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Arsenic-induced DNA hypomethylation affects chromosomal instability in mammalian cells

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Early genetic instability induced in dividing V79-Cl3 Chinese hamster cells by inorganic arsenic, as demonstrated in our previous investigation, was evidenced by aneuploidy and nuclear abnormalities, but not by chromosomal rearrangements. Here we report the results of cytogenetic and morphological analyses performed on the progeny of cells dividing at the end of sodium arsenite treatment after they had been expanded through 120 generations (ASO cells) and then cloned. The acquired genetic instability persisted and was increased by highly unstable chromosomal rearrangements, namely dicentric chromosomes and telomeric associations, which were not seen following acute exposure. A peculiar finding was the preferential involvement of a particular chromosome in dicentric rearrangements observed in some isolated ASO clones. Interestingly, by immunostaining with anti-5-methylcytosine antibodies the genome-wide DNA hypomethylation, induced by arsenic immediately after the acute treatment, was found to affect those ASO clones characterized by aneuploidy and chromosomal rearrangements. These findings demonstrate that short-term exposure to arsenic has long-term effects and suggest that genome-wide DNA hypomethylation enhances genetic instability.

Introduction

Several epidemiological studies performed in different geographic areas have demonstrated that inorganic arsenic is a human carcinogen (1–3). However, the mechanism of its carcinogenicity is not yet known. The lack of cancer induction in *in vivo* animal models (4) makes investigations carried out on *in vitro* cell systems very useful in elucidating the causes of arsenic carcinogenicity. Some studies report that arsenic causes gene amplification, chromosome damage and inhibition of DNA repair as well as global DNA hypomethylation, decrease of DNA methyl transferase activity and protooncogene activation (5,6).

Previously, we reported that V79-Cl3 Chinese hamster cells underwent either early genetic instability or apoptosis when exposed to sodium arsenite (SA). In the observations performed both during and shortly after treatment, genetic instability was manifested in the presence of aneuploid and morphologically abnormal cells, but not by cells with chromosome aberrations (7). As dividing cells turned out to be the most sensitive to SA exposure, due to the arsenic's direct action on the mitotic spindle assembly (8), we later ascertained the fate of genetically unstable cells escaping apoptosis, by harvesting mitotic rounded-up cells at the end of a 24 h treatment. This cell population, examined after ~2 months of subculturing (120 cell generations), was still genetically unstable. In this paper we demonstrate the long-term effects of acute exposure to arsenic and show that the genetic instability was associated with structural chromosome changes and concurrently with DNA hypomethylation.

Abbreviations: 5-MeC, 5-methylcytosine; SA, sodium arsenite.

Materials and methods

Cell culture, arsenite treatment and cloning

V79-Cl3 Chinese hamster cells, referred to as the 'parent' cells, were mutagenized with a moderately lethal dose of sodium arsenite (cell survival >70%) (7); briefly, a population of $>10^7$ V79-Cl3 cells in exponential growth was treated with freshly prepared 10 μM sodium arsenite (SA, Sigma-Aldrich, Milan, Italy) by dissolving it in double-distilled sterile water. After 24 h, the cells were washed twice with phosphate-buffered saline (PBS); the rounded-up mitotic cells were harvested by shake-off and allowed to grow in drug-free medium. These cells were called ASO. After ~2 months of expanded growth, 250 ASO cells were seeded in 90 mm plates; after 10 days, 20 colonies were randomly picked up, marked as ASO 1–20, propagated and then stored in liquid nitrogen.

All cells were routinely cultured in D-MEM (BioChrome, Berlin, Germany), supplemented with 5% fetal calf serum (Sigma-Aldrich), penicillin (100 U/ml) and streptomycin (100 μg/ml); they were subcultured every 3 days. Cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

Cytogenetic analysis

For metaphase preparations, 10^6 cells were cultured in 75 cm^2 flasks for 24 h. Colcemid (0.1 μg/ml, Ciba, Geigy AG, Basel, Switzerland) was added 2 h before cells were harvested by trypsinization. Cultures were processed according to conventional air-drying protocols as described previously (9). Cytogenetic analysis was performed on at least 300 metaphases of ASO population and of each ASO clone; 20 G-banded metaphases of two ASO clones (ASO-13 and ASO-19) were examined to determine the karyotype. Untreated V79-Cl3 cells were also examined.

Morphological observations

Cells (2×10^4) were plated on 20 × 20 mm glass cover slips in 60 mm Petri dishes and, 18 h after seeding, were washed twice with PBS, prefixed with methanol added to the culture medium in the ratio of 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 of each culture were examined to determine nuclear abnormalities.

In situ 5-methylcytosine detection

The technique described by Bensaada *et al*. (10) has been modified as follows: the slides, covered with PBS, were irradiated under a germicidal lamp (15 W) at a distance of 15 cm, at 4°C for 10 h. After the denaturation step, the slides were immediately immersed in PBM (PBS + 2% bovine serum albumin + 0.1% Tween 20) for 15 min at room temperature. Then, the antibody against 5-methylcytosine (anti-5-MeC, Megabase Research Products, NE, USA) diluted 1:10 in PBM was added; the slides were covered with a cover slip and incubated for 60 min in a moist chamber at 37°C. After two careful rinses in PBS, the slides were incubated with fluorescein-conjugated anti-rabbit-IgG (Sigma-Aldrich) diluted 1:50 in PBM for 60 min in a moist chamber at 37°C.

After a brief wash in PBS, the slides were mounted in anti-fade solution without any counterstaining agent.

Immunodetection of 5-MeC was carried out on metaphase chromosomes from 15 ASO clones along with metaphase chromosomes from untreated parent V79-Cl3 cells. Metaphase chromosomes from V79-Cl3 cells exposed to 10 μM SA for 24 h were also examined. As positive controls, metaphase chromosomes from V79-Cl3 cells treated with 18 μM 5-azacytidine (5-azaC, Sigma-Aldrich) for 24 h (11) were examined. The slides were then observed under a Nikon fluorescence photomicroscope equipped with a HBO 100 W mercury lamp and a suitable filter. Photomicrographs were processed using Adobe Photoshop 6.0 software.

Results and discussion

The ambiguous behaviour of arsenic—deadly poison, potent cancerogenic agent and, at the same time, unexpected antitumoral drug (12)—has prompted a lot of interesting research into its mechanisms of action. Several hypotheses have been put forward to explain the basis of arsenic-mediated carcinogenesis; among these, its capability to induce DNA double strand breaks, which could lead to chromosomal aberrations and to the establishment of unbalanced cell clones (13), or its capability to induce changes in DNA methylation levels (14). Most of the reports have been concerned with the prompt effects of arsenic exposure in cultured cells. We investigated the possibility that short-term exposure had long-term effects, by examining ASO cells, the progeny of SA-treated V79-Cl3 Chinese hamster cells.

The results of cytogenetic analysis carried out on ASO cell population after 2 months of subculturing showed that cells with 21 chromosomes (modal chromosome number) were 37.09 versus 60% found in the parent V79-Cl3 cells. Surprisingly, ASO cells exhibited dicentric chromosomes or telomeric associations (1.61 versus 0% in parent cells), rearrangements, which can produce genomic instability and very frequently occur in cancer cells and in cultured senescent cells (15).

Table I reports the results of cytogenetic and morphological investigations performed on all 20 clones, randomly isolated from ASO cell population; most clones bearing dicentric chromosomes were aneuploid with a low modal chromosome number and with micronucleated and multinucleated cell frequency higher than parent V79-Cl3 cells. Therefore, according to Pearson's correlation, we analysed the pooled data from these observations and verified that the frequency of cells with 21 chromosomes correlated negatively ($r = -0.63$ to -0.73) and significantly ($0 < P < 0.002$) with all markers of genetic instability.

Moreover, morphological observations of exponentially growing cells evidenced in these clones the presence of several nucleoplasmic bridges between cells at the completion of telophase. These structures are determined by the segregation of centromeres of dicentric chromosomes or chromatids to opposite poles of the cell at anaphase; this occurrence, determining breakage–fusion–bridge cycles, causes genomic instability (16). No nucleoplasmic bridges were found in parent cells nor in clones without dicentric chromosomes.

Two clones with the highest frequency of metaphases with dicentric chromosomes, ASO-13 and ASO-19, were analysed in particular detail; they showed some cells (15.14 and 16.03%, respectively) with metacentric or submetacentric chromosomes, the long arms of which were fused in only one chromatid. This occurrence is considered one of the mechanisms for the formation of dicentric chromosomes; in fact, it is well known that these rearrangements can originate from the reunion of the broken ends of two different chromosomes or when broken chromatids of the same chromosome join (17).

Table I. Frequencies of cytogenetic and nuclear abnormalities

Clone	Cells with		Micronucleated cells (%)	Multinucleated cells (%)
	21 chrs[a] (%)	Dicentric chrs[a] (%)		
V79-Cl3	60.00	0	0.83	0.47
ASO-1	30.94	8.12	5.30	0.78
ASO-2	50.00	1.30	2.30	1.20
ASO-3	5.21	9.77	3.96	12.85
ASO-4	27.00	9.00	2.36	2.59
ASO-5	35.00	2.33	3.65	0.77
ASO-6	38.03	6.88	0.65	0.79
ASO-7	56.10	0	0.70	0.63
ASO-8	61.00	0	0.35	0.75
ASO-9	60.80	0	0.50	0.49
ASO-10	58.00	0	0.87	0.37
ASO-11	59.21	0	0.63	0.55
ASO-12	54.19	0	0.64	0.32
ASO-13	43.84	10.09	7.74	1.98
ASO-14	61.33	0	1.00	0.61
ASO-15	46.96	7.03	5.87	1.80
ASO-16	57.28	0	0.58	0.34
ASO-17	0	5.12	9.10	6.20
ASO-18	10.93	9.37	2.50	0.50
ASO-19	39.93	10.06	4.80	3.10
ASO-20	38.03	5.90	1.39	1.20

[a]Chromosomes.

Karyotype analysis in GTG banding, according to the standard V79 karyotype reported by Thacker (18), showed that most of the dicentric rearrangements were chromosome-specific and recurrent in both clones. In fact, the submetacentric *h* chromosome was found to be involved in 100% of dicentric chromosomes observed in ASO-13 clone and in 71.4% of dicentric chromosomes analysed in ASO-19 clone. The other chromosome involved in the dicentric formation could be one of two metacentric chromosomes belonging to the first (*a*, *b*) or to the second pair (*c*, *d*), or one of the greatest submetacentric chromosomes (*e* or *g*) (Figure 1). The preferential involvement of a particular chromosome in dicentric rearrangements was found to be a common occurrence in primary Chinese hamster embryonic cells (19) and in several human tumour cells (20). At the moment, we do not know why the *h* chromosome was frequently involved in the formation of dicentric rearrangements in the progeny of SA-treated cells, but we can presume that telomeric repeat sequences, localized only in this chromosome in subterminal position of the long arm (21), were the main target for double-strand breaks leading to chromosome aberrations (22). On the other hand, the presence and propagation of dicentric rearrangements involving *h* chromosome in different ASO cell clones indicate that this rearrangement may have conferred a growth selective advantage.

As genomic instability has been associated with DNA hypomethylation, which in turn affects chromosomal rearrangements (23), we evaluated DNA methylation levels using the indirect immunofluorescence technique with anti-5-MeC antibody. As shown in Figure 2, chromosomes from untreated V79-Cl3 cells showed brilliant grains along the overall length; on the contrary, chromosomes from V79-Cl3 cells observed immediately after treatment with SA were uniformly dull, similar to those from cells treated with 5-azaC, an effective

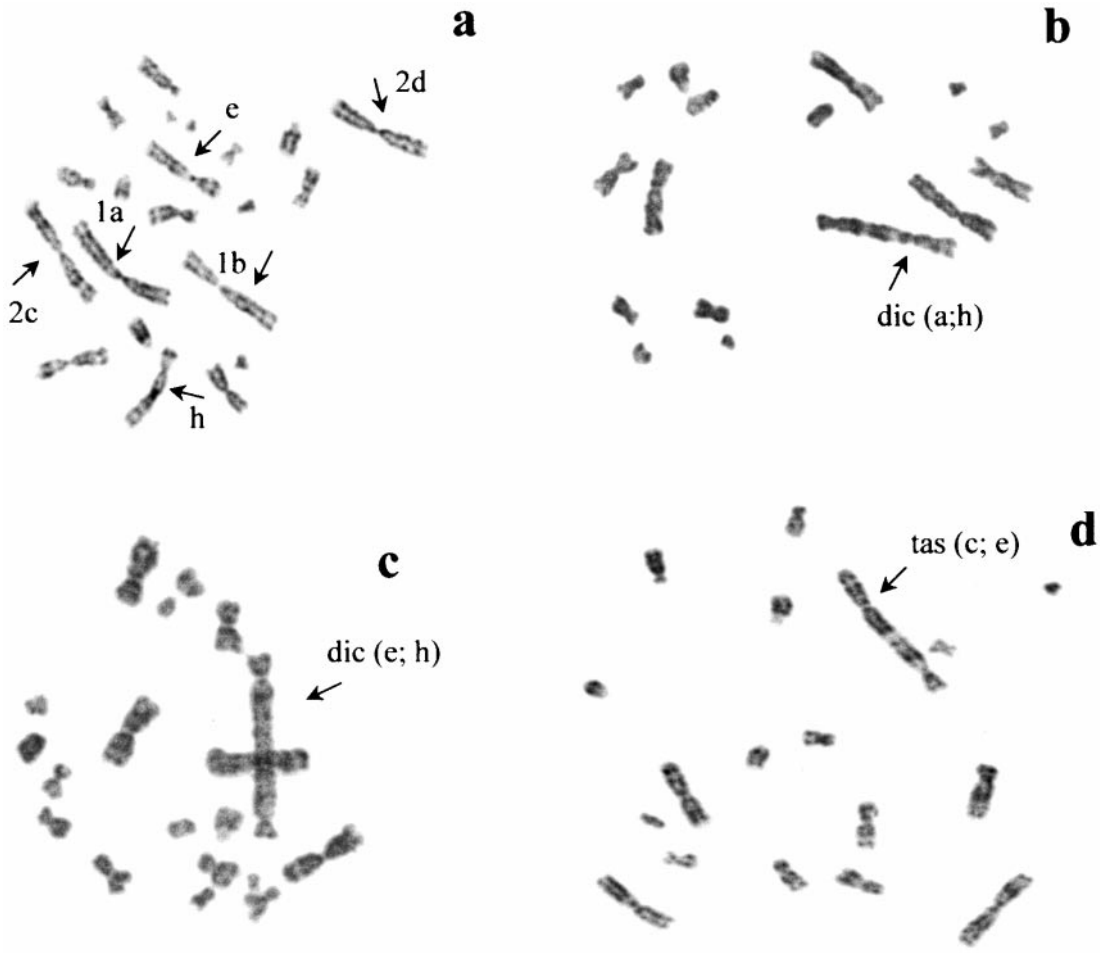

Fig. 1. GTG banded metaphases from: untreated V79-Cl3 cell (**a**); ASO-13 cells showing *h* chromosome involved in dicentric rearrangements (**b** and **c**); ASO-19 cell showing a telomeric association (**d**).

DNA methylation inhibitor. These results showed the hypomethylating effect of arsenic after a short-term exposure. Interestingly, those genetically unstable ASO clones carrying dicentric chromosomes were found to have either uniformly dull (ASO 17 and 20) or faintly fluorescent metaphases with some discrete grains (ASO 3, 4, 5, 6, 13, 15, 18 and 19); this last finding was evidence of a narrow renewal of the epigenetic silencing. On the contrary, genetically stable ASO clones showed brightly fluorescent metaphases (ASO 9, 10, 11, 12 and 14).

Since the relationship between chromosome compaction and DNA methylation is very complex (24) the results of immunostaining experiments could be affected by alterations in chromatin structure unrelated to DNA methylation; however, our cytogenetic investigations on a large number of metaphases (both Giemsa-stained and G-banded) did not show any difference in the chromosome compaction between ASO clones and untreated parent V79-Cl3 cells.

The quantitative evaluation of 5-MeC present in the cells (25) could be of interest to know the actual levels of DNA methylation; however, the consistency of findings obtained from each cell clone supported the hypothesis that the differences in intensity of the anti-5-MeC antibody labelling could be attributed to differences in methylation status. Moreover, the constant detection of dull metaphases in unstable clones and bright metaphases in stable ones suggested a relationship between DNA methylation status and chromosomal instability level. In fact, the Spearman's rank correlation coefficients (rho), calculated by assuming the fluorescence intensity as an ordinal variable with values 0, 1 or 2 for dull, faint or bright fluorescence, respectively, indicated that immunodetected 5-MeC strongly correlated both positively with the frequency of cells with 21 chromosomes and negatively with the frequency of cells with instability markers (Table II).

It has been reported that arsenic induces early genetic instability by a direct effect on the spindle microtubule dynamics (26) as well as DNA hypomethylation by methyl depletion (14). Our results demonstrate that the prompt effects of arsenic exposure were persistent for a long time and support the hypothesis that the interference of arsenic with the spindle assembly could trigger the initial genetic instability leading to production of aneuploid, micronucleated and multinucleated cells, while DNA under-methylation could favour karyotypically detectable rearrangements (27), which enhanced the instability. In fact, a large part of the progeny of ASO cells not only was aneuploid and showed nuclear abnormalities, but also presented novel chromosomal rearrangements, namely dicentric or telomeric associations, which were not seen following acute exposure; interestingly, these chromosome changes occurred in cells affected by global DNA

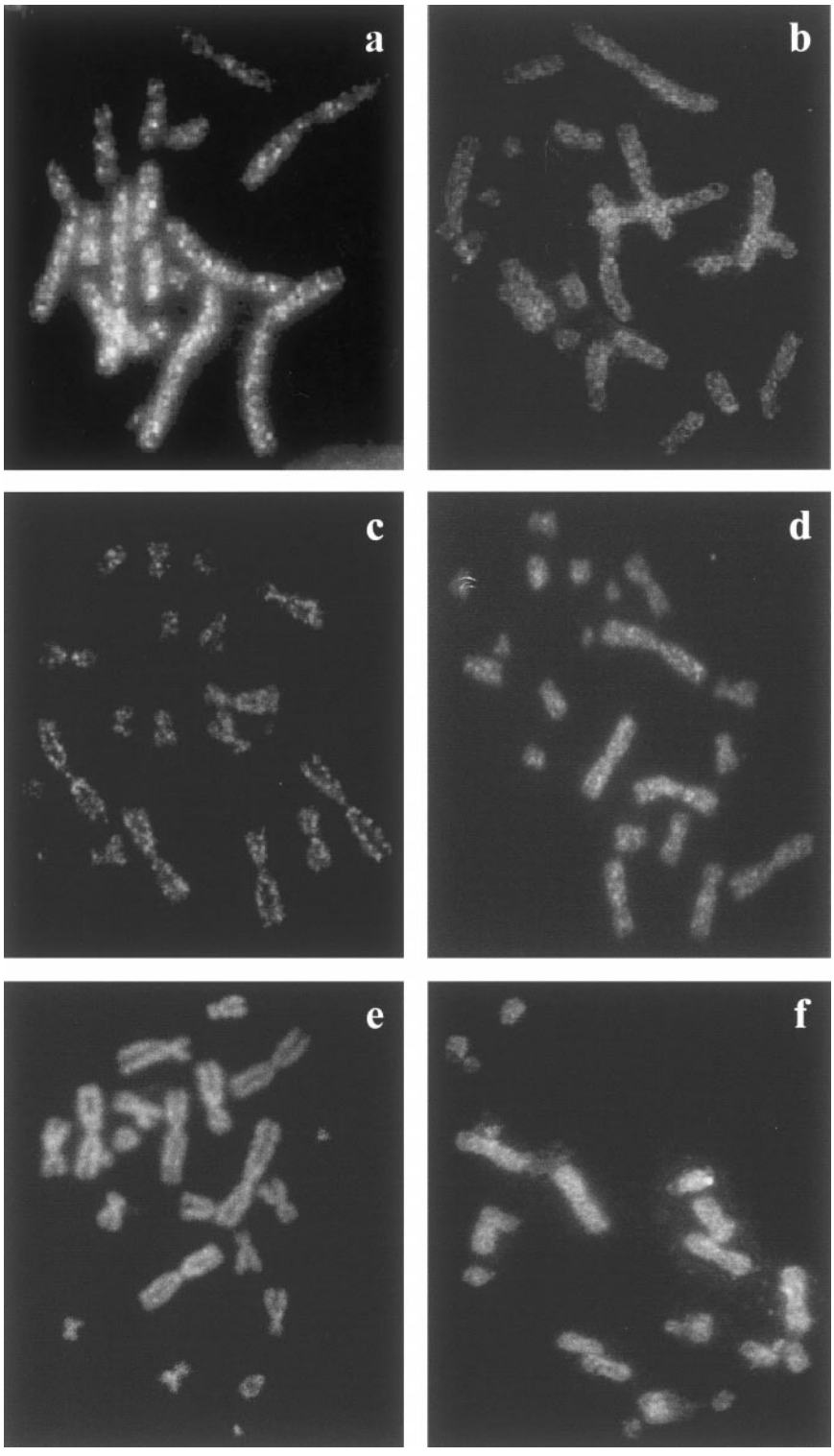

Fig. 2. Immunodetection of 5-MeC in metaphase chromosomes from: untreated V79-Cl3 cell (**a**); V79-Cl3 cell treated with SA for 24 h (**b**); ASO-6 cell (**c**); ASO-19 cell (**d**); ASO-20 cell (**e**); 5-azaC-treated V79-Cl3 cell (**f**).

hypomethylation. The contemporary occurrence of hypomethylated state and genomic instability is likely to confer a selective advantage to cell variants.

One important topic that remains to be clarified is the maintenance of an altered methylation pattern even in the absence of arsenic. Many authors reported results from chronic exposure and showed that arsenic and its trivalent methylated metabolites have the capability to interact with the enzymes of the DNA methylation pathway [references in (28)]. In this study we found that also the acute exposure to arsenic induced

Table II. Correlation between DNA methylation levels and instability markers

		Cells with		Micronucleated cells	Multinucleated cells	
		21 chrs	Dicentric chrs			
5-MeC	rho	0.84		−0.70	−0.73	−0.76
	P	<0.0005		<0.003	<0.001	<0.001

DNA hypomethylation that in some cells turned to heritable trait. Considering that it may be a way for arsenic-mediated carcinogenesis, further investigations into the mechanism that maintains the hypomethylated state merit to be performed.

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