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Short communication

Monomethylarsonous acid: Induction of DNA damage and oxidative stress in mouse natural killer cells at environmentally-relevant concentrations

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ABSTRACT

The carcinogenicity of arsenic has been confirmed in many studies, but its mechanism of action is still unclear. A lymphocyte component of the innate immune system, natural killer (NK) cells are responsible for killing cancer cells. Although inorganic arsenical species are the prevalent forms of arsenic in the environment, monomethylarsonous acid (MMA^{+3}) is the major arsenical species found in immune-system cells, in this 30 d drinking-water-exposure study in mice. Therefore, the effect of MMA^{+3} on NK cells should be studied as a possible contributor to arsenic-induced toxicity at environmental exposure levels. In the mouse drinking-water-exposure model, As^{+3} induces dose-dependent DNA damage in NK cells. In *in vitro* studies, MMA^{+3} inhibited cell growth and induced DNA damage and oxidative stress at low concentration (20 and 50 nM) in isolated mouse NK cells. Strong correlations were found between DNA damage and oxidative stress in MMA^{+3}-treated mouse NK cells. Even at low concentrations relevant to environmental arsenic exposures, MMA^{+3} is genotoxic to primary mouse NK cells.

1. Introduction

Arsenic (As) contamination in food and drinking water is a worldwide public health issue. Many diseases, such as skin lesions, diabetes, cardiovascular diseases, and multiple types of cancers, are associated with environmental exposure to As [1–4]. The inorganic forms of arsenic in the environment, trivalent arsenite (As^{+3}) and pentavalent arsenate (As^{+5}), are metabolized to mono-methylated and di-methylated trivalent and pentavalent organic species *in vivo* [5]. Previous studies indicate that the intermediate form of arsenic during its metabolism, monomethylarsonous acid (MMA^{+3}), is more toxic than other arsenical species [6,7]. Our study in As^{+3}-exposed mice showed that MMA^{+3} is accumulated in primary immune organs, such as the thymus and bone marrow [8]. The accumulation of MMA^{+3} in the thymus and bone marrow was detrimental to the development of both T cells and B cells, the two critical types of lymphocyte in the immune system [9–11].

Nature killer (NK) cells are important members of the lymphocyte family. Key players in the prevention of carcinogenesis in the human body, NK cells are the professional killers of cancer cells in the innate immune system. In contrast to cytotoxic T cells, the killing activity of NK cells is independent of major histocompatibility complex (MHC) recognition [12]. Similar to other types of lymphocytes, NK cells develop in the bone marrow, lymph nodes, spleen, and thymus [13]. NK cell differentiation/activation depends on two cytokines, IL-2 and IL-15, which act to expand the NK population *in vitro* [14–16]. Since NK cells do not express T cell receptors, the pan-T-cell marker CD3 can be used to separate them from NKT cells, a type of T cell with NK-cell properties [17].

The genotoxicity of MMA^{+3} and its molecular mechanisms have been studied by many researchers [18,19] Shen et al., 2013. Our previous studies demonstrated that low to moderate doses of MMA^{+3} induce genotoxicity and suppress cell proliferation in primary B and T cells [20,21]. Since NK cells are also lymphoid cells and develop in the primary lymphoid organs, which accumulate MMA^{+3}, they are likely to be affected by MMA^{+3} in As exposure. Since the carcinogenicity of environmental exposure to As has been confirmed in many studies, study of the toxicity induced by MMA^{+3} in cancer-preventing NK cells may inform understanding of the mechanism of As carcinogenesis [22,23]. In the present study, we have examined DNA damage and oxidative stress induced by environmentally-relevant concentrations of MMA^{+3} and we have analyzed the association between MMA^{+3}-induced DNA damage and oxidative stress in primary mouse NK cells. Further mechanistic studies are being conducted to reveal the

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molecular basis of the genotoxic effects observed in the NK cells.

2. Methods

2.1. Chemicals and reagents

Sodium meta-arsenite (As^{+3}, CAS 774-46-5, $NaAsO_2$, 98.8% purity by HPLC-ICP-MS), RPMI 1640 medium and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monomethylarsonous acid (MMA^{+3}, CAS 25400-23-1, CH_5AsO_2, > 99% purity by HPLC-ICP-MS) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Youkang (Beijing, China). Penicillin/streptomycin (Pen/Strep) was purchased from Mei5 Biotechnology (Beijing, China). Fetal bovine serum (FBS) and 0.5 M EDTA (Cat. No. AM9260G) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant murine IL-2 (Cat. No. 212-12) and murine IL-15 (Cat. No. 210-15) were purchased from Peprotech (Rocky Hill, NJ, USA). The comet assay kit (Cat. No. 4252-040-ESK) was purchased from Trevigen (Gaithersburg, MD, USA). PE-Cy™7 hamster anti-mouse CD3e clone 145-2C11 (Cat. No. 561100) and FITC mouse anti-mouse NK-1.1 clone PK136 (Cat. No. 553164) were purchased from BD Biosciences (San Jose, CA, USA). Dihydroethidium (DHE, Cat. No. 50102ES02), SYBR Green I (Cat. No. 10222ES60) and 0.4% trypan blue (Cat. No. 40207ES60) were purchased from Yeasen Biotech (Shanghai, China). RNeasy mini kit (Cat. No.74104), QuantiTect reverse transcriptionkKit (Cat. No. 205313), Mm_Hmox1_1_SG QuantiTect Primer Assay (Cat. No. QT00159915) and QuantiTect SYBR Green PCR Kit (Cat. No. 204145) were purchased from Qiagen (Valencia, CA, USA).

2.2. Mouse in vivo experiment and the isolation of NK cells

Male C57BL/6 mice were purchased at 7 weeks of age from Cavens (Changzhou, Jiangsu, China). All animal experiments were conducted following procedures approved by our institutional Laboratory Animal Use and Care Committee. Mice were accommodated in our animal facility for 1 week before the 30 d As^{+3} drinking-water exposure started. Drinking water was changed every week during the exposure and the amount of As^{+3} was confirmed by ICP-MS. Mouse spleen cell harvesting procedures were as described in Xu et al. [8]. After organ weight was recorded, a single-cell suspension of mouse spleen cells was prepared by homogenizing the spleen between the frosted ends of two sterilized microscope slides into a cell culture dish containing cold cell-culture medium, 5 mL (500 mL RPMI 1640 plus 50 mL FBS and 100 U/ml pen/strep). Cells were centrifuged at 300*g* for 5 min, aspirated, and washed twice with cold cell-culture medium. Cell number and viability were determined by trypan blue staining on a LIFE Countess II automated cell counter (Thermo Fisher Scientific). From the single cell suspension, 3×10^8 cells were concentrated to 1 mL in cell culture medium and stained with 1 μg CD3e and NK1.1 antibody for 30 min on ice in the dark. Cells were washed twice with cell culture medium and sorted on a BD FACSJazz sorter at 10000 events/s to achieve > 98% purity.

2.3. NK cell in vitro culture and treatment

The sorted NK cells were seeded at 2×10^5 cells/mL on a 24-well plates in cell-culture medium with or without 100 U/mL IL-2 and 50 ng/mL IL-15 [24]. After 2 h seeding, the NK cells in culture with the cytokine supplements were treated with MMA^{+3} for 8 and 18 h *in vitro*. Cells were counted as above. After 18 h treatment, an aliquot (100 μL) of cells from each well was transferred into a 1.5 mL microcentrifuge tube and washed with DPBS for comet assay analysis. One aliquot (400 μL) of cells was transferred into flow tubes for DHE staining and another (500 μL) was used for RNA extraction.

2.4. Comet assay

MMA^{+3}-treated NK cells were immobilized in low-melting-point agarose on a 20-well comet assay slide, according to the Trevigen comet assay kit protocol. Cells were lysed with lysis solution overnight at 4 °C. Next day, the slide was transferred into basic buffer (8 g NaOH + 2 mL 0.5 M EDTA in 1 L deionized water, pH > 13) and left at RT for 45 min to unwind the DNA. After the unwinding step, the comet slide was electrophoresed in ice-cold basic buffer in a Trevigen CometAssay® electrophoresis system at 0.7 V/cm for 45 min. Slides were then washed with double-distilled water, dried, and stained with Sybr Green I (1:10,000 dilution in double-distilled water) and imaged with a Nikon Ti-S fluorescence microscope. Fifty randomly selected cells from each well were scored using CometScore® software provided by TriTek Corp. (Sumerduck, VA, USA). The use of percent tail DNA as index of DNA damage was recommended in Collins [25].

2.5. Oxidative stress analysis

For reactive oxygen species (ROS) detection, DHE was dissolved in DMSO and diluted to 25 μM in cell culture medium as the working solution. Working solution, 100 μL, was added to cell suspension, 400 μL, to achieve final DHE concentration = 5 μM. After 30 min staining at 37 °C, cells were washed twice with cold DPBS and analyzed on an AccuriC6 Plus flow cytometer (BD Biosciences).

For heme oxygenase 1 (Hmox1) expression analysis, RNA from the treated cells was extracted as per the Qiagen RNeasy Mini Kit manual. RNA was then quantified on a Thermo Scientific NanoDrop 3300 spectrophotometer. After the reverse transcription step with the QuantiTect Reverse Transcription Kit on a Bio-Rad T100 thermocycler, cDNA samples were stored in DNase-free water at −80 °C. Real time qPCR reactions with Mm_Hmox1_1_SG QuantiTect Primer Assay and QuantiTect SYBR Green PCR Kit were carried out on a StepOnPlus Real-Time PCR system (Thermo Fisher Scientific) in a 96-well plate. GAPDH was used as the endogenous reference gene. Comparative C_T (the first amplification cycle exceeding threshold) was calculated by the StepOne™ software and applied for quantification of Hmox1 expression.

2.6. Statistics and correlation analysis

Data were analyzed with Excel 2016 and SigmaPlot v12.5 software. Five mice were used in each treatment group for the *in vivo* experiment. Three independent experiments were performed and analyzed for each dose of MMA^{+3} in the *in vitro* studies. One-way analysis of variance (ANOVA) and Dunnett's test were used to determine differences between the control and treatment groups. Pearson correlation and polynomial linear regression were used in the analysis of the correlations between DNA damage and other variables. R^2 was used to evaluate the correlations and was indicated in the figures.

3. Results

3.1. d As^{+3} drinking-water exposure induces DNA damage in mouse NK cells

Our previous study indicated that As^{+3} drinking-water exposure induces DNA damage in mouse spleen, thymus, and bone marrow cells [8]. NK cells develop and mature in these immune organs, and are likely to be affected by drinking water As^{+3} exposures. To test whether As^{+3} exposure induces DNA damage in mouse NK cells, 8-week old C57BL/6 mice (five per group) were exposed to 0, 10, 100, 500, and 1000 ppb As^{+3} via drinking water for 30 d. No significant changes were observed in water intake, mouse weight, or spleen weight between the control and As^{+3}-exposed groups (Table 1). Splenic NK cells of the exposed mice were isolated by cell-surface-marker staining and cell sorting (Fig. 1A). Significant increases in DNA damage were observed in