Toxicology and Applied Pharmacology 244 (2010) 263–272

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Arsenite induces apoptosis in human mesenchymal stem cells by altering Bcl-2 family proteins and by activating intrinsic pathway

Santosh Yadav, Yongli Shi, Feng Wang, He Wang *

Department of Environmental Health Sciences, School of Public Health and Tropical Medicine, Tulane University New Orleans, LA, USA

ARTICLE INFO

Article history:
Received 3 August 2009
Revised 4 January 2010
Accepted 5 January 2010
Available online 18 January 2010

Keywords:
Human mesenchymal stem cells
G2-M arrest
S-phase
TUNEL
Annexin V staining

ABSTRACT

Purpose: Environmental exposure to arsenic is an important public health issue. The effects of arsenic on different tissues and organs have been intensively studied. However, the effects of arsenic on bone marrow mesenchymal stem cells (MSCs) have not been reported. This study is designed to investigate the cell death process caused by arsenite and its related underlying mechanisms on MSCs. The rationale is that absorbed arsenic in the blood circulation can reach to the bone marrow and may affect the cell survival of MSCs.
Methods: MSCs of passage 1 were purchased from Tulane University, grown till 70% confluency level and plated according to the experimental requirements followed by treatment with arsenite at various concentrations and time points. Arsenite (iAs^{III}) induced cytotoxic effects were confirmed by cell viability and cell cycle analysis. For the presence of canonic apoptosis markers; DNA damage, exposure of intramembrane phosphotidylserine, protein and m-RNA expression levels were analyzed.
Results: iAs^{III} induced growth inhibition, G2-M arrest and apoptotic cell death in MSCs, the apoptosis induced by iAs^{III} in the cultured MSCs was, via altering Bcl-2 family proteins and by involving intrinsic pathway.
Conclusion: iAs^{III} can induce apoptosis in bone marrow-derived MSCs via Bcl-2 family proteins, regulating intrinsic apoptotic pathway. Due to the multipotency of MSC, acting as progenitor cells for a variety of connective tissues including bone, adipose, cartilage and muscle, these effects of arsenic may be important in assessing the health risk of the arsenic compounds and understanding the mechanisms of arsenic-induced harmful effects.

Introduction

Mesenchymal stem cells or marrow stromal cells (MSCs) are non-hematopoietic multipotent stem cells, which have shown high therapeutic potential because of their ability to self-renew and ease of their isolation and expansion (Prockop, 2009). MSCs possess a broad spectrum for regenerative medicine due to their potential to differentiate into lineages of mesenchymal tissues such as bone, cartilage and tendon (Lund et al., 2009; Johnstone and Yoo, 1999; Grogan et al., 2009; Schnabel et al., 2009). MSCs reside predominantly in the bone marrow but they are also distributed throughout many other tissues, where MSCs serve as local sources of quiescent stem cells. Moreover, these cells with other non-hematopoietic cells such as osteoblast, adipocyte, fibroblast, reticular and endothelial cells possess a functional role in regulating hematopoiesis (Schofield, 1978; Nemeth et al., 2009). Thus, it is obvious that any cellular stress in MSCs can drastically diminish the hematopoietic system. It has been previously revealed that radiation or presences of toxic substances in hematopoietic environment can damage genetic material of stromal and hemopoietic cells (Ludwika et al., 1996) or may interfere in the process of hematopoiesis through the alteration in stromal layer or in cytokine homeostasis (Ebert et al., 2006). It is, therefore, extremely relevant to explore, carcinogens or toxicant's induced response on MSCs in order to predict whether a functional MSCs is available. In this study, we sought to determine the role of inorganic arsenite on MSCs. Inorganic arsenite (iAs^{III}) is classified as a human carcinogen by the International Agency for Research on Cancer (IARC, 1980). In some areas of the world, arsenic contamination level in the ground water can reach up to several times higher than the safe level, as cited by WHO (1996). In portions of the western United States, domestic and public wells have shown increased arsenic levels which correlate to excess fatality risks estimated to be 1 per 9300 to 1 per 6600 (Kumar et al., 2009).

Arsenite and its metabolites can travel to the fetus through the placental barrier and can compromise the normal developmental process including fetal and neonatal stages (Vahter, 2008; Hill et al., 2009; Lianos and Ronco, 2009; Röllin et al., 2009; Concha et al., 1998). Despite the large number of studies on the developmental toxicology of arsenite, information about its impact on MSCs is still lacking.

* Corresponding author. Department of Environmental Health Sciences, 1430 Tulane Ave., Tulane University, Box TW-2100, New Orleans, LA 70112, USA. Fax: +1 504 988 1726.
E-mail address: hwang2@tulane.edu (H. Wang).

0041-008X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2010.01.001

However, studies have shown the potential immunotoxic effects of iAs^{III} and its methylated metabolites on human cord blood or murine bone marrow derived progenitor cells (Ferrario et al., 2008, 2009; Pessina et al., 2002; Pessina et al., 2005). To extend these studies, we aimed at establishing MSCs from bone marrow as an adult stem cell model for the risk assessment of iAs^{III}. In this study, we have chosen trivalent form of arsenic over the pentavalent, due to its association with the higher cytotoxicity potential (Dopp et al., 2005). This study is focused on apoptotic stimulation in MSCs by altering Bcl-2 family proteins and activating intrinsic apoptotic pathway. Arsenite can inhibit cell cycle progression (McCollum et al., 2005) therefore, we also examined the effect of arsenite on cell cycle profile of MSC. In MSCs, the most of the population exists in G1 phase of the cell cycle in contrast to the cancer cell lines where ample number of cells exists in S-phase, therefore the analysis of arsenite targeted cell cycle phase in MSCs can be interesting aspect of the study.

Materials and methods

Culture of MSCs. Mesenchymal stem cells (MSCs) from adult human bone marrow were obtained from the center for the preparation and distribution of adult stem cells of Tulane University (New Orleans, LA, USA), as frozen vials of passage one (P-1) (http://www.som.tulane.edu/gene_therapy/distribute.shtml) which were well characterized for multipotent differentiation, and these cells were negative for hematopoietic markers (CD34, CD36, CD117, and CD45), and positive for CD29 (95%), CD44 (>93%), CD49c (99%), CD49 (>70%), CD59(>99%), CD90 (>99%), CD105 (>99%), and CD166 (>99%). To culture MSCs, alpha-MEM supplemented with FBS (16.5%) (Atlanta Biologicals, Lawrenceville, GA USA) and 2 mM L-glutamine and penicillin/ streptomycin (1%) (GIBCO, Invitrogen, Carlsbad, CA, USA) was used. Cells were maintained with culture conditions such as 37 °C temperature with 5% CO_2. MSCs ($1X10^6$/per vial) were plated in a 15-cm diameter dish and allowed to reach up to 70–75% confluence level (3–4 days). After appropriate confluency level, MSCs were harvested with Trypsin/1 mM EDTA (0.25%) (GIBCO, Invitrogen), and re-plated in various culture plates according to the experimental design. In the entire study, MSCs from passage 2 to 5 were used from the same donor.

Preparation of arsenite treatment. Inorganic arsenite (iAs^{III}) compound was purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA) with the purity of 99%, as stated by Sigma. In each experiment, fresh iAs^{III} treatment was prepared with the various concentrations, i.e., 1 to 40 μM in α MEM in the absence of FBS.

Analysis of cell viability by MTT assay. The MTT assay, an index of cell functionality, is based on the ability of cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) from a yellow water-soluble dye to a dark blue insoluble formazan product. The MTT assay kit was purchased from Sigma (St. Louis, MO, USA). For the assay, MSCs (1×10^4) were plated in 96 multi-well microtiter plates. After overnight incubation, cells were treated with various concentration of iAs^{III} (1 to 40 μM) for 6–48 h. Then the MTT dye was added to each well for the last 2 h of the incubation; the reaction was then stopped by the addition of solubilization reagent. The optical density in each well was then determined at 570 nm using the *FLUOstar Optima* multi-detection plate reader. Background absorbance of medium in the absence of cells was subtracted from all measured values. Untreated (UT) MSCs were considered as 100 % viable cells.

Morphological analysis. MSCs were seeded in 12-well plates in complete culture medium (α-MEM). After overnight incubation, cells were treated with various concentrations of iAs^{III} (1 μM to 40 μM) for 24 h. After desired treatment duration (24 h), for morphology the images were taken using bright field microscope under 20X magnification.

BrdU assay for cell proliferation. Inhibition in DNA synthesis by iAs^{III} was assessed by bromodeoxyuridine (BrdU) incorporation rate using enzyme-linked immunosorbent assay kit (Chemicon International Inc., Temecula, CA, USA) according to the protocol provided by the manufacturer. In brief, MSCs (1×10^4) were plated in 96 well plate and incubated overnight at 37 °C. Next day, cells were treated with iAs^{III} (1 μM to 20 μM) for 24 h. Subsequently, at 22 h BrdU was added and further incubated for 2 h. After that, cells were fixed for 30 min, at room temperature (RT), followed by washing. For detection, anti-BrdU monoclonal (1:200 dilution) was added and incubated for an hour at RT. Cells were washed, and incubated with goat anti-mouse IgG, peroxidase conjugate for 30 min, at RT. After the final wash, the cells were treated with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate followed by an incubation of 30 min at RT (in dark). Finally, reading was taken by a spectrophotometer at the wavelength of 405 nm (FLUOstar plate reader). All samples were assayed in duplicate.

Cell cycle analysis. The effect of iAs^{III} on cell cycle distribution was assessed by flow cytometry using propidium iodide staining. Briefly, 0.3×10^6 MSCs were plated and treated with iAs^{III} for 24 h. At the completion of the treatment, MSCs were harvested using 0.25% Trypsin/EDTA followed by two times washing in cold PBS (0.1% BSA). After that, MSCs were fixed with ice-cold 70% ethanol for overnight at –20 °C. MSCs were then incubated with propidium iodide containing 80 mg L^{-1} RNase A and 50 mg L^{-1} for 30 min. Cell cycle profile was assessed by BD LSRII analyzer (Becton Dickinson, USA). Analysis of the cell cycle was carried out using ModFIT software (Becton Dickinson, USA).

TUNEL assay. To examine apoptosis by TUNEL assay, cells (1.5×10^4) were seeded on 8 well chamber slides (Nunc). Apoptotic cells were detected by *in situ* cell death detection kit (TMR-red, Roche, IN, USA) according to the manufactures' instructions. In brief, cells were washed twice with PBS (0.2% BSA) at 4 °C. Then, 0.2 mL fixation solution (4% formalin) was added to the each well of the chamber slide followed by 30 min incubation on ice, while shaking, and then washed with PBS. Cells were permeabilized with 0.1% Triton X–100, (freshly prepared in 0.1% sodium citrate), and fixed in 70% ethanol for 30 min at −20 °C. After fixation, cells were washed twice with PBS and incubated in TUNEL reaction mixture (dUTP-FITC) for 60 min at 37 °C. Cells were then washed with PBS (twice) and stained with DAPI (4′,6-diamidino-2-phenyl indole) (Sigma Aldrich, St. Louis, MO, USA) for nuclear localization. Samples were analyzed using fluorescence microscope (LICA model), with the range of 570/620 nm and DAPI with the range of 340/380 nm by measuring TMR-red (dUTP-FITC incorporated fragmented) and DAPI (binding to DNA).

Annexin V-FITC apoptosis detection. MSCs (5×10^5) were plated in 10 cm petri dishes for each data point. After overnight incubation at 37 °C, cells were treated with iAs^{III} (5–20 μM). After 24 h treatment duration, cells were dissociated using 0.25% trypsin/EDTA for 1 min (keeping all floating cells). Annexin V-FITC staining was performed using Annexin V-FITC apoptosis detection kit (Sigma Aldrich. St. Louis, MO, USA) in accordance to the manufacturer's instructions. In brief, cells were centrifuged, re-suspended in 1× binding buffer at a concentration of 1×10^6/mL in 100 μL of the solution, transferred in a 5 mL FACS tube, combined with 5 μL Annexin V/FITC (conjugated with fluorescein isothiocyanate) and 10 μL propidium iodide. After incubation for 30 min at RT in dark, 400 μL of 1× Binding Buffer was added to each tube and flow cytometry analysis was performed immediately. Data acquisition and analysis were performed by BD LSRII analyzer (Becton Dickinson, USA) using "CELL Quest" software. Cells that were Annexin V (−) and PI (−) were considered viable cells. Cells that were Annexin V (+) and PI (−) were considered early-