Persistence of X-Ray-induced Chromosomal Rearrangements in Long-Term Cultures of Human Diploid Fibroblasts

Yoshio Kano and John B. Little

ABSTRACT

As part of a long-term study of mechanisms of human cell neoplastic transformation, we have examined the change in the frequencies of X-ray-induced chromosome rearrangements in density-inhibited human foreskin fibroblasts as a function of subculture time. In nonproliferating cells, the frequency of chromosomal aberrations declined within 24 to 48 hr but still remained at a relatively high level up to 43 days after irradiation. Aberrations disappeared rapidly, however, when the cells were allowed to proliferate, indicating that these lesions are lethal to dividing cells. The frequency of induced translocations, as determined by analysis of G-banded karyotypes, was dose dependent and remained stable up to 20 mean population doublings after irradiation. When subculture of density-inhibited cultures was delayed for 4 hr after irradiation (confluent holding), the frequency of chromosomal aberrations in the first mitosis declined, whereas the translocation frequencies at later passage were elevated as compared with cells subcultured immediately. This correlates with the reported increase in the frequency of transformation under similar conditions. These findings support the hypothesis that chromosomal rearrangements induced by DNA damage may be involved in the initiation of cancer.

INTRODUCTION

Chromosomal rearrangements are associated with most human and rodent cancers as well as with cell transformation in vitro. The low efficiency of complete transformation of human cells in culture by chemical carcinogens (13, 34) has been ascribed to the stability of human chromosomes. Most of the cancer-prone human genetic disorders are associated with chromosomal instability or specific rearrangements, which may be related to the high risk of cancer in these patients.

During the past 2 decades, much cytogenetic evidence has been accumulated which links specific chromosomal abnormalities, especially translocations, to specific forms of human cancer (5, 14, 25). The classic example of these abnormalities is the 9:22 translocation that generates the Philadelphia chromosome associated with chronic myelogenous leukemia (23). Specific chromosome abnormalities have also been associated with Burkitt’s lymphoma (18), acute nonlymphoblastic leukemia (25), salivary tumors (26), and small cell carcinoma of the lung (32). Recent evidence suggests that most human hematopoietic cancers may have associated chromosomal abnormalities (33). Whether these chromosomal rearrangements are causally related to these tumors is, however, not known. The human c-myc gene was mapped to human chromosome 8, band q24, the chromosomal segment involved in the reciprocal Burkitt translocations [(t(8;14), t(8;22), and t(2;8)] (28). In 2 (t(8;14) human Burkitt cell lines, c-myc appears to have been translocated directly into a DNA restriction fragment that also encodes the immunoglobulin heavy chain gene. These data provide a molecular basis for considering the role that specific translocations may play in malignant transformation (28).

Most of the chromosomal abnormalities seen in tumors and other neoplastic cells are probably acquired. They are believed to be the result of the clonal or nonclonal proliferation of cells in which a chromosomal abnormality has arisen by somatic mutation in a cell, the karyotype of which was originally normal. X-Irradiation is a potent inducer of chromosomal aberrations in cultured human diploid cells (21, 22). The cells which are damaged by X-rays in the G1 phase may be classified into 3 major groups: (a) cells with no apparent structural abnormality; (b) cells with chromosomal-type aberrations such as dicentrics, rings, and fragments (a major mode of cell killing by X-irradiation is the mitotic death of cells containing such visible chromosomal aberrations); and (c) cells with chromosomal rearrangements, principally deletions, translocations, and the gain or loss of entire chromosomes. These latter abnormalities are the types associated with a variety of human cancers.

Although the general types of chromosomal aberrations induced in human cells exposed to ionizing radiations (21, 22) as well as the occurrence of chromosomal rearrangement in cancer cells (14, 18, 23, 25, 26, 32, 33) have been known for some time, surprisingly little information is available concerning the frequency of chromosomal rearrangements in X-irradiated human diploid cells in culture. Furthermore, the stability of X-ray-induced chromosomal rearrangements is not known, as they have not been examined at regular intervals in cultures allowed to proliferate for prolonged periods after irradiation. It has been suggested that chromosomal translocations result from either misrepair or genetic recombination following irradiation of G1 cells. In the present study, we observed that X-ray-induced chromosomal translocations are very stable over many population doublings in vitro and that the frequency of stable translocations in X-irradiated human diploid fibroblasts maintained in density-inhibited growth (confluent holding) for 4 hr prior to subculture to low density. We discuss the relationship between chromosomal rearrangements and the mechanism of malignant transformation in human cells by X-irradiation.

MATERIALS AND METHODS

Cells and Culture Conditions. A normal human foreskin-derived cell strain designated AG1522 derived from a 3-day-old male was obtained from the Human Genetic Mutant Cell Repository in Camden, NJ. These cells were used for all experiments. They were grown and routinely maintained by standard techniques in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum and gentamicin sulfate (0.025 mg/ml; Sigma). Cultures were maintained at 37°C in a humidified incubator.
with 5% CO₂:95% air in plastic Petri dishes (Lux). The culture medium was changed every 3 days. Subcultivation was carried out whenever the cultures became confluent (approximately 4 × 10³ cells/100-mm Petri dish at confluence) by adding 0.25% trypsin solution and allowing the culture to stand at room temperature until the cells began to round up. Cells were then suspended in fresh culture medium and dispensed into new dishes as quickly as possible at a 1:4 dilution.

X-Irradiation. Irradiation of confluent cultures was carried out at room temperature with a General Electric Maxima X-ray generator operated at 220 kV and 15 ma, yielding a dose rate of 80 rads/min to the cells. Immediately after irradiation, the cells were either returned to the incubator, subcultured at a dilution ratio of 1:4 for serial subcultivation and analysis of chromosomal changes, or reseeded at low density to measure cell survival.

Confluent Holding. The cells were seeded into 100-mm dishes and allowed to reach confluence. The culture medium was renewed at 24-hr intervals on 3 successive days, and irradiation was performed on the fourth day. Following irradiation, the confluent cultures were either returned to the incubator for a 4-hr holding period or were immediately subcultured in fresh medium to an appropriate lower density for colony formation or chromosome analysis. In some experiments, cultures were held up to 43 days postirradiation in confluence prior to subculture; the nutrient medium was renewed every 3 days. Subculture to low density is a stimulus for the initiation of DNA synthesis and cell proliferation. After the appropriate holding period, cultures were removed from the incubator and subcultured to low density to analyze cell survival and chromosomal damage.

Survival Assay. Surviving fractions were determined by a standard colony formation assay. Plastic 100-mm Petri dishes were seeded with an appropriate number of cells such that, accounting for the cloning efficiency and toxicity of the particular treatment, 50 to 100 macroscopic colonies would develop when the cultures were fixed and stained, approximately 2 weeks later. Five dishes were seeded for each dose or time point, and colonies containing greater than 50 cells were scored as survivors.

Cytogenetic Studies. In order to analyze chromosome aberrations and chromosome rearrangements, the cells were subcultured into eight 100-mm dishes at a density of approximately 1 × 10³ cells/dish. Dose-dependent mitotic delay occurred in the X-irradiated cell population, ranging from 1 to 4 hr following 100 to 400 rads of exposure. To analyze first mitotic cells, Colcemid (final concentration, 0.1 μg/ml) was added to each of 2 of the dishes at 5-hr intervals beginning 28 hr after subculture. The total sampling time thus covered a 20-hr period. Slides were prepared, and chromosome abnormalities were analyzed in the sample harvested at the peak mitotic index. Cells were harvested, treated with a hypotonic 0.075 M potassium chloride solution for 30 min at 37°, and fixed with acetic acid:methanol (1:3). Air-dried preparations were stained by 2% Giemsa solution. Chromosome-type aberrations were analyzed in 100 metaphase figures from each sample according to the criteria of Buckton and Pike (4).

Chromosome rearrangements were analyzed using G-banded karyotypes. In order to attain a high success rate (more than 80%) in G-banding preparations, we used a 2-step procedure. (a) To appropriately aged (1 week to 3 months) slides, 4 drops were added of a solution of 0.3 μ NaCl and 0.03 μ trisodium citrate at pH 7.2, and a coverslip was placed over the cells. These slides were maintained on a slide warmer at 57° for 40 min. The coverslips were then removed, and the slides were rinsed with distilled water, dried, and then warmed again with the NaCl:trisodium citrate solution at 57° for 1 hr. (b) Rinsed and dried slides were cooled to room temperature, covered with cold (0–2°) 0.25% trypsin in Earle’s balanced salt solution for 15 sec, quickly washed with cold salt solution:ethanol, and then dried. The chromosome specimens were stained with 2% Giemsa solution in 0.01 μ phosphate buffer (pH 6.8) at 20° for 8 min and photographed, and karyotypes were prepared. A total of 25 G-banded karyotypes representing cells in first mitosis were analyzed in each experimental group. Reciprocal translocations were scored, such as t(1:5) (p32; q13).

RESULTS

AG1522 cells were irradiated with 400 rads of X-rays at the confluent, density-inhibited phase of growth. A series of dishes was regularly passaged by a 1:4 split regimen, while the others were maintained in confluence without any further passages for up to 43 days. Chromosome-type aberrations (dicentrics, rings, and fragments) were measured at desired times. Chart 1 shows...
the changes observed with time in the frequencies of chromosome-type aberrations after X-irradiation. When cells were subcultured immediately after irradiation to allow cell division to take place, the frequency of chromosome aberrations declined rapidly (Chart 1, 0). Few aberrations were observed at the second subculture, and none was present at subculture 5. This rapid decline suggests that the presence of dicentrics, rings, and some fragments at mitosis is lethal to the cells.

In cells that were not subcultured after irradiation, but maintained in confluence with regular medium changes (Chart 1, O), the frequency of chromosome-type aberrations declined rapidly, corresponding exactly to the recovery kinetics for potentially lethal damage reported previously (20). However, the frequency of chromatid aberrations persisted at a relatively high level (about 30% of the initial frequency) in these cells during holding periods of up to 43 days (Chart 1).

G-banded karyotypes were used to analyze the frequency of chromosome rearrangements including reciprocal translocations, deletions, and inversions following X-irradiation. These results are shown in Table 1. We examined 25 cells each in the first mitosis and at subcultures 5 and 10 after irradiation. At the first mitosis after irradiation, the frequencies of translocations, dicentrics plus rings, fragments, and inversions were 0.48, 0.32, 0.56, and 0.04 per cell, respectively (Table 1). The frequency of translocations in the first mitosis was slightly higher than that of dicentrics and rings. The frequency of fragments was the highest, and inversions were the lowest of the types of chromosome damage induced by 400 rads. In subculture 5 after irradiation, no dicentrics, rings, or acentric fragments were observed, although a rather high frequency of translocations and deletions remained (Table 1, Chart 2). The persistence of deletions at subculture 5 suggests that chromosome fragments change to deletions following cell division, whereas acentric fragments disappear from one daughter cell. Spontaneous chromosome rearrangements were not observed in nonirradiated control cells studied at several passages (Table 1), except in cells nearing senescence (beyond passage 28 which represented approximately 56 mean population doublings).

We also analyzed the frequency of cells which contained translocations only. These data are summarized in Chart 2. Among karyotypes of 25 cells scored at the first mitosis after irradiation with 400 rads, 12 translocations were observed in 9 cells, yielding a translocation frequency of 12 of 25 or 0.48 per cell. Among the 9 cells with translocations, 3 cells had both translocations and dicentrics, 2 cells had both translocations and fragments, one had a translocation and an inversion, and 3 cells had only translocations. The frequency of cells containing only translocations was thus 3 of 25 or 0.12 at the first mitosis. This frequency in cells of subcultures 5 and 10 was 0.08 and 0.16, respectively (Chart 2). These results suggest that cells containing X-ray-induced translocations alone persisted over many cell generations and that such cells are very stable. However, the frequency of cells with only induced fragments at the first mitosis was 0.28, whereas those of cells with deletions alone at subcultures 5 and 10 were 0.08 and 0.04, respectively, suggesting that about 70% of cells containing fragments were eliminated during proliferation. The apparent decline in the frequency of translocations with subculture observed in Table 1 thus appears to reflect the death of cells which also contained deletions and dicentrics. Translocations at all subculture times appeared to be random in type, with no evidence for the appearances and expansion of abnormal clones bearing identical translocations.

The frequency of translocations at the first mitosis after exposure of confluent cultures to 600 rads was about twice as high as that observed in cultures treated with 400 rads (Table 2). The frequencies of dicentrics, rings, and deletions were similarly elevated. The relative elevation in the frequency of translocations was maintained at subcultures 5 and 10 (Table 2).

Chart 3 shows an X-ray survival curve and the results of an experiment in which confluent density-inhibited cultures of

![Chart 2](image-url)

**Chart 2.** The change in the frequency of chromosomal rearrangements per cell with subculture number (numbers in parentheses) and time (days) after 400 rads X-irradiation in proliferating (continually subcultured) cells. O, translocation frequency; A, deletion or fragment frequency; X, dicentric frequency. The cells with translocations (O) include those with dicentrics, inversions, or deletions (fragments) as well; the cells with deletions (A) include those with dicentrics, inversions, or translocations as well. X, frequency of cells which contain only deletions or fragments. A, frequency of cells which contain only translocations; A, frequency of cells which contain only deletions or fragments.

**Table 2**

<table>
<thead>
<tr>
<th>Dose (rads)</th>
<th>Subculture no. after irradiation</th>
<th>Passage no.</th>
<th>No. of cells analyzed</th>
<th>Translocations/cell</th>
<th>Dicentrics and rings/cell</th>
<th>Fragments or deletions/cell</th>
<th>Inversions/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>600</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>25</td>
<td>22 (0.88)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23 (0.92)</td>
<td>28 (1.12)</td>
<td>2 (0.08)</td>
</tr>
<tr>
<td>600</td>
<td>5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
<td>25</td>
<td>11 (0.44)</td>
<td>0</td>
<td>1 (0.04)</td>
<td>1 (0.04)</td>
</tr>
<tr>
<td>600</td>
<td>10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>15</td>
<td>25</td>
<td>14 (0.56)</td>
<td>0</td>
<td>3 (0.12)</td>
<td>2 (0.08)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total number of chromosome-type aberrations or translocations present in 25 cells analyzed.

<sup>b</sup> The first mitosis following 400 rads of X-irradiation.

<sup>c</sup> Numbers in parentheses, average frequency per cell.

<sup>d</sup> The first mitosis after 5 or 10 subcultures following 400 rads of X-irradiation.
AG1522 cells were irradiated with 400 rads of X-rays and then subcultured at low density either immediately or 4 hr later to measure colony-forming ability. Holding the cells in confluence for 4 hr after X-irradiation significantly enhanced their ultimate survival. This phenomenon has been termed recovery from potentially lethal damage (15).

Chart 4 presents the results of experiments in which the frequencies of chromosomal aberrations were measured at the first mitosis and at subcultures 2, 3, 5, and 10 in cells subcultured immediately after irradiation with 400 rads or held in confluence for a 4-hr recovery interval prior to subculture. As can be seen, the aberration frequency observed at the first mitosis declined from 0.8 to 0.45 aberration per cell in cells held for 4 hr prior to subculture, whereas survival in this population was enhanced from 15 to 31% (Chart 3). The aberration frequencies remained unchanged, however, with longer holding times.

As can be seen in Tables 3 and 4, the frequencies of translocations in subcultures 5 and 10 of cells initially allowed 4 hr of recovery prior to the first subculture were higher than in cells allowed no recovery. In the first mitosis, however, the frequencies of translocations were similar in the immediate and 4-hr subculture groups (Table 4).

### DISCUSSION

Most human tumors are characterized by chromosomal rearrangements, suggesting that such rearrangements may be causally related to the induction of cancer. Radiation and chemical carcinogens are known to induce chromosome rearrangements, but the persistence of these rearrangements in proliferating human cells has not been systematically studied. Buckton et al. (3) examined the frequency of cells with unstable and stable chromosome aberrations in peripheral blood lymphocytes of patients treated with 250 kV of X-rays for ankylosing spondylitis. The level of cells with stable chromosome aberrations remained approximately constant over the 20-year period following exposure (3). Similarly, persistent aberrations have been observed in Hiroshima atomic bomb survivors (24) and Syrian hamsters exposed to X-rays (27).

As is seen in Chart 1, the frequency of persistent chromosome-type aberrations in irradiated human diploid cells declined rapidly with cell proliferation after irradiation; by the fifth subculture, aberration frequencies had essentially dropped to control levels. The frequency of chromosome aberration in cells that were not subcultured after irradiation and thus not allowed to proliferate declined rapidly within 24 to 48 hr of exposure, reaching an

### Table 3

<table>
<thead>
<tr>
<th>Dose (rads)</th>
<th>Subculture no. after irradiation</th>
<th>Passage no.</th>
<th>No. of cells analyzed</th>
<th>Translocations/cell</th>
<th>Dicentrics and rings/cell</th>
<th>Fragments or deletion/cell</th>
<th>Inversions/cell</th>
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<tbody>
<tr>
<td>400</td>
<td>1b</td>
<td>6</td>
<td>25</td>
<td>12 (0.48)</td>
<td>5 (0.20)</td>
<td>10 (0.40)</td>
<td>0</td>
</tr>
<tr>
<td>400</td>
<td>5b</td>
<td>10</td>
<td>25</td>
<td>8 (0.32)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>400</td>
<td>10b</td>
<td>15</td>
<td>25</td>
<td>8 (0.32)</td>
<td>0</td>
<td>1 (0.04)</td>
<td>0</td>
</tr>
</tbody>
</table>

- Total number of chromosome-type aberrations or translocations present in 25 cells analyzed.
- The first mitosis following 400 rads of X-irradiation.
- Numbers in parentheses, average frequency per cell.
- The first mitosis after 5 or 10 subcultures following 400 rads of X-irradiation and 4 hr of holding.
apparently stable plateau of about 30% residual aberrations which persisted up to 43 days thereafter. The kinetics of the initial rapid decline is similar to that observed during confluent holding recovery in density-inhibited mouse 10T1/2 cells irradiated with 400 rads (20). The decline in the aberration frequency in noncycling cells is thus probably related to a cellular repair process, whereas in cycling cells, it probably results from cell death at mitosis as, for example, from anaphase bridge formation by dicentric chromosomes at mitosis (1, 6).

Translocations are apparently the result of recombinations of DNA from 2 chromosomes and are probably induced by DNA double-strand breaks and misrejoining. We assume that the mechanism of dicentric formation is probably similar to that for translocation formation. The mechanism for the reduction in the frequency of translocations with cell proliferations as shown in Chart 2 differs from that for the reduction of chromosome aberration in Chart 1. The results in Chart 2 indicate that translocations are not lethal during cell division, and cells survive for at least 10 passages (20 mean population doublings) with stable translocations. The apparent decline in the frequency of translocations seen in Table 1 can be attributed to the occurrence of dicentrics or deletions in the same cells, which are lethal lesions (Chart 2). The frequency of cells with translocations alone remained unchanged (Chart 2). We cannot exclude the possibility that the initial radiation-induced translocations were gradually eliminated from the population, while the remaining translocations were independent of those caused by the radiation exposure itself, leading to an apparently constant level of translocations.

Clones of cytogenetically abnormal cells with chromosome rearrangements have been recognized in fibroblasts cultured from normal human adult skin (12, 17). We observed no such clones in human newborn foreskin fibroblasts under similar culture conditions. The karyotype of nonirradiated control cells remained diploid for prolonged periods, although the percentage of cells with chromosome abnormalities increased quite sharply as the cells approached senescence. It has been reported previously that clones of cells with translocations may be recognized in fibroblasts cultured from patients who have been irradiated (2, 7, 31) or who have one of a small group of genetically determined disorders, including Fanconi’s anemia (10), Bloom’s syndrome (9), ataxia telangiectasia (11), porokeratosis of Mibelli (29), and xeroderma pigmentosum (8). All of these subjects have an increased susceptibility to develop malignant disease. Our findings of a persistent increase in the frequency of cells with translocations following X-irradiation suggest that the frequent occurrence of translocations in cells of cancer-prone individuals might reflect the expression of a similar underlying process as we observe in irradiated cells in culture.

As can be seen in Charts 3 and 4, the relative decline in chromosomal aberrations during the 4-hr confluent holding period closely paralleled the enhancement in survival (recovery from potentially lethal damage) seen during the same interval. Chromosomal aberrations are generally lethal events. They have been shown previously to correlate closely with lethality under several conditions and have been associated with DNA double-strand breaks. As is seen in Tables 3 and 4, however, the changes in the frequencies of translocations during recovery differed markedly from those for survival and aberrations. The higher frequency of translocations at subculture 5 in cells allowed to recover for 4 hr is consistent with the fact that cells allowed a period of recovery prior to subculture show increased survival and a decline in aberrations. The frequency of translocations in first mitotic cells after 4 hr of recovery was similar to that in the 0-hr group (Table 4), whereas the frequency of aberrations declined by nearly 50% (Chart 4). Thus, the frequency of cells containing translocations only should increase by nearly 2-fold in the 4-hr, as compared with the 0-hr, recovery group. The nearly 2-fold rise in the translocation frequency in subculture 5 is consistent with this hypothesis and would thus result from the decreased probability that cells with stable translocations will also have lethal chromosome aberrations.

Terzaghi and Little (30) observed that the frequency of malignant transformation induced by X-ray frequency was enhanced when mouse 10T1/2 cells were held in confluent, densely inhibited growth for 2 to 4 hr after irradiation. In similar experiments, a parallel increase in survival (15) and in the frequency of sister chromatid exchanges (19, 20) was also found during the first 4 hr of holding. These findings have been interpreted in terms of the hypothesis that misrepair might occur in mammalian cells exposed to X-rays and that mitotic recombination reflected by sister chromatid exchanges might be an important step in the expression of radiation damage in several conditions.

In the present investigation, we have observed that the frequency of stable translocations is enhanced in X-irradiated human cells allowed 4 hr of recovery prior to subculture. We hypothesize that these findings are related and, on this basis, that genetic recombination and chromosomal rearrangement induced by DNA damage may be an important process in the initiation of cancer.

**ACKNOWLEDGMENTS**

We thank Erika Matkin for expert technical assistance.

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*Cancer Res* 1984;44:3706-3711.