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Original Article

Arsenic Induces DNA Damage via Reactive Oxygen Species in Human Cells

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Abstract

To elucidate arsenic-induced oxidative DNA damage, the genotoxicity of arsenic in human cells was comparatively studied with single cell gel electrophoresis (SCGE) assay in combination with the observation of the protective effects of dimethyl sulfoxide (DMSO) and catalase. Arsenic, at the concentration of 2.4 μM by coincubation for 24 hours, significantly induced DNA damage in HL60, a human promyelocytic leukemia cell line. In contrast, significant DNA damage was found in human mononucleocytes at the concentration of 4.8 μM or above. The cells were incubated separately with DMSO (12 mM/l), a well-known hydroxyl radical (OH^-) scavenger, and catalase (1,300 U/ml), a hydrogen peroxide (H_2O_2) scavenger, for 6 hours and then further coincubated with various concentrations of arsenic for 24 hours at 37°C and 5% CO_2. The findings showed that both DMSO and catalase significantly reduced the arsenic-induced tail moment, a parameter of total damaged DNA, in HL60 and mononucleocytes. Hence our findings indicate that arsenic, with micromolar concentrations, induces typical and various extents of DNA damage in human cells via reactive oxygen species in a dose-dependent manner.

Key words: single cell gel electrophoresis (SCGE) assay, DNA damage, arsenic, reactive oxygen species, human cells

Introduction

Arsenic is widely distributed in the environment and is responsible for human multiple malignant tumors and internal diseases[1)]. However, its carcinogenic effects as well as noncarcinogenic effects are still not fully understood (for a review, see Rossman et al.[1)]). Studies have shown that its carcinogenesis may be attributed to inhibition of DNA repair by inhibiting repair enzymes of dithiol groups[2–4)]. Recently, it has been associated with generation of reactive oxygen species[5–7)]. A study using an earlier version of the alkaline elution method has indicated that arsenic could induce DNA strand breaks in human fetal lung fibroblasts[8)]. The cells with different differentiation or dividing ability may differ in response to arsenic-induced genotoxicity, which is little reported. Single cell gel electrophoresis (SCGE) assay is a rapid, reliable and sensitive method that detects DNA damage and repair efficiency in individual cells[9)]. We comparatively studied the genotoxicity of arsenic in human promyelocytic HL60 and unstimulated human lymphocytes from peripheral blood of a healthy male using this technique, and simultaneously observed the protective effects of DMSO, a well-known hydroxyl radical scavenger, and catalase, a hydrogen peroxide scavenger, for elucidation of the genotoxicity of arsenic in human cells and its mechanisms.

Materials and Methods

Cell and reagents

HL60 cells were supplied by the Japanese Cancer Research Resources Bank (Japan). Mononucleocytes were separated from whole blood of a healthy male using a Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Sweden). Dimethyl sulfoxide (DMSO) and arsenic trioxide (As_2O_3, prepared in Ca^{2+}/Mg^{2+}-free PBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI 1640 was obtained from Nikken (Japan). Penicillin and streptomycin were purchased from Gibco-BRL (USA). Ethidium bromide was from Sigma (St. Louis, Mo, USA), and catalase from Boehringer Mannheim GmbH (Germany).

Cell treatment

HL60 cells were grown in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Mononucleocytes were separated from peripheral blood of healthy males using a Ficoll-Hypaque solution. Briefly, 5 ml of blood was taken, mixed in 5 ml of PBS and centrifuged over 10 ml of Ficoll-Hypaque solution. Mononucleocytes above the Ficoll-Hypaque layer were washed twice with PBS and cul-

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tured in RPMI 1640 complete culture medium. About 95% of the cells present in the fraction were mononucleocytes with 95% viability, and about 60% lymphocytes from the original blood sample with the separation method described in Ficoll-Paque instructions. They are collectively referred to as mononucleocytes, and were used immediately for experiments.

The cells (2.5×10^6 cells/60-mm dish) were pre-treated with DMSO (12 mM) and catalase (1,300 U/ml) separately for 6 hours, and then incubated with various concentrations of arsenic (0, 1.2, 2.4, 4.8, 9.5 and 19 μM) in RPMI 1640 complete culture medium for further 24 hours at 37°C and 5% CO_2. Equal volumes of solvent, Ca^{2+}/Mg^{2+}-free PBS, were supplied to the cells for incubation under the same conditions as the controls. After treatment, the cells were washed twice with ice-cold PBS and diluted with 37°C-warmed medium at concentrations between 5×10^5 and 1×10^6 cells/ml for SCG assay.

For determination of cytotoxicity of arsenic, the cells were coincubated with various doses of arsenic for 24 hours. The cell population was determined with a hemocytometer before further assay.

Each experiment was performed in duplicate, and repeated at least three times.

SCGE (single cell gel electrophoresis) assay

The method was employed according to Singh et al[9]. Briefly, 0.5% normal agarose (Sigma, St. Louis, Mo, USA) and 0.75% low melting-point agarose (LMA, Nusieve GTG, FMC BioProducts, USA) were melted in PBS separately in a microwave oven. Pretreatment of regular microscopic slides (Matsunami Glass INO, Ltd., Japan) was carried out by coating a thin layer of 0.5% normal agarose (about 20 μl) for firm attachment of the following layers. Then the slide was quickly dried by heating. When still warm, the slide was applied with the first layer of the gel by spreading 80 μl of 0.5% normal agarose and then sealed with a coverslip (20×50 mm). Slides were then left at 4°C for 10 min for consolidation of the gel. Then 25 μl of cell suspension was suspended in 0.75% LMA at 37°C. The second layer of the gel was prepared with 75 μl suspension of the cell-LMA, and the third layer was made with only 80 μl LMA as before.

After consolidation of the third layer at 4°C for 10 min, the coverslip was gently removed. Slides were immediately immersed in alkaline lysis solution (100 mM Na_2-EDTA, 2.5 mM NaCl, 1% Na Sarasinate, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO added fresh) at 4°C for 1 hour to remove cellular proteins. Slides were removed from lysis solution, drained and placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (300 mM NaOH and 1 mM Na_2-EDTA, pH 13) to a level of 0.25–0.5 cm above the slides to allow DNA to unwind for 20 min. Electrophoresis was conducted for the following 20 minutes at 25 V and 300 mA using an electrophoresis compact power supply (ATTO, Japan). Electrophoresis was performed under the conditions of darkness and ice-cold surroundings to avoid further damage to DNA. After electrophoresis, slides were drained and flooded with two changes of neutralization buffer (400 mM Tris-HCl, pH 7.4) for 5 min each. The slides were then stained with 50 μl ethidium bromide (20 μg/ml) and covered with a coverslip for image analysis.

Observation and DNA migration analysis

Observation was made at a magnification of 20×10 under a fluorescence microscope (Olympus BX50) connected with an HCC-600 color camera system and SCG image analysis software (DHS-SCG, Ver.1.0, 1998, KEIO Electronic Ind., Co., Ltd., Japan). One hundred cellular nuclei were measured at random for tail moment, a parameter of the percentage of total DNA in the tail (tail length × tail intensity)[10,11] with a computer software measuring system. The 100 cellular nuclei were recorded simultaneously for frequencies and classes of the comet tail to fit each measuring, based on a 5 classification system of the comet tail according to Kobayashi and Hayashi[11].

Statistics

The SCGE data were transferred into Microsoft Excel (Japanese Windows 98 version), and pooled from the repeated experiments. The statistics was either done with Excel or with SPSS (Japanese Windows version 7.5). Tukey multiple ANOVA comparisons and nonparametric Mann-Whitney multiple comparisons were done. The values are expressed as the Mean±SE or the Mean±SD as the figure legends indicate.

Results

Genotoxicity of arsenic

Fig. 1 shows that arsenic induced typical and various extent of tail moment that represents different extend of DNA damage in both HL60 and mononucleocytes. Arsenic, at the concentration of 2.4 μM, significantly induced DNA strand breaks in HL60 cells (Fig. 2). In contrast, significant changes in DNA tail moment in mononucleocytes were found at 4.8 μM or above. In addition, HL60 showed a higher extent of tail moment than that observed in mononucleocytes at low doses of arsenic. Fig. 2 also shows that arsenic induces DNA damage in a dose-dependent manner with micromole concentrations, both in HL60 (correlation coefficient $r=0.968$, $p<0.001$) and mononucleocytes ($r=0.907$, $p<0.05$).

Protective effects of DMSO and catalase

The protective effects of DMSO and catalase against arsenic-induced DNA damage expressed as tail moment are presented in Fig. 3 and Fig. 4, respectively.

From Fig. 3 it can be seen that DMSO significantly reduced the tail moment caused by arsenic. It was shown that DMSO basically reduced the tail moment to the control levels in all the treated cells with low concentrations of arsenic (≤4.8 μM), and decreased more than 70% of the tail moments in the cells treated with high concentrations of arsenic (9.5 μM). Similarly, catalase equally and significantly reduced the tail moment caused by arsenic (Fig. 4). A similar reduction pattern of the tail moment in the cells incubated with catalase to that in the cells treated with DMSO was observed. No significant difference in tail moment was found between DMSO-As or catalase-As coincubated cells and the control. These findings show that DMSO or catalase basically abolished most of the arsenic-induced DNA damage.

Comparison between the findings from counting the comet tail and from measuring tail moment

The relationship between tail moment and the visual classes of the comet tail in human mononucleocytes is shown in Fig. 5. A very close correlation was found between the finding from counting the comet tail, based on the 5 grading system[11], and that from measuring the tail moment using computer software ($r=0.9231$,

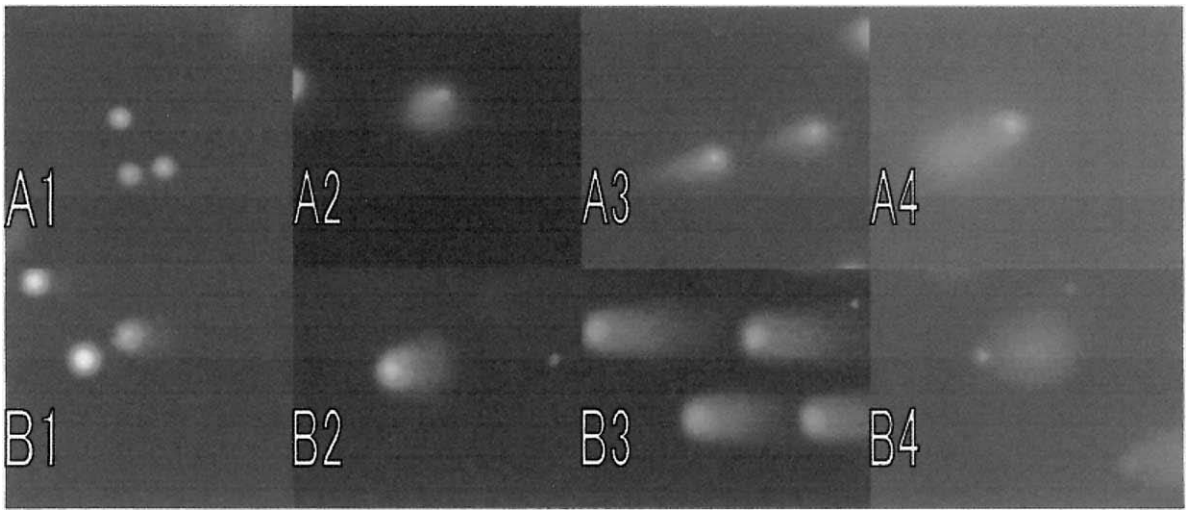

Fig. 1 Various extents of DNA migration (comet tail) induced by arsenic in mononucleocytes (A) and HL60 (B). The 5 classification system of the comet tail was employed according to Kobayashi and Hayashi[11].
A1 shows mononucleocytes of Class 1 (normal) comet tail; A2 shows a Class 2 comet tail; A3 and A4 show Class 3 and Class 4 comet tail, respectively. B1 shows two normal HL60 and a Class 2 comet tail; B2 shows a Class 3 HL60 nucleus; B3 shows a group of Class 4 nuclei that responded to arsenic genotoxicity identically; and B4 shows a Class 5 comet tail. This classification is characterized by the comet tail without clear head. Most of the comet tail nuclei were unable to be measured by the computer software due to the low intensity (see discussion).

$p<0.001$).

Fig. 6 shows the distribution of frequencies and classes of the comet tail in each group of human mononucleocytes treated with various concentrations of arsenic for 24 h.

It can be seen that less than 15% of positive tail moment was observed in normal human mononucleocytes, and in addition, no comet tail of Class 5 was observed. Most significantly, we found a higher correlation between the frequency of Class 5 and doses of arsenic ($r=0.966$, $p<0.001$), than that between the total number of positive comet tails (Class 2 or above) and doses of arsenic ($r=0.867$, $p<0.05$). Using nonparametric Mann-Whitney multiple comparisons, we obtained similar levels of significance to those using parametric Tukey multiple comparisons as shown in Fig. 2.

Discussion

It has been reported that arsenic concentrations in red blood cells of residents exposed to arsenic from drinking water in Taiwan and Blackfoot prevalent areas could reach 1.2 and 15 μM, respectively[12]. We used this range of arsenic concentrations to treat human cells to examine the effect of arsenic on DNA integrity, and found that arsenic significantly induced DNA strand breaks in a dose-dependent manner in HL60 and mononucleocytes, respectively. Accordingly, we confirmed that arsenic induces DNA damage in human cells with micromolar concentrations.

DMSO, a well-known hydroxyl radical ($^{\cdot}OH/OH^{-}$) scavenger, significantly reduced arsenic-induced DNA damage in the present study. Catalase, a hydrogen peroxide (H_2O_2) scavenger,

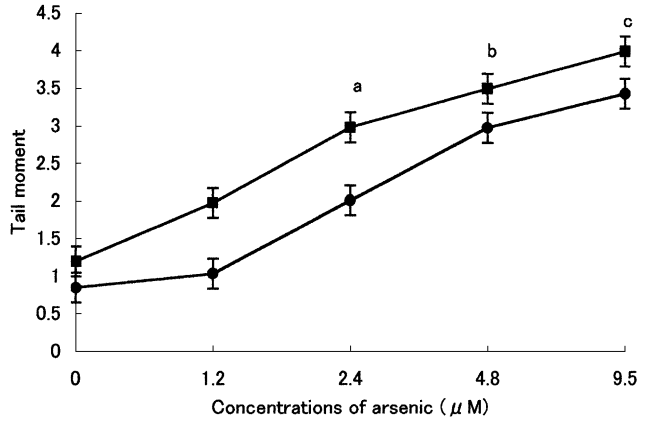

Fig. 2 Arsenic-induced DNA damage expressed as tail moment detected by SCGE assay in HL60 and mononucleocytes by coincubation for 24 h. Values are expressed as the Mean±SE. Black squares: HL60; Black circles: Mononucleocytes.
a, $p<0.05$ (compared between HL60 and controls, Tukey multiple ANOVA comparisons); $p<0.05$ (between HL60 and mononucleocytes); b, $p<0.01$ (between HL60 and controls); $p<0.05$ (between mononucleocytes and controls); c, $p<0.001$ (between HL60 and controls); $p<0.01$ (between mononucleocytes and controls).

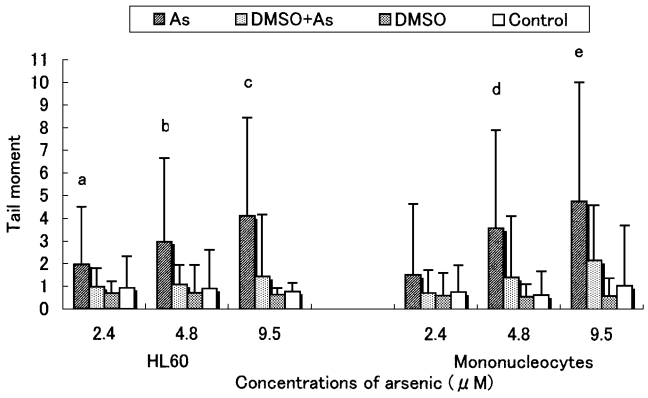

Fig. 3 Protective effects of DMSO against arsenic-induced DNA damage expressed as tail moment in HL60 and mononucleocytes. The cells were incubated with 12 mM DMSO for 6 hours and further co-incubated with arsenic in the presence or absence of DMSO for 24 hours. The solvent was supplied to the cells as the control. Values are expressed as the Mean+SD.
a, $p<0.05$ (compared with DMSO+As, Two-way ANOVA and Tukey multiple comparisons); b, $p<0.001$ (as above); c, $p<0.001$ (as above); d, $p<0.05$ (as above); e, $p<0.05$ (as above).

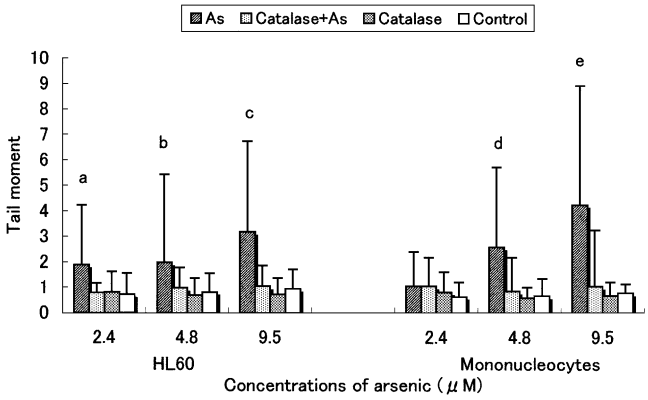

Fig. 4 Protective effects of catalase against arsenic-induced DNA damage expressed as tail moment in HL60 and mononucleocytes. The cells were incubated with catalase (1,300 U/ml) for 6 hours and further coincubated with arsenic in the presence or absence of catalase for 24 hours. The solvent was supplied to the cells as the control. Values are expressed as the Mean+SD.
a, p<0.05 (compared with Catalase+As, Two-way ANOVA and Tukey multiple comparisons); b, p<0.01 (as above); c, p<0.01 (as above); d, p<0.01 (as above); e, p<0.001 (as above).

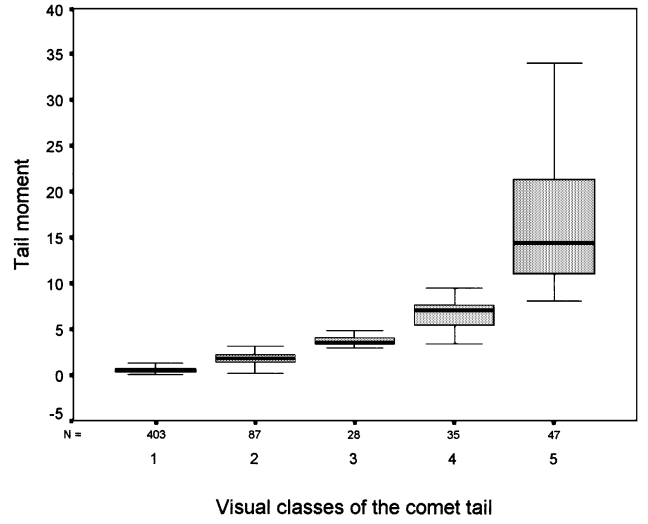

Fig. 5 Relationship between tail moment and visual classes of the comet tail in human mononucleocytes treated with arsenic for 24 h.
Tail moment was measured with computer software, and simultaneously visual classes were recorded, based on the 5 classifications of the comet tail[12], in all the tested concentrations of arsenic from 0, 1.2, 2.4, 4.8, 9.6 to 19 μM, with each group of 100 nuclei examined at random. The boxplots show the median range, and outliers show extreme cases of individual variables of the detected tail moment fitted to the visual classes. Regression equation ws obtained as: $Y=3.30X-3.14$ ($R^2=0.838$, $p<0.001$, $n=600$).

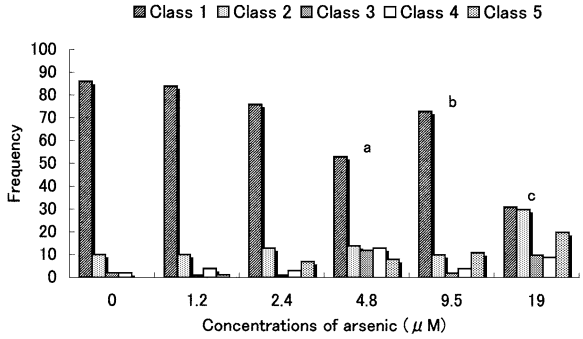

Fig. 6 Distribution of frequencies and classes of the comet tail in human mononucleocytes treated with various concentrations of arsenic for 24 h.
a, p<0.05 (compared with control, nonparametric Mann-Whitney multiple comparisons); b, p<0.01 (as above); c, p<0.001 (as above).

was also shown to significantly reduce arsenic-induced DNA damage. It is suggested that exogenous addition of catalase inhibits extracellular accumulation of H_2O_2 and then reduces intracellular production of the oxygen oxidant. H_2O_2 can diffuse across the plasma membrane during oxidant stress[13]. Inhibition of catalase was shown to increase extracellular H_2O_2 release, which, together with exogenously added H_2O_2, simultaneously increased intracellular H_2O_2[13,14]. Exogenously added catalase was also shown to significantly inhibit intracellular H_2O_2 formation in cells induced by H_2O_2[14,15], and abolish H_2O_2-generated DNA strand breaks[16]. Arsenic was shown to increase both extracellular and intracellular levels of H_2O_2 and superoxide (O_2^-)[17–19]. DMSO has been reported to be one of the strongest scavengers for ·OH-induced DNA strand breaks[16,20]. Both DMSO and catalase significantly inhibited the induction of arsenic-induced DNA strand breaks in HL60 and mononucleocytes in a similar manner in the present study, indicating that H_2O_2 was the main reactive oxygen species released in human cells stimulated by micromolar arsenic. O_2^- is probably involved since hydroxyl radicals can be formed from both H_2O_2 and O_2^-. Hence, the present findings indicated that arsenic induces DNA strand breaks via reactive oxygen species (ROS) in human cells.

ROS-induced DNA damage in cells involves a Fenton reaction that requires two basic components; H_2O_2 and metal ions (e.g. Fe^{2+} or Cu^+). The resulting damaged species of this reaction appear to be hydroxyl radicals, very powerful oxidants, although metal ion peroxide complexes cannot be ruled out[21]. Hydroxyl radicals tend to react with DNA bases not by electron transfer but instead to form adducts, for example by addition to carbon-carbon double bonds (i.e. the 5,6-bond in pyrimidines)[20]. Hydroxyl radical adducts of the bases in DNA can lead to formation of strand breaks. The lesions may have deleterious consequences for cells, such as mutagenesis, carcinogenesis, aging, and apoptosis or lethality[22]. In a biological setting, DNA damaging Fenton reactions must occur in close proximity to DNA because hydroxyl radicals react rapidly with nearly all biological compounds and, thus, do not diffuse very far from their site of generation. Therefore, the formation of oxidative DNA damage in cells is suggested to depend on the rate of production of free radicals and oxidants, the probability that these species diffuse to DNA and the movement of reactive metal ions from cellular sources to DNA. In addition, free radicals and oxidants induce the release of metal ions, which in turn generate more reactive species, such as reactive ions liberated from heme-containing compounds, ion storage proteins and proteins containing ion-sulfur clusters, which can also contribute to oxidative DNA damage[23].

Here we showed that arsenic induced DNA damage through mediation of ROS in human cells. However, the exact mechanisms by which arsenic stimulated or enhanced intracellular accumulation of ROS are still unclear. Previous studies indicated that arsenic might inhibit the activities of catalase and glutathione peroxidase to result in accumulation of H_2O_2[17,24]. It may stimulate superoxide dismutase (SOD) to produce O_2^- and increase activity of heme oxygenase to release reactive ions[17]. It was reported that

arsenic could stimulate cell signaling and activate transcription factors to enhance production of H_2O_2 and O_2^{-}[19]. Other recent studies have suggested that arsenite activates NADH oxidase to produce O_2^{-}, which then causes oxidative DNA damage[25,26]. These studies suggest that arsenic may trigger oxidative stress through multiple pathways, but H_2O_2 and O_2^{-} are the main ROS chiefly involved in arsenic-induced DNA damage, as indicated in the findings of the present study.

Oxidative DNA damage in living cells takes the forms of single strand breaks (SSBs), double strand breaks (DSBs) and alkali-labile sites as well as base damage, and SCGE assay is able to detect these forms of damage[27]. Arsenic was shown to produce alkali-labile sites using the traditional alkaline elution method[2,8]. When DNA is heavily damaged, or alkali-labile sites are accumulated in large numbers in the parental strands, DNA replication may then generate breaks in the undamaged daughter strands, and DNA double strand breaks may consequently occur[2]. Here we provide evidence, with detection by the alkaline comet assay and observation of the protective effects of ROS scavengers, that arsenic probably induces these forms of damage, which encourages further study.

We additionally observed that cells of different dividing ability had different responses to arsenic-induced DNA damage. HL60, a cell line of differentiated dividing cells *in vitro* showed higher sensitivity to lower doses of arsenic-induced genotoxicity, while non-dividing cells such as unstimulated lymphocytes from peripheral blood were relatively resistant to the damage. It was shown that the damage derived from hydrogen peroxide to the cells of efficient repair or dividing cells was easily detected by SCGE assay, and non-dividing cells generally have a relative resistance to low-level damage[27]. The phenomenon could be the feature of non-dividing cells. This could be one of the mechanisms by which the cells exert self-protection and then maintain efficient treatment of low-level damage *in vivo*.

SCGE assay can detect apoptosis. Apoptotic cells are largely made up of fragmented DNA, which forms the typical appearance of the comet tail classified as "without clear head but just visible tail" through conventional SCGE assay[28,29], as either shown in Fig. 1B4 in the present study. It has been reported that arsenic trioxide induces apoptosis in myeloid leukemia cell lines including HL60[24,30]. We found that about 30% of the apoptosis-prone human leukemia cell line HL60 cells were Class 5 in the high dose group of arsenic. The classification of Class 5 is not a confounding factor when assaying for genotoxicity with SCGE assay. Fig. 1 shows that the arsenic-induced DNA damage in HL60 is a serial process from mild to most severe DNA damage that depends on dosage. Time and dose-dependent DNA damage caused by a potent mutagen in HL60 was shown to be consistent with apoptosis detected by alkaline filter elution, SCGE and morphological analysis[31]. A similar finding was also observed in rat neurons with the same technique[32]. These findings, and those of the present study indicate that apoptosis is equally a category of genotoxicity with SCGE assay.

However, when DNA is heavily damaged, it is disassociated in the gel to form the comet tail of Class 5 appearance characterized by "without clear head but just visible tail" through SCGE (Fig. 1B4), most of which cannot be measured by computer software due to its low intensity compared with the background under the present experimental conditions (i.e. concentrations of agarose, and unwinding and electrophoresis time). With increased severity it becomes formless, which results in a high intensity of background but with too few formed nuclei to allow measurement. This suggests either severe DNA damage or the high cytotoxicity of the test agent. In this condition, repeated experiments are recommended by lowering the doses of the test agent or making observations with multiple populations of cells for evaluation of the cytogenetic effect.

SCGE assay is a sensitive, rapid and economical method for detecting cytogenotoxicity. It can be applied to all levels of studies and monitoring. In observation and analysis, using nonautomatic scoring methods of counting the frequencies or numbering the classes of the comet tail could achieve a similar result to that from measuring the DNA migration[27,28]. Here we show that counting the frequencies or numbering the classes of the comet tail could obtain the same results as those from measuring tail moment, a comprehensive index of total DNA damage. A higher correlation was shown between the comet tail of Class 5, than that between total positive comet tail, and doses of arsenic. In addition, a similar significance was obtained between using nonparametric Mann-Whitney multiple comparisons for classes of the comet tail and using parametric multiple comparisons for the tail moment. These findings undoubtedly widen the applications of the technique. This counting method is a time-saving and efficient way to record SCGE-detected DNA damage (counting 100 nuclei takes less than 15 min for a slide, while measuring 100 nuclei takes more than 40 min). It can meet most requirements, especially for quick environmental and human monitoring.

Acknowledgements

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