Chromosome Alterations in Syrian Hamster Cells Transformed in vitro by Sodium Bisulfite, a Nonclastogenic Carcinogen

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INTRODUCTION

Sodium bisulfite, a nonmutagen at neutral pH, induces neoplastic transformation of cultured Syrian hamster fetal cells. Morphologically transformed fibroblast colonies were isolated, and derived cell lines formed anchorage-independent colonies in agarose and progressively growing s.c. fibrosarcomas in nu/nu mice. Five tumorigenic cell lines analyzed by G- and C-banding were chromosomally abnormal with numerical deviations and structural alterations. Three tumors that developed in nude mice had the chromosome constitution of the inoculated transformed cell as well as secondary changes associated with tumor progression. Transformed cell lines had either a predominantly near-diploid or a near-tetraploid population with consistent chromosome gain and loss. Monosomy of the chromosome 13 observed in three cell lines was a nonrandom numerical alteration. Four lines had abnormal chromosomes resulting from deletions, unbalanced translocations, or centric fusions, and one cell line had a chromosome with a homogeneously staining region. Changes of chromosomes 1 and X were observed in three lines. The breakpoints on X chromosome nonrandomly involved the region q5 which is frequently affected in hamster cells transformed by other carcinogens and may result in loss of genes essential for the maintenance of a normal phenotype. The formation of abnormal chromosomes cannot be directly attributed to the initial DNA damage as bisulfite concentrations effective in causing neoplastic transformation induced a significant but minimal increase in sister chromatid exchanges and failed to cause chromosome aberrations. Bisulfite inhibition of DNA replication might be a contributing factor in the occurrence of abnormal chromosomes. This cytogenetic analysis provides the first evidence that neoplastically transformed cells by a nonclastogenic carcinogen exhibit persistent chromosome rearrangements, a genetic alteration essential to the process of malignant transformation.

MATERIALS AND METHODS

Cell Cultures. The quantitative HFC model and the culture conditions have been described (11, 12). Cells seeded for colony formation from a secondary culture were exposed 24 h later for a period of 15 min to 20 mM sodium bisulfite in Dulbecco’s phosphate-buffered saline. After being incubated for a total of 7 days, morphologically transformed colonies, identified under phase microscopy by their lack of growth control and criss-crossing pattern of cells, were ring isolated and expanded. Within 6 wk cell populations were injected s.c. into nu/nu mice to determine tumorigenicity. Five mice were given injections for each cell line.

SCE Analysis. SCE frequencies were determined on logarithmically growing and stationary HFC. Secondary HFC plated at 1 × 10^6 cells/dish were treated 24 h later with bisulfite diluted in PBS for 15 min. After treatment, cells were incubated in complete medium with BrdUrd (10 μg/ml of medium) for 24 h until chromosome fixation for SCE visualization.

For SCE analysis of stationary cultures, HFC were plated at 1 × 10^6 cells/dish in complete medium. This medium was replaced 24 h later with AGDM, supplemented with 0.2% fetal bovine serum, and cultures were incubated for an additional 72 h. At this time less than 5% of the cells were synthesizing DNA. Cultures were released from AGDM arrest by complete medium, and 10 h later, when the majority of the cells were in S phase, the medium was removed and bisulfite diluted in PBS was added for 1 h followed by BrdUrd for 36 h until chromosome fixation. Student’s t test was used to determine the level of statistical significance for SCE frequency data.

Chromosome Aberrations Analysis. Secondary HFC were plated at 1 × 10^6 cells/dish, and 24 h later, cells were treated as above with bisulfite diluted in PBS for 15 min. After treatment, cells were incubated in complete medium. Chromosomes were prepared 6 and 24 h after bisulfite treatment. Colcemid (0.13 μg/ml of medium) was added 4 h before cell harvest.

Chromosome Banding Analysis of Transformed and Tumor Cell Lines. Cultures from transformed or tumor-derived cell lines were subcultured 1:5 and used 24 h later for chromosome preparation. Colcemid was added 4 h before harvesting. The cells were mechanically detached from the surface of dishes and exposed to 0.075 M KCl for 15 to 20 min. The cells were fixed with a 3:1 mixture of methanol/acetic acid; the fixative was changed 3 times before making slides. To obtain G-bands, chromosome preparations were stained with Giemsa or Wright stain after trypsin pretreatment. Other slides were processed for C-banding. Chromosome number was determined on 50 conventionally stained metaphases, and 25 karyotypes, prepared according to the previous G-band karyotype and ideogram (13, 14), were examined for identification of structural or numerical chromosome changes.
RESULTS

Bisulfite Induction of SCE and CA on Normal HFC. The effect of bisulfite on chromosomes of normal HFC capable of undergoing in vitro neoplastic transformation was determined by analyzing the induction of SCE and CA. Exponentially growing and stationary HFC exposed to 10 μg of BrdUrd/ml of medium had 9.6 and 17 SCE per cell, respectively (Tables 1 and 2). Bisulfite caused statistically significant increases in SCE frequencies as determined by t test in both exponentially growing and stationary cultures at concentrations of 10, 20, and 40 mM, respectively (Tables 1 and 2). CA analysis at first (6 h) and second (24 h) mitosis after 10, 20, or 40 mM bisulfite showed no significant increases in aberrations over the controls (Table 3).

Cytogenetic Data of Transformed and Tumor Cell Lines. Five cell lines originating from individually isolated transformed colonies produced tumors with a latency period of 15 to 20 days after s.c. inoculation of 5 × 10⁶ cells into nude mice. Cell lines were examined cytogenetically 5 to 15 passages after colony isolation, and tumor cell lines, at the first or second passage in culture. All lines had a mixture of cells with a near-diploid and near-tetraploid chromosome number; two of the lines also had a third minor population with an octoploid chromosome number (Table 4). All five cell lines were chromosomally abnormal, four with both structural and numerical deviations and one only with consistent chromosome loss (Table 4). The number of normal chromosomes varied for each cell line from one to as many as six (Table 4). Structural alterations involving chromosomes X and 1 were observed in all cell lines. The karyotype of the transformed parental cells was preserved even though new alterations may have occurred (Table 4).

Line A had two karyotypically distinct populations with a near-diploid chromosome number (Table 4). One population had three copies of apparently normal X chromosomes and four abnormal chromosomes originating from deletions or translocations and a mid-size subtelocentric chromosome uniformly stained on G-band preparations (Fig. 1) and positively stained on C-band preparations. The positive C-band staining of this chromosome suggests that its origin is from either the heterochromatic arm of the X or the Y chromosome. However, its size and morphology are not consistent with either possibility. The second population had a similar if not an identical homogeneously stained chromosome, but these cells had only one abnormal chromosome, a deleted X chromosome.

The tumor-derived cell line was examined after the second in vitro subculture had a single tetraploid population exhibiting four copies of each autosome, two deleted X chromosomes and two copies of a homogeneously stained subtelocentric chromosome. Therefore, the tumor developed from the transformed cell population with less complex structural alterations.

Cell line D also had two populations: 68% of the cells had a near-diploid chromosome number, and 32% were tetraploid with 86 to 90 chromosomes (Table 4). Both populations had three identical abnormal chromosomes resulting from deletion of the heterochromatic long arm of the X-chromosome and deletions of chromosomes 1 and 6. Chromosomes 1, 6, 17, and 19 were trisomic, and chromosome 13 was monosomic. This line was also examined after colony growth in agarose. Cell cultures expanded from colonies developed in agarose exhibited the same karyotype as the original cell line, except for a new isochromosome 6.

Line E was the only line in which the majority (87%) of the cells were tetraploid with no detectable structural alterations even by high resolution prometaphase banding analysis. All the cells had monosomy 13, and chromosome 6 was monosomic in 50% of the cells (Table 4).

Line F had 32% and 68% of the cells with near-diploid and near-triploid chromosome number, respectively (Table 4). Both populations had the same chromosome changes consisting of an abnormal chromosome 17 and an extra chromosome 9 (Fig 2). To assess the tumorigenicity potential of the near-diploid and near-tetraploid cells, the two populations were cloned and inoculated into nude mice. Both near-diploid and near-tetraploid cell lines produced tumors with karyotypes identical to the inoculated parental cells.

Line G was analyzed at the fifth passage after isolation of transformed colonies and at passage 13 prior to the inoculation of the cells into nude mice or selection in agarose. The tumor-derived line was also analyzed at the second subculture. Cell cultures at the fifth passage had an equal number of normal and abnormal cells. The latter had deletions of the long arm of the X chromosome, a translocation involving chromosomes 1

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Table 1  SCE induction by bisulfite in exponentially growing HFC

<table>
<thead>
<tr>
<th>Dose (mM)</th>
<th>Range of SCE</th>
<th>SCE metaphases%</th>
<th>SCE chromosomes%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-15</td>
<td>9.65 ± 0.67</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>13.65 ± 0.79</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>14.00 ± 1.11</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

* A minimum of 50 cells of each sample was examined.

Table 2  SCE induction after bisulfite treatment in HFC arrested in G0 by AGDM

<table>
<thead>
<tr>
<th>Dose (mM)</th>
<th>Range of SCE</th>
<th>SCE metaphases%</th>
<th>SCE chromosomes%</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-28</td>
<td>17.00 ± 1.09</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16.60 ± 0.96</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>20.40 ± 0.76</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>22.35 ± 1.53</td>
<td>0.51</td>
<td></td>
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</table>

* A minimum of 50 cells of each sample was analyzed.

Table 3  Frequency of chromosome aberrations in HFC after treatment with bisulfite

<table>
<thead>
<tr>
<th>h after treatment</th>
<th>Dose (mM)</th>
<th>Gap</th>
<th>Break</th>
<th>Exchange</th>
<th>% of chromosome aberrations</th>
<th>% of chromosome aberrations</th>
<th>% of metaphases with aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>2</td>
<td>0</td>
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<td>2</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>2</td>
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<td>24</td>
<td>10</td>
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<td>1</td>
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<td>0</td>
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<td>1</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>40</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Cell cultures were harvested for chromosome analysis at the intervals indicated, and 200 metaphases per sample were examined.
The table below provides cytogenetic data of HFC cell lines transformed by bisulfite.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ploidy (%)</th>
<th>Range of chromosome no.</th>
<th>Chromosome</th>
<th>Structural abnormalities (markers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>92 8 0</td>
<td>43-46</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>A(t)</td>
<td>0 100 0</td>
<td>91-94</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>D</td>
<td>68 32 0</td>
<td>42-47</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>D(ag)</td>
<td>80 20 0</td>
<td>42-45</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>E</td>
<td>10 87 3</td>
<td>84-89</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>F</td>
<td>30 68 2</td>
<td>42-45</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>F(t)</td>
<td>76 20 4</td>
<td>43-46</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>G</td>
<td>85 15 0</td>
<td>43-44</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
<tr>
<td>G(t)</td>
<td>100 0 0</td>
<td>44-45</td>
<td>X</td>
<td>3, 19, 20, 21</td>
</tr>
</tbody>
</table>

* Chromosome analysis of five cell lines was done 10 to 15 passages after isolation of individual transformed colonies. Cell lines derived from tumors are indicated by letter "t," and cell lines examined after growth in agarose are indicated by the letters "ag."

* The ploidy was determined on relative counts of 200 chromosome spreads.

* The chromosome number was determined by counting 50 metaphases.

* Chromosome gain and loss relative to the ploidy level.

* Chromosome gain and loss relative to the ploidy level.

* At least 25 G-banded karyotypes were examined. The karyotypes were arranged according to the previously reported karyotype (13) and ideogram (14).

Fig. 1. G-band karyotype from bisulfite-transformed cell line A with 44 chromosomes. The chromosomes X and 3 are trisomic and monosomic, respectively. Four abnormal chromosomes are indicated by arrows. An abnormal chromosome placed on the left side of the karyotype exhibits homogeneous staining on its entirety.
and 6, identified in a fraction of the cells, and trisomy of chromosome 19. The proportion of cells with a normal and abnormal karyotype remained unchanged eight passages later when the cells were tested for and exhibited anchorage-independent growth in agarose and produced tumors in nude mice. The examination of the tumor-derived cell line showed that the tumor contained only the cells with an abnormal karyotype. The chromosome constitution of the tumor was similar to that of the parental transformed line even though a new large abnormal chromosome 13 carrying an apparent HSR was present in all the cells (Fig. 3).

**DISCUSSION**

Five tumorigenic cell lines transformed *in vitro* by sodium bisulfite were chromosomally abnormal with numerical and structural alterations. Tumors developed in nude mice preserved the karyotypic changes of the inoculated transformed cells even though secondary alterations associated with tumor progression may have occurred. Transformed cell lines had either a predominantly near-diploid or a near-tetraploid population. Chromosome gain or loss was consistent in each cell line with monosomy 13 being identified in three cell lines. This numerical deviation has not been previously observed in any Syrian hamster transformed cell line, and thus it represents a new nonrandom alteration. Interestingly, none of the bisulfite-transformed cell lines had monosomy 15, a frequent alteration associated with Syrian hamster cell transformation which had been found in the early 1970s in 25% of the cell lines transformed by diverse chemical carcinogens or oncogenic viruses (9, 10).

Subsequently, monosomy 15 was reported in cells transformed by oncogenic viruses (15) or by transfection with viral oncogenes (16). It has been postulated that monosomy 15 is essential to the transformation process as it may result in a loss of cellular genes required for the expression of neoplasia (16). Such an interpretation, however, is in conflict with the proposed chromosome balance mechanism for Syrian hamster cell transformation, as chromosomes 15 and 11 putatively carry genes for the expression of malignancy and should be in excess relative to chromosome 16, which carries the gene for suppression of malignancy (17).

*In vitro* mammalian cell systems are uniquely suited for assessing the role of carcinogen insult to the formation of
persistent chromosome alterations during the process of neoplastic cell transformation. The current data on the acute effect of bisulfite on chromosome damage and the cytogenetic characteristics of transformed cells are relevant to this critical issue. Present cytological evidence, as well as biochemical data generated with HFC, conclusively demonstrates that bisulfite at concentrations effective in causing malignant transformation induced low frequencies of SCE, had no effect on the induction of CA or excision of postreplication repair, and did not induce strand breaks in parental or nascent DNA and DNA adducts (18). Despite this lack of or limited DNA-damaging potential, all bisulfite-transformed lines had structural rearrangements common for HFC transformed by potent clastogenic carcinogens (9, 10). This indicates that persistent structural abnormalities can occur as a result of molecular and cellular changes not directly caused by bisulfite treatment. According to a recent hypothesis, an imbalance of DNA and protein synthesis could be the cause of a damaging effect on the chromosomes (19, 20). Bisulfite induced a dose-dependent decrease in the rate of DNA synthesis of HFC due to an apparent reduction of functioning replicons (18). The inhibitory effect of bisulfite on DNA replication might be a contributing factor in the formation of chromosomal abnormalities as an important step in the acquisition of the neoplastic transformation. Such a mechanism would explain and is consistent with our previous interpretation of the nature of persistent structural alterations in human fibroblasts exposed to a chemical or physical carcinogen as indirectly related to the initial carcinogen induction of DNA lesions (21).

Several forms of human cancer exhibit specific structural chromosome alterations (22). The breakpoints in these alterations affect a limited number of locations and frequently occur at sites of cellular protooncogenes (23–25).

Recurrent structural alterations in HFC transformed by either chemical carcinogens or oncogenic viruses have not been identified despite a sustained search in several laboratories studying the mechanism of cell transformation (26–36). However, our cumulative statistical analysis of the breakpoint distribution from various translocations and deletions revealed that certain sites, such as Xpa5, Xqa5, 2q11, 11pa2-a4, 15qa2, and 17qa4, are nonrandomly affected (not shown). In this series of bisulfite transformations, structural alterations of chromosomes 1 and X were identified in three cell lines. Deletions of Xqa5 observed in three bisulfite-transformed cell lines were also reported in cells transformed by simian adenovirus 7 (SA7) (10), SA7 in conjunction with a chemical carcinogen (10, polyoma (27), and herpes viruses (4)).

Mammalian X chromosome was considered as lacking oncogenic potential due to its paucity of protooncogenes (37). However, recently two protooncogenes have been localized on the human X chromosome (38, 39). To our knowledge, no protooncogene has been localized on Syrian hamster chromosomes. Possibly the long arm of the Syrian hamster X chromosome carries genes important in cell growth regulation and tumorigenesis, and by analogy with human neoplasia, deletions of this chromosome could result in homozygosity of certain genes or may remove a suppressor gene of malignancy. Chromosome alterations of chromosome 1 involved two sites, qa4 and qb1, the latter resulting in an unbalanced translocation...
with chromosome 2. The breakpoint on chromosome 2 occurred at band 2q11, a site most frequently affected in cells transformed by several chemical carcinogens or oncogenic viruses (9, 10, 15). In certain forms of human cancer, protooncogenes can be relocated due to chromosome rearrangements near enhancing or promoter sequences, resulting in changes in protooncogene regulation (40, 41). Possibly chromosome translocations identified in these transformed cells have similar consequences in structure or activity of certain genes controlling cell growth.

The chromosomes with HSR observed in one transformed cell line and one tumor-derived cell line indicate gene amplification, a genetic alteration commonly associated with tumor progression or drug resistance (40–43). Significantly, these bisulfite-transformed cell lines, despite their similarities and differences in chromosome structures and numbers, exhibit a remarkable specificity in terms of polypeptide pattern (44), suggesting that a common genetic alteration other than visible chromosomeosomal mes may have also contributed to the induction of malignancy.

REFERENCES

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