Early induction of genetic instability and apoptosis by arsenic in cultured Chinese hamster cells

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In order to assess at what time from the beginning of exposure inorganic arsenic can give rise to genetic instability and trigger apoptosis, V79-C13 Chinese hamster cells were treated with 10 µM sodium arsenite for 24 h. Under these conditions, cell survival was >70% and cells showed neither an increase in chromosome aberration frequency nor a delay in cell cycle progression. Investigations, which were carried out every 6 h during the treatment, revealed an early appearance of genetically unstable cells, namely micronucleated, multinucleated and mononucleated ‘giant’ cells, as well as apoptotic cells. Indirect immunostaining using anti-β-tubulin antibody showed severe alterations in spindle morphology after only 6 h treatment, when cells with small spindles whose poles were inside the metaphase plate appeared, and after 12 h treatment, when cells in which spindle assembly had completely failed were observed. These cells, unable to complete mitosis, underwent apoptosis. In fact, cells which turned out to be positive in the TdT-FragEL test had condensed chromatin arranged in metaphase-like plates; their maximum frequency was reached after 24 h treatment. A cytogenetic study was conducted at the end of the period of exposure to arsenic and after post-treatment incubation in fresh medium for up to 5 days. It showed that the percentage of cells with 21 chromosomes (modal number of the cell line) decreased, making way for aneuploid cells. Arsenic, therefore, induced early genetic instability or apoptosis in dividing cells. However, while apoptosis tended to cease when arsenic was removed from the culture medium, the acquired instability remained and propagated within the cell population.

Introduction

Arsenic is widely distributed in nature, being found in food, the soil, water and airborne particles; it derives from both natural and human activities. Human beings are therefore inevitably exposed to it. Many epidemiological studies have confirmed that exposure to arsenic and its compounds can have adverse effects on human health. Inhaling As can cause lung carcinomas in particular, while when ingested in the form of drugs or in food and water it can provoke not only skin, respiratory system, liver and bladder tumours, but is also associated with cardiovascular, neurological and diabetic diseases (Goering et al., 1999). Nevertheless, the reasons why As is carcinogenic have still to be clarified. Many authors have referred to the fact that As can have co-genotoxic effects, interfering with repair systems (Jha et al., 1992; Hartwig et al., 1997; Lynn et al., 1997; Hu et al., 1998), that it can modify the state of DNA methylation (Mass and Wang, 1997; Zhao et al., 1997; Chen, H. et al., 2001) and that it can affect cell proliferation (Gonsebatt et al., 1994; Hamadeh et al., 1999). Other authors have reported that As can induce chromosome aberrations and aneuploidy (Kochhar et al., 1996; Radha and Natarajan, 1998) and that it can also perturb functioning of the mitotic spindle apparatus (Ramirez et al., 1997; Huang and Lee, 1998). Recently, it has also been reported that in HeLa S3 cells treated with As mitosis is arrested and a process of cell death by apoptosis begins (Huang et al., 2000).

To investigate whether As-induced genetic instability occurs at the same time as As-induced apoptosis and whether the two phenomena depend on presence of the poison in the culture medium, we treated V79-C13 Chinese hamster cells with a moderately lethal dose of sodium arsenite and undertook morphological observations, indirect immunostaining, a test for apoptosis and chromosome counting during the period of exposure to As and in the period following its removal.

Materials and methods

Cell culture and arsenite treatment

The V79-C13 Chinese hamster cell line was routinely cultured in D-MEM (BioChrome) supplemented with 5% foetal calf serum (Sigma), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cultures were maintained at 37°C in a 5% CO₂ humidified incubator. These cells have a modal chromosome number of 21, as previously determined (Sciandrello et al., 1996) and regularly checked, with a doubling time of 12–15 h.

Treatments with sodium arsenite (SA) (Sigma) were carried out 18 h after seeding for 24 h; SA was freshly prepared by dissolving in double distilled sterile water. For morphological, immunostaining and apoptosis analyses, 2.0×10⁶ cells were plated on 20×20 mm glass coverslips in 60 mm Petri dishes. For metaphase preparations, 10⁶ cells were cultured in 75 cm² flasks. In each experiment, untreated cells were utilized as controls.

Cell survival

Three hundred cells were plated in P-6 dishes and exposed to increasing doses (5, 10, 20, 30, 40, 50 and 60 µM) of SA in complete medium. After 24 h, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium for 10 days; the colonies were then stained with 0.1% methylene blue. Treatments were performed three times and cell survival was expressed as a percentage of untreated cells.

Morphological observations

Observations were performed every 6 h during the 24 h exposure to 10 µM SA and after post-treatment incubation in fresh medium for 6, 24 and 48 h. The cells were washed twice with PBS, prefixed with methanol added to the culture medium in the ratio 1:1 for 5 min at room temperature, fixed with methanol for 7 min and then stained with 2.5% Giemsa. At least 1000 cells were examined each time and three independent experiments were performed to determine abnormal cell frequencies.

Indirect immunostaining

For the microtubule analysis, cells, washed and fixed as reported above, were incubated with fluorescein (FITC)-conjugated anti-mouse IgG (Sigma) diluted 1:32 for 30 min at 37°C. After washing, the preparations were mounted in

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Fig. 1. Cell survival in increasing concentrations of sodium arsenite. Data are presented as the mean ± SD percentages of three independent experiments.

antifade solution (1 mg/ml p-phenylenediamine dihydrochloride in 1 part PBS and 9 parts 87% glycerol, pH 8.0) containing 2 µg/ml propidium iodide as a counterstaining agent. The slides were then observed under a Nikon fluorescence photomicroscope equipped with a HBO 100 W mercury lamp and a suitable filter.

Apoptosis assay
Cells, washed and fixed as reported above, were assayed for apoptotic chromatin condensation every 6 h during the 24 h exposure to 10 µM SA and after post-treatment incubation in fresh medium for 6, 24 and 48 h. The apoptotic cells were detected using a TdT-FragEL kit (Oncogene), according to the recommendations of the manufacturer. At least 200 cells were scored in each sample and three independent experiments were performed to determine the frequencies of apoptotic cells.

Chromosome counts
Cytogenetic observations were performed in cells exposed to 10 µM SA at the end of the 24 h treatment and after post-treatment incubation in fresh medium for 24, 48 and 120 h. Colcemid (0.1 µg/ml; Ciba) was added 2 h before cells were harvested by trypsinization. Cells were treated with 75 mM KCl for 15 min at 37°C, fixed twice with 3:1 methanol/glacial acetic acid, dropped onto chilled slides, air dried and stained with 2.5% Giemsa. At least 200 metaphases for each sample were scored and three independent experiments were performed to determine the frequency of cells with different numbers of chromosomes. The range of chromosome numbers constituting each ploidy level was calculated according to the ISCN Guidelines for Cancer Cytogenetics (Mitelman, 1991).

Image processing
All photomicrographs for morphological observations, immunofluorescence analysis and apoptosis assay were processed using Adobe Photoshp 3.0 LE software.

Results

Cell survival
Figure 1 shows the survival of V79-Cl3 cells after exposure to increasing doses of SA. The cytotoxic response was dose dependent: at a dose of 5 µM SA cell survival was unaffected, whereas at 10 µM SA cell survival was 76.3 ± 2.61% of untreated cells. This concentration was chosen for further experiments.

Morphological observations
The results of morphological observations performed during the treatment with SA and up to 48 h after release from treatment are shown in Figure 2. Cells with nuclear abnormalities, namely micronucleated and multinucleated cells, steadily increased during the treatment. After only 6 h exposure to SA micronucleated cell frequency was significantly different (P < 0.05, according to Student’s t-test) in comparison with untreated cultures; this difference became more significant (P < 0.02) after 24 h treatment (Figure 2a); 48 h after release from SA treatment (Figure 2a’) a further increase in micronucleated cell frequency was observed (P < 0.001).
Comparison of multinucleated cell frequencies showed a highly significant difference ($P < 0.001$) at the end of the treatment (Figure 2b) and during the period of recovery (Figure 2b').

Some cells with a very large nucleus, termed by us ‘giant’ cells as they were much larger than normal Chinese hamster fibroblasts, grew in size in the presence of SA (Figures 2c and 3a and b); their frequency was highly significantly different ($P < 0.001$) in comparison with untreated cultures after 12 h exposure to SA and for the whole period of treatment. These ‘giant’ cells were not found after SA had been removed from the culture medium (Figure 2c'). In fact, during recovery we saw only those large cells spontaneously present in the untreated cell population.

Round cells with either vacuolated cytoplasm or a condensed and markedly pycnotic nucleus appeared after only 6 h exposure. The frequency of vacuolated cells increased in the period up to 12 h exposure, after which their frequency decreased, while pycnotic cells increased throughout the whole treatment (Figures 2d and 3c). After SA had been removed from the culture medium round cells with vacuolated cytoplasm were not found and the frequency of pycnotic cells showed a dramatic decrease (Figure 2d').

**Apoptotic cells**

A TdT-FragEL kit was used for the apoptosis assay. This allowed us to distinguish apoptotic cells, coloured dark brown, from normal cells, coloured blue-green. Observed under a microscope, the TdT-positive cells were round and small, with condensed chromatin often arranged in such a way as to resemble a metaphase plate. These cells appeared after the first 6 h of exposure and were stained a very dark brown (Figure 4). A quantitative analysis demonstrated that the frequency of TdT-positive cells increased during treatment, showing a significant difference ($P < 0.05$) after only 6 h exposure in comparison with untreated cells (Figure 5). This frequency gradually decreased once SA had been removed.

**Immunostaining**

Indirect immunostaining using anti-β-tubulin antibody highlighted various alterations in the mitotic spindles. In particular, observations conducted after 6 h exposure to SA showed mitotic cells with small spindles inside metaphase plates in which the chromosomes did not connect with the spindle microtubules (Figure 6a and b). After 12 h treatment, mitotic cells in which anti-β-tubulin antibody decorated the whole surface were seen, revealing that spindle assembly had completely failed (Figure 6c). Moreover, multipolar spindles were observed in large cells (Figure 6d).

**Chromosome count**

Chromosome counts carried out after a 24 h SA treatment revealed that the frequency of cells with the modal chromosome number was much lower than for the untreated cells, falling from 60 to <30%. In contrast, the frequency of hyperdiploid cells was higher than for the untreated cells (12.1 versus 3.5%); this percentage remained more or less the same even up to 120 h after treatment had been terminated (14.5%) (Figure 7). The hyperdiploid cells included cells with a near triploid (33 ± 5) and near tetraploid (44 ± 5) chromosome number.

Moreover, the cytogenetic study showed that the chromosome aberration frequency (abs, 2.3%) and the mitotic index (MI, 9.4) of exposed cells did not differ significantly ($P < 0.2$) from those of the untreated cells (abs 1.5% and MI 11.2).

**Discussion**

For a long time As has aroused a particular, perhaps morbid, interest because it has been used as a lethal poison in several
Fig. 4. Normal green cells and arsenite-induced apoptotic dark brown cells, revealed by TdT assay, after (a) 18 h and (b) 24 h exposure, at a higher magnification. Bars represent 10 µm.

Fig. 5. Frequencies of TdT-positive cells during the 24 h treatment with 10 µM SA. Bars represent SD of three independent experiments.

Fig. 6. Examples of arsenite-induced aberrant spindles following indirect immunostaining with anti-β-tubulin antibody. (a and b) Small spindles with scattered chromosomes; (c) a normal spindle and a cell with peripheral fluorescent decoration (in the inset an enlargement of the same abnormal cell shows the chromatin arranged in a metaphase-like shape); (d) tetrapolar spindle in a large cell. DNA was counterstained with propidium iodide. Bars represent 10 µm.

real life stories and in detective thrillers. It is well known that As is ubiquitously present in the environment and may act as a carcinogen, but recently it has been successfully used as a chemotherapeutic agent in treating acute promyelocytic leukemia (Chen, G.-Q. et al., 1997) and multiple myeloma (Munshi, 2001). Therefore, it seems that As has passed from the role of killer to the role of life saver. However, the mechanisms responsible for its anti-carcinogenic effects as well as the cellular and molecular interactions that influence its carcinogenic activity have still to be understood (Pott et al., 2001). Accumulated evidence has shown that As exercises its action on cells which are about to divide, perhaps due to the way As disrupts spindle microtubule dynamics (Huang and Lee, 1998); the derangement of spindle assembly results in apoptosis (Li and Broome, 1999) and this could account for the therapeutic efficacy of As. On the other hand, spindle disturbance can produce aneuploidy, essential in neoplastic development (Pihan and Doxsey, 1999).

Our results, confirming that mitotic spindle assembly is one of the main targets of As, demonstrate that such interference can produce two very different results: cells in the mitotic phase whose mitotic apparatus has been damaged by As promptly either undergo a process of genetic instability or die by apoptosis. Presumably this depends on the concentration of poison in the cells.
Genetically unbalanced cells, namely micronucleated, multinucleated and ‘giant’ cells, were conspicuous after only a few hours of exposure and for the duration of the SA treatment. The multinucleated cells probably arose from mitotic cells with multipolar spindles, in which nuclear envelopes reform and cytokinesis did not occur, while the mononucleated ‘giant’ cells were most likely cells which underwent several DNA replication cycles not followed by cell cleavage.

Genetic instability was maintained for a long time after SA had been removed. Morphological observations carried out up to 48 h after release from treatment showed a further increase in the frequency of micronucleated cells and a constant frequency of multinucleated cells. Similarly, cytogenetic investigations conducted for up to 5 days after treatment revealed that the cells had a wide range of chromosome counts and a constant percentage of them were in a hyperdiploid condition. Therefore, the percentage of cells with the modal chromosome number found for the untreated cells was not recovered. Most likely, the fraction of aneuploid cells was also increased by aberrant division of the ‘giant’ cells, which were no longer found once SA had been removed. This suggests that As can also induce mitotic slippage and polyploidy and, when it is removed, polyploid cells undergo aneuploidy.

The process of programmed cell death was also triggered early, as highlighted by the appearance of round cells with vacuolated cytoplasm, a first sign of apoptosis, or with condensed chromatin often arranged in metaphase-like plates. These apoptotic cells were arrested in mitosis, presumably due to an abortive mitotic apparatus. We found that apoptosis was linked to the presence of SA and gradually decreased when cells were maintained in fresh medium.

It is worthwhile noting that the As dose utilized in this study had no effect on biological end points, such as chromosomal aberrations and cell cycle progression, but it led to apoptosis and, above all, it induced a persistent aneuploidy in V79-C13 cells. As the permanent missegregation of chromosomes in mitosis represents a potential cause of neoplastic cell transformation, we think that further studies are necessary to investigate the effects that relatively low As doses, namely those present in environmental pollution, can have on living organisms.

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References


