BIOCHEMICAL PHARMACOLOGY 72 (2006) 761–769

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Involvement of hydrogen peroxide in the differentiation and apoptosis of preosteoclastic cells exposed to arsenite

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ARTICLE INFO

Article history:
Received 5 May 2006
Accepted 8 June 2006

Keywords:
Arsenite
Hydrogen peroxide
Osteoclasts
Apoptosis
Tartrate-resistant acid phosphatase (TRAP)
Caspase-3

Abbreviations:
AsO_2, sodium arsenite
DPI, diphenylene iodonium chloride
ERK, extracellular signal-regulated protein kinases
JNK, Jun N-terminal kinase
RANKL, ligand for receptor activator of nuclear factor-κB
RANK, receptor activator of NF-κB
PMA, 4β-phorbol-12-myristate-13-acetate
ROS, reactive oxygen species
TRAP, tartrate resistant acid phosphatase
TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

ABSTRACT

Long-term exposure to sodium arsenite (AsO_2) promotes the development of various cancers. Paradoxically, arsenic also induces pro-myelomonocytic leukemia cell differentiation, and at higher concentrations, apoptosis. The present study investigated the effects of AsO_2 on preosteoclasts. When treated with 2.5–5 μM AsO_2, RAW264.7 cells underwent osteoclast differentiation as evidenced by an increase in the number of multinucleate cells expressing tartrate resistant acid phosphatase (TRAP). The appearance of these phenotypic markers was preceded by a low level increase in extracellular production of H_2O_2 and was prevented by the addition of catalase (4.5 μg/ml), an enzyme that removes H_2O_2. Only at high concentrations (10–25 μM) of AsO_2 was a significant loss of cell viability and a high level increase in H_2O_2 production (1.5 μM) observed. Apoptosis was blocked by pretreatment with diphenylene iodonium chloride (2 μM), a NAD(P)H-flavoprotein inhibitor, suggesting the involvement of NADPH-oxidase. The data show that AsO_2, dose-dependently, stimulates increasing amounts of H_2O_2 production. Moreover, at concentrations found in tissues of individuals exposed to geochemical AsO_2, osteoclasts underwent an H_2O_2-dependent differentiation. Therefore, chronic exposure to low-level amounts of AsO_2 could result in increased bone resorption and contribute to bone related pathologies.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.06.027

1. Introduction

Arsenic is a highly toxic element that is found ubiquitously in soil and water. Substantial amounts of arsenic are released into the air and water as a consequence of geological activity, the disposal of industrial waste products, the combustion of coal and from the use of arsenical herbicides, insecticides, and rodenticides [1]. The primary routes of arsenic exposure are through drinking contaminated water and eating contaminated foods.

General health effects associated with extended arsenic exposure include cardiovascular and peripheral vascular disease, hematologic disorders (anemia, leukopenia, and eosinophilia) and multiple cancers of the skin, lung, liver, urinary bladder, kidney, and colon [2]. The development of these seemingly diverse disorders involving cell transformation, proliferation, and cell death is highly dependent on the level, duration and chemical species of arsenic exposure. There are several organic and inorganic species of arsenic that are found in the environment. Of these, inorganic arsenate and arsenite are considered to be the most hazardous, with arsenite being more harmful than arsenate. Arsenate is an inorganic molecular analog of phosphate and as such would be expected to accumulate in the apatite lattice of bone. Indeed, an accumulation of both species was detected in the femoral head of 68.7 ± 8.7 year old people living in southern and central Poland, regions known to have elevated arsenic levels [3]. Although the accumulation and long-term effects of environmental arsenic on bone is of only recent interest, a 1974 report indicated that individuals working with arsenic evidence bone changes that affect locomotion [4]. Based on this report, Lever suggested that the widespread use of arsenic by the cotton industry might account for the abnormal geographic variations in prevalence rates of Paget's disease of bone in Lancashire [5]. Although speculative, he suggested that long-term exposure to elevated levels of arsenic might be a contributing factor in osteoclast-mediated bone resorption in Paget's disease.

Arsenite has been reported to induce signaling from the cell surface to the nucleus by reacting with the sulfhydryl groups and crosslinking GPI-anchored proteins on membrane rafts [6,7]. The initial crosslinking of GPI-anchored proteins on membrane rafts triggers the activation of several signal transduction pathways, including the activation of PI3K, Src family kinases, G-proteins, and Rac1 [6–9]. This interaction can lead to the activation of NADPH-oxidase and the generation of superoxide anion, which either spontaneously or enzymatically is converted to H_2O_2, a membrane permeant ROS.

Hydrogen peroxide has been implicated as an important component in osteoclast differentiation. For example, we and others have reported that H_2O_2 is produced by preosteoclasts and it affects osteoclast differentiation [10–14] and resorption efficiency [10,11,15–22]. Additionally, it has been shown that H_2O_2 is produced by preosteoclasts in response to RANKL treatment [2,23,24]. Thus, at lower concentrations, ROS play a role in receptor activated signaling pathways in osteoclasts [12,22–24]. At higher concentrations of, ROS, cellular components are oxidized, which may result in cell death. Because ROS are a group of highly reactive molecular forms of oxygen, they can oxidize lipids, proteins, and DNA causing severe damage to cell structures and impairing cell functionality [16,25].

The direct effects of arsenic on osteoclasts have not been studied. However, because arsenic compounds are also used therapeutically to treat patients with pro-myelomonocytic leukemia, cells that share at least some characteristics with osteoclast precursor cells, we know that at the cellular level arsenic can initiate differentiation, cause cell cycle arrest, or act as an apoptogen [26–30]. Moreover, arsenic can induce cell signaling.

The understanding of the signaling pathways necessary for osteoclast differentiation has advanced tremendously over the last few years and new proteins and other intracellular mediators continue to be discovered; exposure to arsenite may trigger osteoclast differentiation. However, at present, no one has evaluated the direct effects of arsenic on osteoclast differentiation from myelomonocytic precursor cells. Given the presence of arsenic in the peripheral blood and bone, the direct effects of arsenic on pro-myelomonocytic leukemia cell differentiation and apoptosis and the production of ROS by other cell types in response to arsenic, we explored the influence of arsenic on osteoclast differentiation and apoptosis.

2. Materials and methods

Diphenylene iodonium chloride (DPI), an NADPH-oxidase inhibitor [31,32], catalase, which removes H_2O_2 by converting it to water and oxygen [33], phorbol 12-myristate 13-acetate (PMA), a known activator of NADPH-oxidase, sodium arsenite (AsO_2), neutral red, and sodium tartrate were purchased from Sigma–Aldrich, St. Louis, MO.

2.1. Cell culture

The RAW264.7 murine macrophage cell line was used for the current studies (ATCC (no. TIB-71)). This cell line undergoes osteoclast differentiation in response to receptor activator of NF-κB ligand (RANKL) [11,34] (Angeles, CA). Cell were maintained in 75-cm^2 tissue culture flasks in DMEM (Cellgro Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C and incubated in a humidified atmosphere of 5% CO_2. Cells were passaged once a week before reaching confluency by transferring 0.3 ml of the 1 ml of trypsinized cells to 20 ml of fresh medium. After 18 passages, cultures were replaced with cells from early passage stocks that were stored in liquid nitrogen.

For the current experiments, cells were plated at a density of 3×10^4 cells/well in a 24-well tissue culture plate (Costar, Corning Inc., Corning, NY) in DMEM phenol red free supplemented with 10% heat-inactivated fetal bovine serum.

2.2. Sodium arsenite preparation and exposure

AsO_2 was diluted in distilled water at a concentration of 1 mM and was stored at 4 °C. Fresh stock solutions were made for each experiment. The amounts of AsO_2 used in this study were within the range of the plasma levels (0–25 μM) found in the population of Northeastern Taiwan [35] and those achieved in acute pro-myelomonocytic leukemia patients [36]. Cells were

exposed to AsO_2 (0–25 μM) for 24 h in DMEM containing heat-inactivated 2.5% FBS. After 24 h, fresh DMEM containing heat-inactivated 10% FBS without AsO_2 was added to the cells.

2.3. Extracellular H_2O_2 measurements

Extracellular H_2O_2 production was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes; Eugene, OR) according to manufacture's recommendations. Cells were simultaneously incubated in the presence of Amplex Red (10-acetyl-3.7-dihydroxyphenoxazine in PBS supplemented with 4.5 mg/ml glucose; Cellgro Mediatech, Inc., Herndon, VA) and either PMA (50 ng/ml) or AsO_2 (0–25 μM). The production of H_2O_2 was monitored from 0 to 3 h after the addition of PMA or AsO_2 as a change in fluorescence at 560 nm excitation and 595 nm emission (Tecan plate reader, Research Triangle Park, NC). A reagent H_2O_2 standard curve was used to calculate H_2O_2 concentration.

2.4. Tartrate-resistant acid phosphatase (TRAP) histochemistry

Cells were treated with AsO_2 (0–25 μM) for 24 h ± catalase (4.5 mg/ml) to remove H_2O_2. After 24 h, the medium was replenished and the cells were incubated for an additional 3 days in the absence of AsO_2 ± catalase. On day 5, TRAP activity was determined using the Acid Phosphatase Assay Kit (Sigma Diagnostics Inc., St. Louis, MO) according to manufacture's recommendations, with the exception that cells were fixed in 2% paraformaldehyde for 15 min at room temperature, rinsed, and then incubated in ice cold methanol for several minutes, additionally fresh sodium tartrate was prepared and added at a final concentration of 50 mM. A red precipitate was observed in cells containing TRAP activity.

2.5. Cell number and viability

Cells were exposed to AsO_2 for 24 h and cell counts were determining using hemacytometer. Cell viability was measured using the neutral red assay. This assay is based on the ability of live cells to take up the dye [37]. In brief, neutral red (50 μg/ml) was added to the medium and the cells were incubated at 37 °C. After the media was removed, cells were washed with 1% formaldehyde–1% $CaCl_2$ solution, and the dye was extracted with 0.2 ml of 1% acetic acid and 50% ethanol for 10 min. Optical density of the extract solution was determined in triplicate at 540 nm in a 96 well plate. Since the amount of cell-associated neutral red is proportional to the number of live cells, the decrease in absorbance at 540 nm provides a direct measurement of cell death.

2.6. Cellular apoptosis

Cells were treated with AsO_2 (0–25 μM) and apoptosis was measured by the TUNEL assay. The TUNEL assay takes advantage of the fact that during apoptosis, between nucleosomes, nuclear endonucleases digest genomic DNA into fragments of multiples of approximately 200 bp. To measure the fragmented DNA, the nucleotide ends were labeled using the Klenow FragEL Kit (Ocogene Research Products, Cambridge, MA) according to manufacturer's instructions and the oxidized diaminobenzidine (brown precipitate) product was visualized by light microscopy as described above. To improve detection of TUNEL positive cells, cells were not counterstained [23,38].

2.7. Caspase-3 activation assay

Caspase-3 is a downstream effecter of the apoptotic response. To confirm that AsO_2 induced preosteoclast apoptosis, we evaluated the activity of this enzyme in treated cells using the caspase substrate, PhiPhiluxG1D2 (OncoImmunin, Inc., Gaithersburg, MD). The proteolytic cleavage of PhiPhiluxG1D2 generates a fluorescent product that is proportional to caspase-3 activation and activity. Cells were treated for 15 min with either 2.5 or 25 μM AsO_2. Some wells were pretreated with 2 μM DPI for 30 min and then 2.5 or 25 μM AsO_2 was added to medium containing fresh DPI to determine if inhibition of H_2O_2 production by NADPH-oxidase would alter caspase-3 activity. Cells were then washed twice and incubated with 10 μm PhiPhiluxG1D2 for 1 h at 37 °C. Excess substrate was removed, cells were washed twice and the cellular fluorescence captured.

2.8. Image acquisition, capture and analysis

The images were acquired on an Olympus IX50 inverted microscope (Olympus, Japan) with either a SPOT digital cooled CCD camera (Diagnostic Imaging Inc., Sterling Heights, MI) or an Olympus Fluoview inverted confocal microscope (Olympus, Melville, NY) equipped with a long-distance lens with a specialized cap to permit evaluation of fluorescence through the plastic dish. Multiple fields were acquired and analyzed with Image Pro Plus Software (Media Cybernetics, Silver Springs,

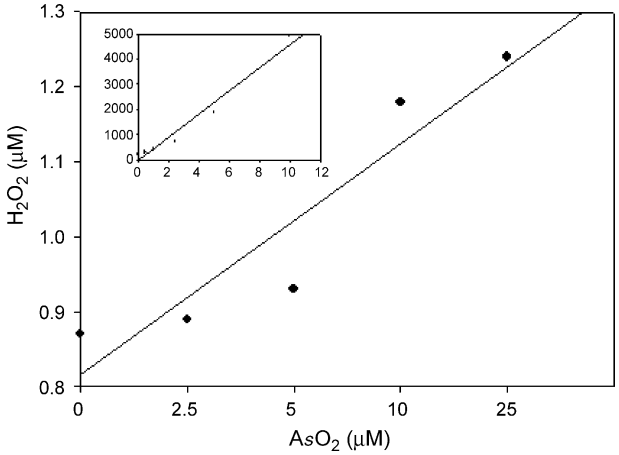

Fig. 1 – H_2O_2 production by RAW264.7 cells in response to AsO_2 or 4beta-phorbol-12-myristate-13-acetate (PMA), a known activator of NADPH-oxidase. Extracellular H_2O_2 production was determined using the Amplex Red assay using the H_2O_2 standard curve (inset). The amount of H_2O_2 produced in response to 0–25 μM AsO_2. The increase in H_2O_2 production was proportional to the amount of AsO_2. Representative data are presented from one experiment done in triplicate. Three independent experiments were performed. Data are expressed as the mean ± S.E.M.

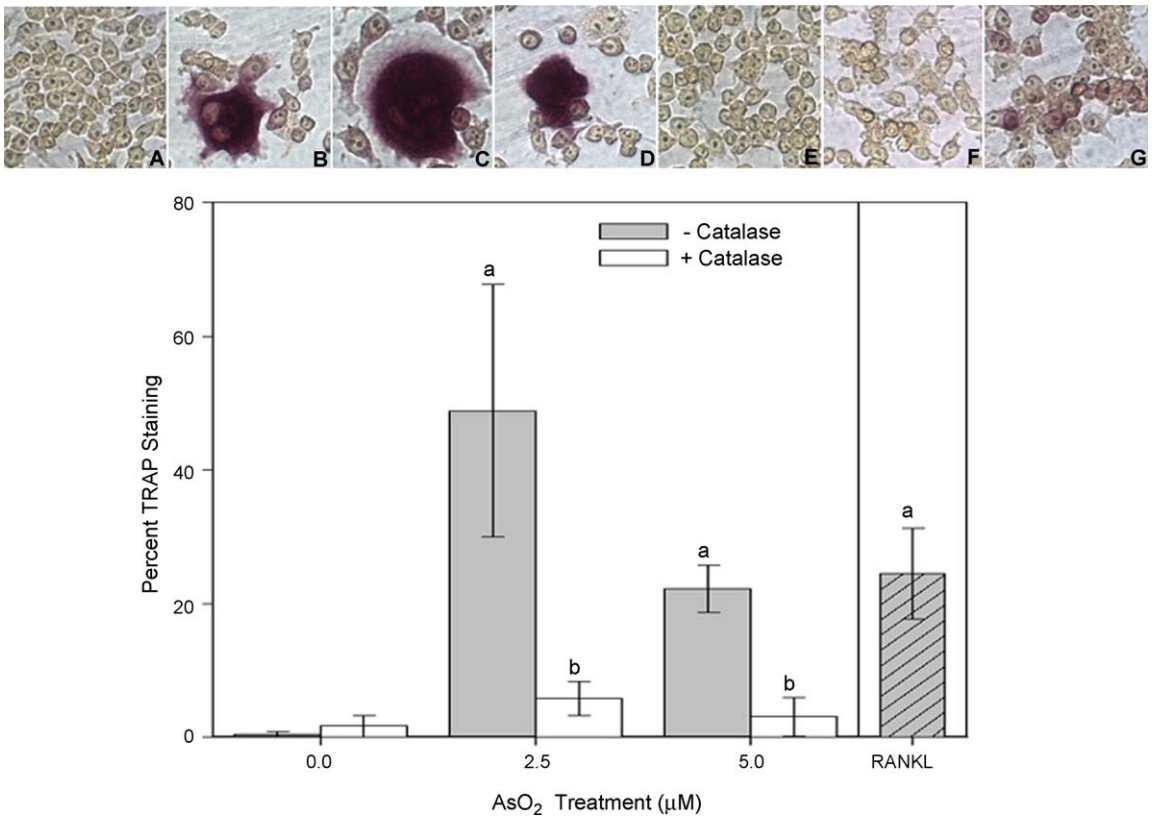

Fig. 2 – Tartrate-resistant acid phosphatase (TRAP) histochemical stain for enzyme activity and multinucleate cell formation after exposure to AsO_2. RAW264.7 cells were exposed to (A) 0 μM AsO_2, (B) 2.5 μM AsO_2, (C) 5 μM AsO_2, (D) RANKL (35 ng/ml), (E) 0 μM AsO_2 plus catalase (4.5 μg/ml), (F) 2.5 μM AsO_2 plus catalase, (G) 5 μM AsO_2 plus catalase, or (H) TRAP quantitation of percent positive staining. After 24 h, fresh medium without AsO_2 was added to the cells. Fresh medium with catalase or RANKL was added to (D–G). TRAP histochemistry was performed on day 4. In cell preparations that did not receive AsO_2 (control), an occasional multinucleate cell and some TRAP activity was observed. Treatment of the cells with AsO_2 increased multinucleate cell formation and both the number of TRAP expressing cells and the amount of TRAP per cell as compared to control. The mean increase in TRAP activity in response to 2.5 μM AsO_2 was higher than the 5 μM AsO_2 mean, which was comparable to RANKL induced TRAP activity. However, there was no statistical difference between the three treatments. The observed increase in TRAP in response to AsO_2 exposure was inhibited by the addition of catalase, an enzyme that catalyzes the conversion of extracellular H_2O_2 to water and oxygen. Significant differences: a, significant increase as compared to control $p < 0.05$; b, significant decrease as compared to AsO_2 treatment $p < 0.05$. Images (60×) displayed are representative of triplicate field observations from three independent experiments.

MD). Exposure time and all microscope settings were determined by imaging the experimental group with the highest staining intensity first and then duplicating the conditions and time for all other samples. TRAP quantitation of percent positive staining was determined by area of positive colorimetric staining by TRAP divided by total cell area. Density mean was determined in both the caspase-3, after background non-specific fluorescence was subtracted, and the TUNEL assay by defining each individual cell area after which density measurements were performed by Image Pro Plus software. The density mean is the mean of all the cells per time point.

2.9. Statistical analysis

All experiments were repeated 3–5 times. Similar results occurred in each replicate. Data are expressed as the mean ± S.E.M. or as a percentage of control activity. Data are shown as the mean ± S.E.M. of the percentages and reported as fold increase over control. Data presented here were analyzed using a one-way ANOVA and a Tukey post hoc test, with the exception of the TRAP image analysis and caspase-3 image analysis, which were analyzed using a Kruskal–Wallis one-way ANOVA on ranks with a Dunn's method multiple comparison procedure. Significance was assessed when $p < 0.05$. Correlation between cell counts and neutral red uptake was calculated using Pearson's r.

3. Results

3.1. H_2O_2 production by preosteoclasts after exposure to sodium arsenite

To evaluate the response of preosteoclast exposure to AsO_2, the extracellular production of H_2O_2 was measured. Preosteoclastic RAW264.7 cells were exposed to a known

NADPH-oxidase activator, phorbol myristate acetate (PMA) or AsO_2 at concentrations ranging from 1 to 25 μM. Cells incubated under the same conditions in the absence of AsO_2 served as controls. The amount of H_2O_2 produced by cells was calculated based on arbitrary fluorescence units of a 0–10 μM H_2O_2 standard curve (Fig. 1; H_2O_2 standard curve inset). There was a small increase in extracellular H_2O_2 production by 2 h in response to 2.5–5 μM AsO_2 (0.89 ± 0.10 and 0.93 ± 0.12 μM, respectively) as compared to 0 μM AsO_2 (0.87 ± 0.09 μM) (Fig. 1). At higher concentrations of AsO_2 (10–25 μM) the amount of H_2O_2 detected in the medium was increased (1.18 ± 0.19 and 1.24 0.18 μM, respectively) and was similar to the level of H_2O_2 produced by cells stimulated with 50 ng/ml PMA (1.51 ± 0.10 μM).

3.2. TRAP expression and activity after exposure to sodium arsenite

We and others have reported that the production of H_2O_2 initiates an early stage of osteoclast differentiation characterized by high levels of TRAP expression and activity, and multinucleate cell formation [10–14]. In the present study, when exposed for 24 h to AsO_2 at concentrations that induce low and extended levels of H_2O_2 production, RAW264.7 cells showed increased TRAP activity and became multinucleate (Fig. 2). These cells had similar numbers of TRAP positive and multinucleate cells as those treated with 35 ng/ml of RANKL. Cells incubated in the absence of AsO_2 did not contain measurable TRAP activity. The percent of TRAP staining in cells exposed to 2.5 μM, 5 μM AsO_2 or 35 ng/ml of RANKL was significantly increased above the control cells.

To remove H_2O_2, catalase (4.5 μg/ml) was added. The percent of TRAP staining in cells exposed to 2.5 or 5 μM AsO_2 in the presence of catalase was decreased to essentially control cell amounts, indicating the involvement of H_2O_2 in osteoclast differentiation.

3.3. Cell viability and number after exposure to sodium arsenite

To investigate the toxicity of AsO_2, preosteoclasts were treated with AsO_2 at concentrations ranging from 2.25 to 25 μM for 24 h and the percent inhibition of cell viability, as indicated by neutral red uptake, was calculated relative to control (Fig. 3). There was a dose-dependent decrease in neutral red absorbance. Cell viability in response to 25 μM AsO_2 was significantly lower than in response to 10 μM AsO_2, just as the viability for cells exposed to 10 μM was significantly less than that observed in response to 2.5 and 5 μM. Finally, cell counts were also performed and showed significant correlation with the neutral red results ($r^2 = 0.867$; p, 0.05).

3.4. TUNEL staining analysis of cells exposed to sodium arsenite

To assess whether cells treated with AsO_2 were undergoing apoptosis, cells were stained by the TUNEL procedure. TUNEL staining was performed on cells pre-treated for 24 h with 0, 5, or 25 μM AsO_2. Data presented in Fig. 4 show that the untreated cells (control) are flattened, indistinct and TUNEL negative. Cells treated with 5 μM AsO_2 show a diffuse stain that is not localized to the nucleus. After treatment with 25 μM AsO_2, RAW264.7 cells exhibit TUNEL positive nuclei. The increase in mean stain density in cells exposed to 25 μM AsO_2 was significantly greater than staining intensity in control cell samples and cells exposed to 5 μM AsO_2.

3.5. Caspase-3 activation analysis of cells exposed to sodium arsenite

To determine further the involvement of apoptosis in mediating preosteoclast cell death, caspase-3 activation was measured. Cells were treated with AsO_2 for 15 min prior to incubation for 1 h with the caspase-3 substrate, PhiPhilux G1D2 (Fig. 5). Note, that, while a few cells exposed to 5 μm AsO_2 stain positively, the staining is punctate. The mean fluorescence density showing caspase-3 activation in cells exposed to 5 μM AsO_2 was comparable to control cell fluorescence. With 25 μM AsO_2 treatment, the stain becomes diffusely distributed throughout the cytoplasm and a greater percentage of the cells contain caspase-3 activity as evidenced by the doubling in the mean fluorescence density as compared to non-exposed controls.

To determine the involvement of H_2O_2 in the AsO_2-induced caspase-3 activation, some cells were pre-treated with DPI (2 μM) to prevent H_2O_2 production. Cells pre-treated with DPI

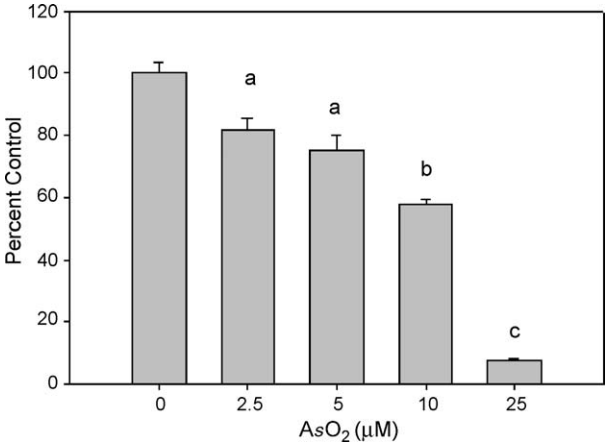

Fig. 3 – Cells were counted and cell viability after exposure to AsO_2 was determined using neutral red uptake by live cells. Cells were exposed to 0–25 μM AsO_2 for 24 h in DMEM containing 2.5% FBS. Cells not exposed to AsO_2 but incubated in the same medium served as controls. These cells were continued to proliferate during the 24 h incubation. There was a dose-dependent decrease in neutral red absorbance as compared to the control. Cell viability in response to 25 μM AsO_2 was significantly lower than in response to 10 μM AsO_2, just as the viability of cells exposed to 10 μM AsO_2 was significantly less than that observed in response to 2.5 or 5 μM AsO_2. Significant differences: a, significant difference from control $p < 0.05$; b, significant difference from control and 2.5 and 5 μM AsO_2 $p < 0.05$; c, significant difference from all four $p < 0.05$. Data are expressed as the mean percentage of control ± S.E.M. for three independent experiments.

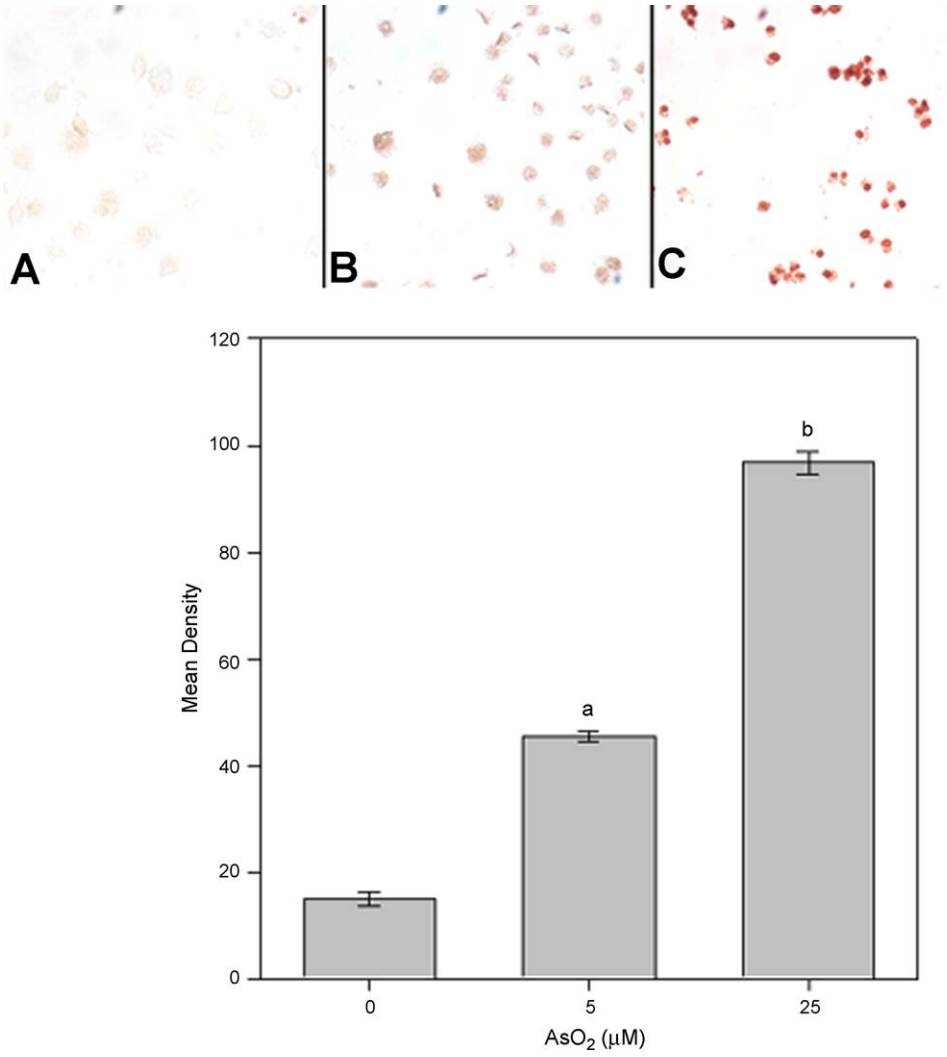

Fig. 4 – Apoptosis after cell exposure to AsO_2 was determined using the TUNEL assay. This assay detects fragmented DNA as a sign of programmed cell death. 1.2×10^5 cells were exposed to (A) 0 μM AsO_2, (B) 5 μM AsO_2, or (C) 25 μM AsO_2 for 24 h. (D) The data are presented as the mean density of TUNEL staining. In cell preparations that did not receive AsO_2 (control), no TUNEL positive cells were observed. The nuclear localization of the TUNEL stain after exposure to 25 μM AsO_2 showed death by apoptosis. Significant differences: a, significant difference from control $p < 0.05$; b, significant difference from control and 5 μM AsO_2 $p < 0.05$. Data are expressed as the mean percentage of control ± S.E.M. Images (20×) displayed are representative of triplicate field observations from three independent experiments.

did not show caspase-3 activation and the mean fluorescence densities for cells exposed to AsO_2 or H_2O_2 were lower than those of the non-exposed control. The addition of H_2O_2 (1.5 μM) to both DPI pretreated and untreated cells served as positive controls. Although added as a bolus, it shows that an H_2O_2 concentration of 1.5 μM will initiate cellular apoptosis.

4. Discussion

The results of our investigation show that the production of H_2O_2 in response to AsO_2 is due to the activation of NADPH-oxidase and that H_2O_2 production induces the differentiation of RAW264.7 cells into osteoclast-like cells. The amount of H_2O_2 produced was dependent on the concentration of AsO_2 to which the cells were exposed. Low micromolar amounts of AsO_2 (2.5–5 μM), within the range detected in peripheral blood and bone, stimulated the production of low micromolar amounts of H_2O_2, which induced multinucleate cell formation and TRAP activity; an enzyme that is highly expressed in osteoclasts during the differentiation of these cells. In contrast, high amounts of AsO_2 (10–25 μM) stimulated an increase in H_2O_2 production equivalent to the amount of H_2O_2 produced in response to PMA. It was only at these elevated concentrations that the preosteoclasts underwent a caspase-3 related apoptosis. Both the expression of TRAP and the activation of caspase-3 were dependent on the production of H_2O_2, which precedes these two events. Therefore, the amount of AsO_2 and the threshold of ROS production determine the susceptibility of the cells to differentiate or undergo apoptosis.

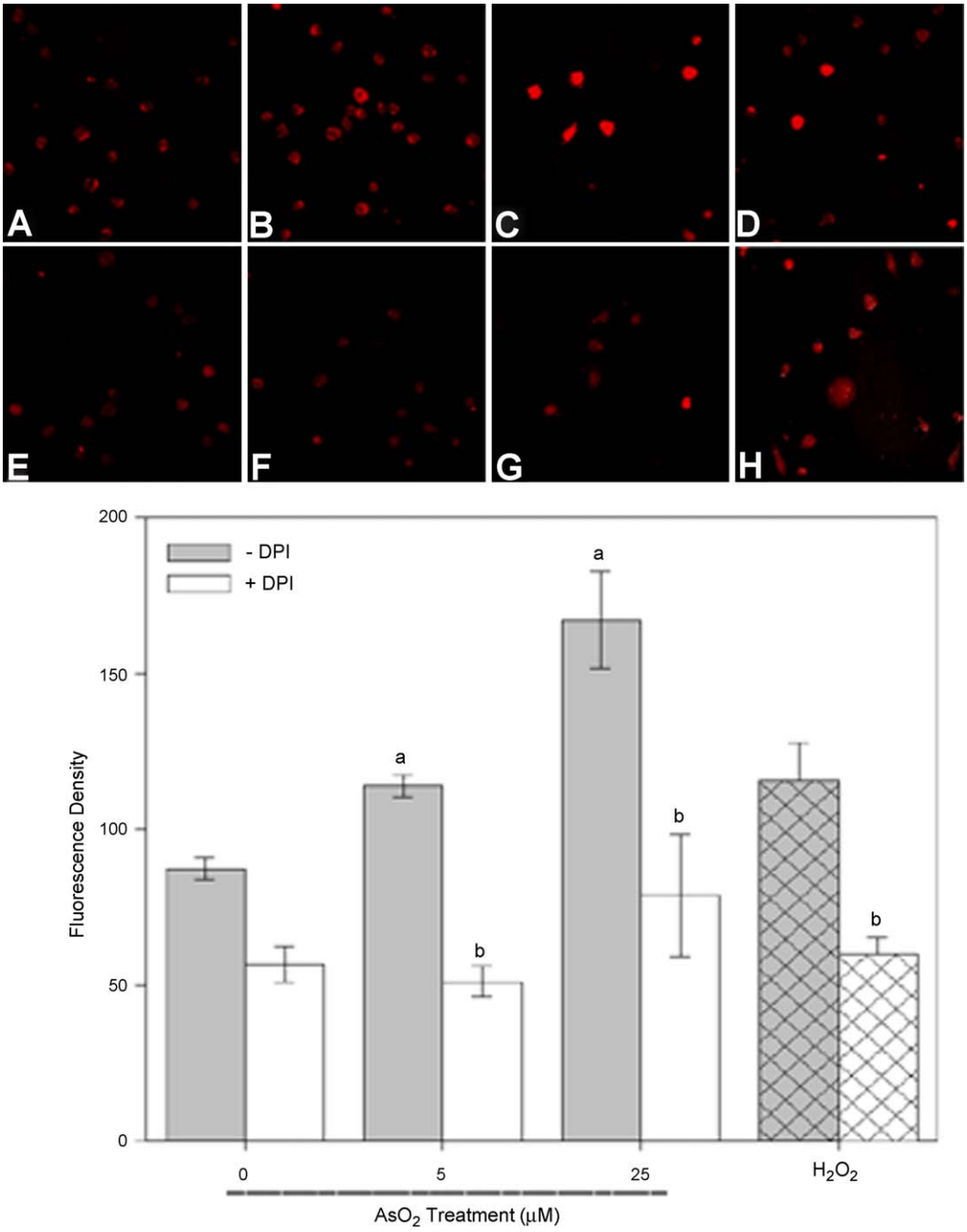

Fig. 5 – Caspase-3 activation after cell exposure to AsO_2 was determined using the caspase substrate, PhiPhiluxG1D2. 1.2×10^5 cells were exposed to (A) 0 μM AsO_2, (B) 5 μM AsO_2, (C) 25 μM AsO_2, (D) 1.5 μM H_2O_2, (E) 0 μM AsO_2 plus 2 μM DPI, or (F) 5 μM AsO_2 plus 2 μM DPI, (G) 25 μM AsO_2 plus 2 μM DPI or (H) 1.5 μM H_2O_2 plus 2 μM DPI for 15 min and substrate was added for an additional hour. In cell preparations that did not receive AsO_2 (control), no caspase-3 activity was observed. Cells exposed to 25 μM AsO_2 or 1.5 μM H_2O_2 were bright red indicating caspase-3 activation. To inhibit H_2O_2 production, cells were pretreated for 30 min with DPI. The data show that AsO_2 through an H_2O_2-mediated process activated a caspase-3 dependent apoptosis and that DPI inhibited caspase-3 activation. Significant differences: a, significant increase as compared to control $p < 0.05$; b, significant decrease as compared to AsO_2 or H_2O_2 treatments $p < 0.05$. Images displayed are representative of triplicate field observations from three independent experiments and data are expressed as the mean percentage of control ± S.E.M.

Our findings agree with others, that arsenic has multiple affects depending on the concentration to which the cells are exposed. In other cell types, arsenic can initiate cell differentiation, act as an apoptogen, affect cell transformation or cause cell cycle arrest [26–30]. McCabe et al. [28] showed that low concentrations of arsenic (≤1 μM) potentiated vitamin-D_3-induced differentiation of U937 myelomonocytic leukemia cell line [28]. Whereas, concentrations of >10 μM rapidly induced

cell death irrespective of cell cycle phase. Intermediate concentrations of arsenic (5–10 μM) were cytostatic initially, but after cell division induced caspase-3 dependent apoptosis. Yi et al. [29] showed that ROS are involved in determining the susceptibility of pro-myelomonocytic leukemia cells to arsenic trioxide induced apoptosis [29]. Chou et al. [39] reported that arsenic induces the expression of NADPH-oxidase in NB4 pro-myelomonocytic leukemia cells and that this enzyme plays a major role in ROS production and cytotoxicity [39]. The RAW264.7 cells that were used in our study already contain NADPH-oxidase and produce H_2O_2 in response to PMA, so further expression is not required. Thus, the RAW264.7 cells are a more mature cell line as compared with the NB4 cells and produce H_2O_2 after exposure to AsO_2. Further studies are needed to determine the H_2O_2-dependent mechanism by which AsO_2 induces osteoclast differentiation.

The present study was designed to investigate whether AsO_2 affected preosteoclast differentiation or apoptosis and the involvement of H_2O_2 production in these processes. Our findings show that preosteoclasts produce H_2O_2 after exposure to AsO_2 and dependent on the concentration of AsO_2 and consecutively the amount of H_2O_2 produced, these cells undergo differentiation or apoptosis. At reported in vivo concentrations of <3 μM, AsO_2 induced an H_2O_2-dependent differentiation of osteoclasts but not apoptosis. Thus, chronic low level arsenite exposure could increase bone resorption/remodeling by promoting osteoclast differentiation. It is therefore possible that arsenic could contribute to the development of Paget's disease or other osteoclast dependent bone related diseases, such as osteoporosis.

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