Sodium arsenite-induced chromosomal aberrations in the Xq arm of Chinese hamster cell lines

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Non-cytotoxic concentrations (1.5, 3 and 6 µM) of sodium arsenite (SA) were used to study its cytogenetic effects with special reference to the frequency and nature of chromosomal aberrations on the X chromosome at Xq21 and at centromeric regions in CHO9, EM-C11, V79, V-H4 and CHE cell lines using Giemsa and FISH techniques. A high frequency of chromosomal breakpoints was distributed on the X chromosome localized at the secondary constriction region of the q arm (Xq21) and to a lesser degree at the centromeric region (c band-positive region), showing a high degree of fragility of these regions. This phenomenon was observed in all cell lines except for V79, where aberrations were localized only in the Xq21 region, and CHO9, where the observed breakage frequency was lowest in comparison with other cell lines and breaks were mostly located in the centromeric region rather than at Xq21. Homozygous expression of the breakpoint at Xq21 (fragile site) and in the centromeric region was also confirmed using a female Chinese hamster embryonic (CHE) cell line, which showed a similar high frequency of breakpoints at Xq21 and in the centromeric region of both X chromosomes. Further, a detailed cytogenetic study in CHO9 and its ligase-deficient mutant EM-C11 cell line showed slightly higher sensitivity to SA in a cell survival assay. No difference was found for chromosomal aberrations in Giemsa stained preparations. For SCEs a higher spontaneous frequency was evident in EMC-11. SA significantly increased the frequency of SCEs in CHO9, but no effect was found in EM-C11. Further, we observed an increased number of abnormal cells with pulverised chromosomes, decondensed chromatin, isochromosomes and hyperploidy in CHO9 and EM-C11 cell lines at all doses of SA.

Introduction

Preferential breakage of the q arm of the X chromosome in CHO and V79 cells induced by various chemicals (Galloway et al., 1987; Loveday et al., 1989; Sen et al., 1989; Yu et al., 1992; Zwanenburg and Pujadas, 1994) suggested the presence of one or more fragile sites in that region (Tommerup, 1987; Turleau et al., 1989; Fasth et al., 1990) (Chinese hamster genome). This has been confirmed and the region mapped to Xq21 (Slijepcevic and Natarajan, 1995).

Expression of fragile sites may be a consequence of a change in tissue culture conditions (Sutherland, 1991), may occur spontaneously in human cells (Heim et al., 1989) and may be induced by metal salts (Sen et al., 1989) and by chemicals (Galloway et al., 1987; Yunis et al., 1987; Loveday et al., 1989; Yu et al., 1992; Zwanenburg and Pujadas, 1994) in rodent and human cells. It has been reported that fragile sites arise as a result of: (i) failure of replication at chromosomal points; (ii) its unusual sensitivity to interference during DNA synthesis (Laird et al., 1987); (iii) recombinogenesis (Glover and Stein, 1987). They may participate in inter- and intrachromosomal rearrangements induced by mutagens in vitro (Yunis et al., 1987).

The long arm of the Chinese hamster X chromosome is heterochromatic and has a secondary constriction at the Xq21 region, which is susceptible to a high frequency of breakage, possibly due to its loose chromatin packing, and is also a late replicating region of the X chromosome (Galloway et al., 1985). This loose chromatin packing was reported to be the sites in the heterochromatin region with the appearance of chromosomal breaks or gaps without the loss of continuity of the chromatin (Haaf and Schmid, 1990).

Various chromosomal aberrations shown to be involved in X chromosome breakage, such as two-branched chromosomes (triradials with identical distal parts) in V79 cells and multi-branched chromosomes at certain fragile sites in lymphocytes of immunodeficiency patients (Turleau et al., 1989; Fasth et al., 1990), were reported to be due to an indirect mechanism, such as disturbance of nucleotide pools (Zwanenburg and Pujadas, 1994), or one or more fragile sites involved in the breakage events, such as that reported at the human fragile site Xq27 after BrdUrd treatment (Tommerup, 1987).

Sodium arsenite (SA) was used in our studies because arsenic is one of the few environmental chemicals which has been shown to be carcinogenic in the human but not in animals and hence needs further study. Cytogenetic investigations on the influence of arsenic have, so far, yielded conflicting results. Increased frequencies of chromosomal aberrations were reported in humans and negative results have also been reported in this regard (Burgdorf et al., 1977). Burgdorf et al. (1977) and Wen et al. (1981) showed an enhanced frequency of SCEs in human lymphocytes treated with arsenic. The mechanism(s) by which arsenic induces changes in the genetic material is still unclear and therefore further study is needed.

Hence a study was undertaken using non-cytotoxic concentrations of SA to determine its cytogenetic effects with special reference to the frequency and nature of aberrations on the X chromosome at Xq21 and at the centromeric region in CHO9, EM-C11, V79, V-H4 and CHE cell lines. A comparative study of CHO9 and its mutant ligase-deficient EM-C11 cell line was undertaken to detect differential cell type-specific sensitivity, if any, to SA. For this a survival assay and the frequency and pattern of chromosomal aberrations (CA) and sister chromatid exchanges (SCEs) were analysed using Giemsa and FISH techniques. The analysis of chromosomal aberrations at Xq21 and in the centromeric region was carried out in all the

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thus making scoring easier. The concentration of BrdU was reduced by the concentration of BrdU was reduced to reduce the level of SCEs per cell, Sister chromatid exchange (SCE)

As the baseline frequency of SCEs in EM-C11 was very high (87 per cell), Only CHO9 and EM-C11 cell lines were used in the SCE and CA studies and the cells were fixed.

was added for the duration of one cell cycle according to the cell type treated with BrdU:thymidine (1.3) and then SA (at three different concentrations) for CHO9, V79, VH-4 and CHE and -18 h for EM-C11) in complete medium treatment. The cell cultures were grown for the time of one cell cycle (-12 h arsine (CAS no. 7784-46-5; Sigma) were freshly prepared at the time of the same slide. Three different concentrations (1.5, 3 and 6 U.M) of sodium The experimental design and chromosome preparation protocols of Natarajan (1983) were followed and accordingly SCEs and CAs were scored from the dishes were rinsed with NaCl (0.9%), air dried, stained with methylene blue (0.25%) and colonies were counted. Each point on the survival curve represents the mean value ± standard deviation of five independent experiments.

**Materials and methods**

**Cell culture**

A Chinese hamster ovary cell line (CHO9) and its ligand-deficient mutant EM-C11 cell line, V79 and its mitomycin C-sensitive mutant V-H4 and a female Chinese hamster embryonic cell line (CHE) were used in this study. These cell lines were routinely cultured in 9 mm Petri dishes in Ham's F10 medium supplemented with 15% newborn calf serum and standard antibiotics. Incubations were carried out at 37°C in a 5% CO2 atmosphere

Survival assay Cultures in exponential growth were trypsinized and 200-300 cells were seeded into P94 dishes (5 dishes/dose). The cells were exposed for 24 h to three different concentrations of SA (1.5, 3 and 6 μM), washed with phosphate-buffered saline (PBS) and further incubated in fresh medium. After 7-8 days the dishes were rinsed with NaCl (0.9%), air dried, stained with methylene blue (0.25%) and colonies were counted. Each point on the survival curve represents the mean value ± standard deviation of five independent experiments (Figure 1).

**Methods of chromosome preparation for evaluating chromosomal aberrations and sister chromatid exchange**

The experimental design and chromosome preparation protocols of Natarajan et al (1983) were followed and accordingly SCEs and CAs were scored from the same slide. Three different concentrations (1.5, 3 and 6 μM) of sodium arsenite (CAS no. 7784-46-5; Sigma) were freshly prepared at the time of treatment. The cell cultures were grown for the time of one cell cycle (~12 h for CHO9, V79, VH-4 and CHE and ~18 h for EM-C11) in complete medium with BrdU/thymidine (1.3) and then SA (at three different concentrations) was added for the duration of one cell cycle according to the cell type treated and the cells were fixed.

**Sister chromatid exchange (SCE)**

Only CHO9 and EM-C11 cell lines were used in the SCE and CA studies. As the baseline frequency of SCEs in EM-C11 was very high (87 per cell), the concentration of BrdU was reduced to reduce the level of SCEs per cell, thus making scoring easier. The concentration of BrdU was reduced by addition of thymidine at a ratio of 1:3. This decreased the baseline frequency of SCEs from 87 to 52.2 SCEs/cell in EM-C11 (Table I and Figure 2). The same ratio of BrdU/thymidine was also used in the CHO9 cell line. All experiments were repeated twice and the results presented are the mean values ± standard deviation of two experiments.

**Chromosomal aberrations (CA)**

For chromosome aberration studies 100 first division metaphases were scored per treatment after fluorescence plus Giemsa (PG) staining (Perry and Wolff, 1974) and all types of chromosome aberrations were recorded. All experiments were repeated twice and the results presented are means of the two experiments.

**Fluorescence in situ hybridization (FISH)**

In this study all five cell lines (CHO9, EM-C11, V79, V-H4 and CHE) were used. Qx breaks were analysed in a minimum of 200 cells (except in 6 μM-treated CHE cells) for each experimental point. In order to score only first division cells chromosome preparations were incubated in Hoechst 33258 solution (2.5 mg/100 ml) for 20 min and then exposed to UV light for 20 min. The slides were then processed for in situ hybridization, following the protocol of Xiao et al (1996).

Telomere- and X chromosome-specific probes, PCR labelled with biotin-16-dUTP (Sigma) and digoxigenin-11-dUTP (Boehnnger Manheim, Germany) respectively, were utilized. The slides were counterstained with DAPI (0.15 μg/ml) mixed with antifade Vectashield (Vector Laboratories) mounting medium. X Chromosome aberrations were analysed using a Zeiss Axioskop fluorescence microscope equipped with FITC and TRITC filters.

**Statistical analysis**

Student's t-test was used for statistical analysis of variance to define the sensitivity of CHO9 and EM-C11 cell lines following treatment with SA.

**Results**

**Survival assay**

The efficiency of cell killing by SA was determined by the colony forming ability of the cells after treatment. The data are presented in Figure 1, which shows that the cloning efficiency of EM-C11 was 61-85% and CHO9 was ~82-100%. EM-C11 was slightly more sensitive to SA treatment than CHO9, showing a differential effect of SA on different cell lines. Each point in Figure 1 represents the mean ± standard deviation of the results from five independent experiments (P < 0.05).

**Sister chromatid exchange**

Data are presented in Table I. The spontaneous frequency of SCE was ~6-7 times higher in EM-C11 than in wild-type cells (CHO9). Preliminary experiments showed that the levels of SCEs decreased proportionally on decreasing the concentration of BrdU with thymidine. The results for SCEs presented in Table I and Figure 2 represent the mean ± standard deviation of two independent experiments. In our experiment a BrdU/thymidine ratio of 1:3 was used to reduce the spontaneous SCE frequency from 87 to 52.2 per cell. A significant dose-dependent increase was observed in the frequencies of SCEs following SA treatment in CHO9 cells (P < 0.03), while no effect (P < 0.07) was found in EM-C11.
Sodium arsenite-induced chromosomal aberrations

Chinese hamster cell lines

Fig. 2. Chromosomal aberrations observed in the X chromosome of Chinese hamster cell lines after SA treatment.

Table II. Frequency of chromosomal aberrations/100 cells in CHO9 and its mutant EM-C11 cell lines following treatment with SA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dose (μM)</th>
<th>Gaps</th>
<th>ctb</th>
<th>cte</th>
<th>dic</th>
<th>chd</th>
<th>Total aberrations</th>
<th>Hyperploids</th>
<th>Cells with pulverised chromosomes</th>
<th>Total abnormal cells*</th>
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<td>52</td>
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</table>

*Includes hyperploids, cells with pulverised chromosomes and decondensed chromatin.

Abnormal cells observed included hyperploids and cells with pulverized chromosomes, decondensation of the chromatin and breaks in X chromosomes.

Analysis of X chromosome aberrations in all cell types

Although aberrations in the X chromosome are easily recognizable, the fragments often separate from the X chromosome and can be mistaken for a common fragment unless the X chromosome is specifically identified. By application of the FISH technique it was confirmed that the aberration did indeed occur in the q arm of the X chromosome and not in other chromosomes (Table III and Figure 2).

The Chinese hamster embryonic cell line (CHE) (Table III and Figure 3e–g). The female CHE cell line was chosen to check whether both X chromosomes responded similarly in production of Xq21 aberrations (homozygous expression, which is typical of common fragile sites). In 1.5 μM SA-treated cells out of 200 cells 50 (25%) showed aberrations in the Xq arm. In 40 of those 50 cells (80%) the aberrations were in the Xq21 region and in the remaining 10 cells they were in the centromeric region. Of the 40 cells showing aberrations in the Xq21 region both X chromosomes were affected in 20 cells (50%). In the 10 cells showing aberrations in the centromeric region both X chromosomes were affected.

Aberrations were localized at Xq21 region in 66 cells. In 14 cells both X chromosomes were affected. Of the 16 cells showing aberrations in the centromeric region both X chromosomes were affected. Decondensed chromatin associated with X chromosome aberrations was observed in four of 84 cells.

The V79 cell line (Table III and Figure 3a). In this cell line at the concentration of 1.5 μM SA 48 of 210 cells, at the concentration of 3 μM 66 of 256 cells and at the concentration of 6 μM 54 of 274 cells showed Xq aberrations and all aberrations were localized at Xq21.

There was a dose-dependent increase in the frequencies of aberrations in the Xq21 region both X chromosomes were affected in 20 cells (50%). In the 10 cells showing aberrations in the centromeric region both X chromosomes were affected.

The frequency of homozygous fragility observed at Xq21 and in the centromeric region of the X chromosome was high.

The V79 cell line (Table III and Figure 3a). In this cell line at the concentration of 1.5 μM SA 48 of 210 cells, at the concentration of 3 μM 66 of 256 cells and at the concentration of 6 μM 54 of 274 cells showed Xq aberrations and all aberrations were localized at Xq21.

There was a dose-dependent increase in the frequencies of
aberrations. The decrease in aberration frequency at the highest SA concentration (6 μM) may be due to increased cytotoxicity of SA for that cell line.

The V-H4 cell line (Table III and Figure 4h). This is a mitomycin C-sensitive mutant of the V79 cell line. The percentage of spontaneous Xq aberrations was lower than that of its parental cell line (V79).

In 1.5 μM SA-treated cells 24 of 216 cells (11%) showed aberrations on the q arm of the X chromosome, of which 90% (22) of the aberrations were localized at Xq21, while four cells (16.7%) showed aberrations in the centromeric region.

In 3 μM SA-treated cells a slight increase in the frequency of Xq aberrations was observed when compared with cells treated with 1.5 μM SA. Localized aberrations on the q arm were observed in 34 out of 320 cells (10.6%), of which 22 (64.7%) showed aberrations in the Xq21 region and 12 (35.3%) had aberrations in the centromeric region. Decondensation of the chromatin in the q arm of the X chromosome was observed in two cells.

In 6 μM SA-treated cells 38 of 200 cells (19%) showed aberrations on the q arm of the X chromosome and all of them were localized in the Xq21 region.

The EM-C11 cell line (Table III and Figure 3d). This is a ligase-deficient mutant of the CHO9 cell line. In cells treated with 1.5 μM SA 16 of 200 cells (8%) showed aberrations on the q arm of the X chromosome. Localized aberrations in the Xq21 region were observed in eight of 16 cells and an equal number of chromosomal breakpoints were localized in the centromeric region.

In the 3 μM SA-treated cells 18 of 200 cells (9%) showed aberrations on the q arm of the X chromosome. Only four of 18 cells showed aberrations in the Xq21 region, however, an increased percentage of cells (14 of 18) showed aberrations in the centromeric region, indicating a higher sensitivity in the centromeric region than in the Xq21 region for treatment at that concentration of SA. Decondensation of chromatin on the q arm of the X chromosome was observed in six cells.

However, when cells were treated with 6 μM SA 28 of 200 cells had aberrations on the q arm of the X chromosome. Twenty of 28 cells carried aberrations in the Xq21 region and only eight of 28 cells had aberrations localized in the centromeric region of the X chromosome.

The Chinese hamster ovary cell line (CHO9) (Table III and Figure 3b and c). This cell line was the least sensitive with regard to Xq21 aberration, whereas its centromeric region was as sensitive as other cell lines to SA treatment.

In 1.5 μM SA-treated cells while six of 200 cells showed aberrations in the Xq region, only two of six showed aberrations in the Xq21 region, whereas in four of six cells the aberrations were localized in the centromeric region.

While in cells treated with 3 μM SA 26 of 340 cells (7.6%) had aberrations on the q arm, only a low percentage (four out of 26 cells) had aberrations localized in the Xq21 region, whereas 22 out of 26 cells showed aberrations localized in the centromeric region. Isochromosomes or Robertsonian fusions which could be formed from breaks at the centromeric region were included along with centromeric aberrations for data analysis.

While in cells treated with 6 μM SA 30 of 300 cells showed aberrations on the q arm of the X chromosome, only six of 32 cells exhibited aberrations in the Xq21 region. However, there was an increase in the percentage of cells (24 of 30) having aberrations localized in the centromeric region. Decondensed chromatin of the q arm was observed in two cells.

In our experiments only CHO9 showed an increase in the frequency of aberrations in the centromeric region in comparison with the Xq21 region. A dose-dependent increase in these aberrations was also observed.

**Discussion**

SA at all its non-cytotoxic concentrations (1.5, 3 and 6 μM) is effective in producing an increase in the frequency of aberration on the X chromosome in Chinese hamster cells. This excess of SA-induced aberrations on the X chromosome is much higher than could be expected based on its DNA content and random induction. A dose-related increase in SA-induced Xq aberrations was observed in all four cell lines except V79.
Interestingly, we observed that the aberrations induced by SA were non-randomly localized on the X chromosome in general and in the Xq21 region (secondary constriction) in particular, as well as in the centromeric region. There are similar reports showing a non-random localization of aberrations in the Xq 21 region in Chinese hamster cells after treatment with metal salts (Sen et al., 1989) and other chemicals (Natarajan and Schmid, 1971; Galloway et al., 1987; Loveday et al., 1989; Yu et al., 1992; Zwanenburg and Pujadas, 1994; Slijepcevic and Natarajan, 1995).

This high frequency of non-random aberrations on the heterochromatic q arm and to a lesser degree in the centromeric region was observed in all cell lines except in CHO9, where no significant increase in the frequency of Xq21 aberrations was observed. On the other hand, CHO9 cells showed an increased frequency of aberrations in the centromeric region. V79, a cell line highly sensitive in the Xq21 region, showed no aberrations in the centromeric region. Preferential fragility of the centromeric region was localized in all cell lines studied except in V79. This shows that the fragility observed in the centromeric region was cell type-specific. Similar results were reported by Yu et al. (1992) using U-68,553b in CHO cell lines, where the test chemical induced expression of fragile site on chromosome 1 in CHO-K1 but not in CHO-WBL. The degree of sensitivity in the Xq21 region was at variance in the cell lines studied by us and was in the following order (from least to most sensitive): CHO9 < EM-C11 < V-H4 < V79 < CHE.

The female CHE cell line, which was chosen to determine the nature of the fragile site at Xq21, showed that both X...
chromosomes were affected in 21–80% of cells having Xq21 aberrations showing homozygous expression, which is typical of common fragile sites (Sutherland, 1991), in contrast to rare fragile sites. This was supported by a similar observation by Slijepcevic and Natarajan (1995) in the CHE cell line. In the same way the non-random breakpoint in the centromeric region of the X chromosome was present on both X chromosomes in 75–100% of cells having centromeric aberrations.

The EM-C11 mutant, in contrast to wild-type cells (CHO9), exhibits an ~6- to 7-fold elevated frequency of spontaneous SCEs. This compares well with the observations of Zdzienicka et al. (1992). This high SCE frequency was decreased by reducing the extent of BrdU substitution, from 87 to 52 SCEs/cell. The effect of SA on production of SCEs in CHO9 cells was dose-dependent. No significant effect of SA was observed in EM-C11 cells. This could be interpreted as due to the already existing defect in DNA ligase not being enhanced by SA, which is known to inhibit the activity of DNA ligase.

The spontaneous CA in the EM-C11 cell line showed an increase of 2-fold over its parental cell line. After SA treatment the same level of increase in CA (1- to 1.6-fold) was also found in CHO9 and EM-C11 cells.

Further, SA induced hyperploidy and pulverisation of chromosomes. Hyperploid cells could arise from interference by SA with non-disjunction, probably due to the affinity of SA for SH group-containing enzymes. The observed pulverization of chromosome segments represents premature condensation of the heterochromatic arm of the X chromosome induced by SA, by interfering with replication. This could also be due to hypomethylation of these normally hypermethylated regions, as SA has recently been shown to induce hypomethylation by depleting the enzyme methyltransferase.

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