) expression as well as a significant decrease in anchorage-independent growth compared to non-shRNA transduced cells (3 mo CTL), meaning that IL-8 has an important role in regulating the growth rate of 3 mo cells and their ability to grow anchorage-free.

As was shown, the increased gene expression of MMP-9, VEGF and bcl-2 that was previously found in UROtsa cells exposed for more than 3 mo (3 mo CTL cells), was reversed in cells that were silenced for IL-8. In these cells, gene expression of MMP-9 was reduced by 81.36 % on average, Bcl-2 by 51.1 % and VEGF by 76.5% in 3 mo IL-8(-) cells when compared to 3 mo CTL that express “normal” levels of IL-8. These results suggest that IL-8 could play an important role in the modulation of these genes and therefore in tumor progression and invasion. This notion was further supported by the fact that tumors were decreased up to 55.5 % in size when SCID mice were inoculated with IL-8(-) cells.

The relevance of IL-8 signaling in cancer is not new. Prior to our work, Inoue et al. (2000) demonstrated that the use of IL-8 anti-sense in prostate cancer cells led to decreased neovascularity, tumorigenicity, and metastasis in tumor cells after orthotopic implantation in athymic nude mice. Shamaladevi et al. (2009) and Singh et al. (2009a,b,c) showed that when IL-8 or its co-receptor CXCR1 was silenced, prostate cancer cell proliferation was inhibited due to cell cycle arrest at G(1) phase. They also found a decrease in cyclin D1, ERK1/2,

CDK4, and phospho-Rb expression compared to non-silenced cancer cells. What is particularly relevant in our studies is the link between the acquisition of a malignant transformed phenotype in urothelial cells chronically exposed to MMA(III), the activation of signaling pathways and transcription factors related to cancer progression, and the modulating effect of IL-8, which is induced early as a consequence of exposure to MMA(III). In other words, these results suggest that one possible mechanism for the induction of malignant transformation of UROtsa cells by MMA (III) involves the positive regulation of IL-8 expression, which in turns activates a variety of cellular pathways, including those related to tumor progression and inflammation. Under such conditions, it is likely that as more pro-inflammatory cytokines are produced and released into the medium, more ROS are produced, inducing new inflammatory responses in a never-ending cycle that eventually and irreversibly commit the cells to malignant transformation even when the MMA(III) is no longer present in the cell culture medium.

These results encourage further studies in exposed populations to evaluate whether bladder cancer development induced by chronic exposure to arsenicals is mediated through IL-8 over-expression, to use the inflammatory response as a molecular target for prevention or treatment in arsenic-exposed humans.

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Highlights

- IL-8 is over-expressed in human MMA(III)-exposed urothelial cells.
- Internal CXCR1 and tumor progression markers are also increased.
- IL-8 silencing decreased malignant transformation markers in MMA(III)-exposed cells.

Figure 1. Chronic exposure of UROtsa cells to 50 nM of MMA(III) leads to sustained IL-8 gene over-expression. The bars represent the mean of four independent biological samples processed under the same conditions. Asterisks indicate a significant difference [(***), $P < 0.001$] compared to non-exposed cells.

Figure 2.

IL-8 production profile after chronic exposure of UROtsa cells to 50 nM MMA(III). The bars represent the mean $\pm$ SD of three independent experiments. Asterisk indicates a significant difference [(***) $P < 0.001$] compared to nonexposed cells as shown by two-way ANOVA followed by Bonferroni's test.

Figure 3.

IL-8 is expressed in tumors derived from heterotransplanted UROtsa cells. Tissue sections were assayed for IL-8 and Ki67 (proliferation marker) by specific IHC. Tumors derived from cells that were exposed to 50 nM MMA (III) for 3 mo showed higher IL-8 production with an intra-cytoplasmic localization in the basal layer. IL-8 production was also found in the superficial layer, compared to that produced from tumors derived from cells that were not exposed to MMA(III), which was found only in the basal layer with a peri-nuclear localization. These findings were consistent with the expression of the cell proliferation marker Ki67 and tumor size.

Figure 4.

UROtsa cells constitutively express CXCR1 and CXCR2. Untreated UROtsa cells were processed to determine the expression of surface and intracellular CXCR1 and CXCR2. The receptors were found to be mainly intracellular, suggesting IL-8 autocrine activation. A) Histogram of surface and intracellular CXCR1 determination. B) and C) Percentage of intracellular and surface CXCR1 and CXCR2 on untreated UROtsa cells and the lymphoblastoid cell line LCL18564, respectively.

Figure 5.

Increased internalization of CXCR1 in MMA(III)-exposed UROtsa cells. Flow cytometry analysis showed that the cells exposed to 50 nM MMA(III) for 3 or more months had an increased internalization of the IL-8 receptor CXCR1. The bars represent the mean $\pm$ SD of at least three independent experiments. Asterisks indicate a significant difference [(***), $P < 0.001$], compared to nonexposed cells as shown by two-way ANOVA followed by Bonferroni's test.

Figure 6.

UROtsa cells over-expressing IL-8 showed an increase in the expression of genes associated with tumor growth and invasion. RNA from cells exposed to MMA (III) for 3 mo was isolated and transformed to cDNA, and the expression of MMP-9, cyclin D1, VEGF and bcl-2 was assayed by real-time PCR. For comparative purposes, RNA recovered from UROtsa cells treated with 10 ng/ml rIL-8 for 24 h was processed under the same conditions to determine gene expression. Bars represent mean $\pm$ SD of four independent biological samples. Asterisks indicate a significant difference [(***) $P < 0.001$, (*) $P < 0.05$], compared to nonexposed UROtsa cells as shown by ANOVA followed by Bonferroni's test.

Figure 7.

The expression of IL-8 gene was silenced in MMA(III)-exposed UROtsa cells by shRNA lentivirus transduction particles. A) IL-8 gene expression was reduced 99.7 % in 3 mo (IL8-) cells. B) Protein production was reduced 60% compared to non-IL-8 sh RNA transfected cells that were exposed to MMA(III) for 3 mo and cells transfected with non-target control transduction particles (NTC).

Figure 8.

IL-8 gene silencing in MMA(III)-exposed UROtsa cells leads to a decrease in growth rate and in colony formation ability. After IL-8 silencing, UROtsa cells exposed to MMA(III) for 3 mo (3 mo IL8 (-) cells) were assayed for cell proliferation (A) as well as growth in soft agar (B). Bars represent the mean $\pm$ SD of three independent experiments. Asterisks indicate a significant difference [(***), $P < 0.001$], compared to cells exposed to MMA(III) for 3 mo, which express normal IL-8 quantities, and to NTC-treated cells, as shown by ANOVA followed by Bonferroni's test.

Figure 9.

The expression of MMP-9, cyclin D1, bcl-2, and VEGF coding genes was significantly decreased in UROtsa cells exposed to MMA(III) for 3 mo, after IL-8 gene silencing compared with non-transduced cells or NTC-transduced cells expressing normal quantities of IL-8. The bars represent the mean $\pm$ SD of four different biological samples, and significant differences are shown with asterisks [(***), $P < 0.001$].

A)

Cell lines	Tumor incidence (number of tumors/number of mice). n=4	Tumor volume on day 60 post cells implantation (mm ³)	Change from control tumor volume (%)
3mo CTL	4/4	1278 ± 106	0
3mo NTC	4/4	915 ± 148	72
3mo IL8 (-)	4/4	570 ± 72	41

B)

Figure 10.

IL-8 gene silencing decreases tumor growth in MMA(III)-transformed UROtsa cells. UROtsa cells exposed chronically to 50 nM MMA (III) for 3 mo or stably transduced 3 mo MMA (III)-treated UROtsa cells (10×10^6 cells in 0.1 ml PBS) were subcutaneously injected into the flank of SCID mice. (A) Tumor incidence represents the number of animals that developed tumors; tumor volume on day 60 after tumor injection (n=4). (B) Tumor size of 3 mo IL-8(-) decreases significantly compared to 3 mo Ctl cells that express “normal” IL-8 levels or with non target control (NTC) stably transfected cells. Results are shown as mean $\pm$ SEM. *Significantly different from controls ($P \leq 0.05$) as indicated by ANOVA followed by Bonferroni’s test.