Extension of *In vitro* Life-span of γ-irradiated Human Embryo Cells Accompanied by Chromosome Instability

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**Low dose γ-rays/Life-span extension/Chromosome bridge/Chromosome instability/Human embryo cells**

We studied the effect of repeated irradiation with a low dose rate (about 0.0012 cGy per min) of \(^{60}\)Co γ-rays on the *in vitro* life-span of human embryo (HE) cells. HE cells were cultured in an incubator that was set in a \(^{60}\)Co γ-ray-irradiation room, and the irradiation was repeated throughout the life-span of the HE cells (for about 150 to 160 days) on every day base. During this period, the cells accumulated 106 to 123 cGy. The life-span of the irradiated cells prolonged 1.14 to 1.35 times when compared to that of non-irradiated cells. The incidence of cells with chromosome bridge and micronuclei significantly increased in the irradiated cells. Although the number of chromosomes gradually changed with repetition of culture in both non-irradiated and irradiated cells, the frequencies of aneuploid cells in irradiated cells were about two times higher than that in non-irradiated cells. These results indicate that repeated irradiation with a low dose of γ-rays produces chromosome instability, and it may be a cause of numerical chromosome abnormalities and life-span extension of irradiated cells.

**INTRODUCTION**

Biological effects of low doses and low dose rates might be different from those of high doses and high dose rates. According to epidemiological study, the frequencies of chromosome aberration of residents who lived in high background area in China were significantly higher than in normal area\(^{12}\). However, the cancer mortality showed no meaningful difference in both\(^2\).

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We have previously reported that the frequency of morphologically transformed foci increases in the repeatedly irradiated cells with low dose of γ-rays, but the frequency of mutants does not. These contradict the traditional hypothesis that DNA damage causes chromosome aberration, mutation, and transformation. To elucidate the contradiction, we need to do a more detailed examination about the mechanisms of biological effects of low doses and low dose rates of radiation. It is also important to evaluate a risk of low dose radiation.

Normal human diploid cells placed in culture have a finite proliferative life-span and non dividing state termed senescence, which is characterized by altered gene expression. Generally, human cells, different from rodent cells, are hard to acquire unlimited life-span and to transform in vitro by radiation. However, we have found that repeated irradiation of low dose γ-rays or X-rays prolonged the life-span of human cells. In the previous studies, we found that morphologically transformed foci frequently appeared in irradiated cultures, and that transformed cells are accompanied by numerical abnormality of chromosome. However, mutation frequency of irradiated cells at hprt gene was not elevated compared with non-irradiated cells. These cells were not able to acquire unlimited life-span in vitro finally. In contrast, other studies have shown that the life-span of human cells irradiated with a low dose γ-rays is the same as that of non-irradiated cells or is even shortened compared with control cells. Results about genetical effects with repeated radiation contradict. In addition, the irradiation in the above studies have been carried out with relatively higher dose rates, such as 0.27 to 140 cGy per min. These dose rates are too high to suppose biological influence of environment radiation.

Therefore, in the present study, we examined the biological effects of irradiation with 60Co γ-rays at a very low dose rate (about 0.0012 cGy per min), which was 10² to 10⁵ times lower than those used in the previous experiments on primary human embryo cells.

**MATERIALS AND METHODS**

**Cells**

HE cells derived from three different donors (HE17, HE35, and HE50) were used. Cells were grown in Eagle’s minimum essential medium (MEM) supplemented with 0.2 mM serine, 0.2 mM aspartate, 1mM pyruvate, and 10% fetal bovine serum containing 0.3% 2-[4-2-Hydroxyethyl]-l-piperazinyl] ethan-sulfonic acid (HEPES, Dojin Chem., Co. Ltd., Kumamoto) in an incubator at 37°C.

**Irradiation**

One million cells were inoculated in a plastic T75 flask (75 cm²) for γ-irradiation. The flasks were placed in an incubator that was set in a 60Co γ-ray irradiation room (RIKEN, Wako) and were irradiated at 37°C for 10 hours per day (from 8:00 p.m. to 6:00 a.m.). Dosiometry was carried out with a thermoluminescence dosimeter (TLD) (UD-170A Matsushita Electric Co., Osaka). The average dose rates through the life-span at the positions of the three HE cells measured were 1.4 × 10⁻³ cGy per min for HE17, 1 × 10⁻³ cGy per min for HE35 and 1.3 × 10⁻³ cGy per min for HE50. There was no difference in growth speed and the cloning ability of both
irradiated under the above conditions and non-irradiated cells.

**Life-span determination**

Cells were subcultured at an inoculum of $10^6$ cells per T75 flask every 5 day. During subcultivation, the number of cells in the flask ($n$) was counted with a Coulter Counter, and the mean population doubling number (PDN) was calculated as follows:

$$\text{PDN} = \frac{\log(n/10^6)}{\log2}$$

The total population doubling number (TPDN) which was calculated by summation of the PDNs represents the life-span of the cells.

**Analysis of chromosome bridge and micronuclei**

Cells were inoculated onto cover slips in 35-mm plastic dishes with 2 ml of culture medium. The cells were incubated for 2 days in an incubator. The cells were then fixed with ethanol for 5 min and stained with 50 mg/ml of propidium iodide solution containing 200 mg/ml of RNase for 30 min at 37°C. Chromosome bridge and micronuclei were counted under the fluorescent microscope (Olympus AX80, Tokyo).

**Chromosome analysis**

To examine the chromosome aberrations, chromosome samples were prepared by a standard air-drying method$^3$. The number and karyotype of chromosomes in 100 metaphases per sample were scored.

**RESULTS**

Figure 1 shows the growth curves of the three HE (HE17, HE35 and HE50) cell strains. Cells stopped growth at around 150–160 days in culture.

The irradiated cell cultures accumulated 1.06 to 1.23 Gy at senescence. The total population doubling number (TPDN) in irradiated cells was significantly larger than that in control cells in each of the three different HE cells. The results are summarized in Table 1. TPDN of irradiated cells were increased to 114–135% of the non-irradiated cells. These results indicate that the life-span of the cell population is prolonged by repeated low-dose irradiation. This phenomenon is consistent with that of previous reports$^{3,18}$. Surprisingly, in cell groups repeatedly irradiated, the cell with micronuclei (A in Fig. 2) and chromosome bridge (B and C in Fig. 2) was found in high frequency. The results are summarized in Table 2. The incidence of cells with micronuclei increased by at least 3.1–4.8 times in repeatedly irradiated cells (Table 2). Also, the incidence of cells with chromosome bridge in the irradiated cells increased to 11–19 times higher than those in the non-irradiated cells.

As culture time became long, both non-irradiated and irradiated cell populations showed numerical abnormalities in chromosome, rather than structural abnormalities such as rearrange-
Fig. 1. Effects of repeated irradiation with a low dose rate of $^{60}$Co $\gamma$-rays on life-span in vitro of three different HE cell cultures. Open symbols, non-irradiated cells; closed symbols, repeatedly irradiated-cells with a low dose rate (about 0.0012 cGy/min) of $\gamma$-rays. Circles, HE17 cells; Triangles, HE35 cells; Squares, HE50 cells.

Fig. 2. Appearance of micronuclei (A) and chromosome bridge (B and C) in repeatedly irradiated-cells with a low dose rate of $^{60}$Co $\gamma$-rays at about 40 PDN level of HE 35 cells. The cells were stained with propidium iodide solution and micronuclei and chromosome bridge were counted under the fluorescent microscope (see Materials and Methods).
### Table 1. Accumulated dose and extension of life span of human embryonic cells irradiated with repeated low dose of $\gamma$-rays and non-irradiated cultures

<table>
<thead>
<tr>
<th>Cell</th>
<th>Total dose accumulated until senescence$^a$ (Gy)</th>
<th>Total population doubling number until senescence$^a$ (TPDN)</th>
<th>Degree of life span extension$^b$ (TPDN-irradiated / TPDN-non-irradiated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE17</td>
<td>0</td>
<td>41.3</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>1.18</td>
<td>53.1</td>
<td></td>
</tr>
<tr>
<td>HE35</td>
<td>0</td>
<td>47.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.06</td>
<td>53.9</td>
<td>1.14</td>
</tr>
<tr>
<td>HE50</td>
<td>0</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.23</td>
<td>45.9</td>
<td>1.35</td>
</tr>
</tbody>
</table>

$^a$Data show the average of two independent experiments.

$^b$Degree of life span extension was calculated by dividing total population doubling number of irradiated cells by that of non-irradiated cells.

### Table 2. Incidence of cells with chromosome bridge and micronuclei in chronically low dose-irradiated cultures at late passages

<table>
<thead>
<tr>
<th>Cell</th>
<th>Population doubling number at time of test (Gy)</th>
<th>Dose received at time of test (Gy)</th>
<th>% of cell with chromosome bridge$^a$</th>
<th>% of cell with micronuclei$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE17</td>
<td>32.3</td>
<td>0</td>
<td>&lt; 0.5 (0)</td>
<td>0.34 (34)</td>
</tr>
<tr>
<td></td>
<td>38.4</td>
<td>0.67</td>
<td>6.5 (13)$^c$</td>
<td>1.05 (105)$^c$</td>
</tr>
<tr>
<td>HE35</td>
<td>34.0</td>
<td>0</td>
<td>0.5 (1)</td>
<td>0.27 (27)</td>
</tr>
<tr>
<td></td>
<td>38.8</td>
<td>0.50</td>
<td>9.5 (19)$^c$</td>
<td>0.85 (85)$^c$</td>
</tr>
<tr>
<td>HE50</td>
<td>18.9</td>
<td>0</td>
<td>&lt; 0.5 (0)</td>
<td>0.25 (25)</td>
</tr>
<tr>
<td></td>
<td>33.3</td>
<td>0.70</td>
<td>5.5 (11)$^c$</td>
<td>1.18 (118)$^c$</td>
</tr>
</tbody>
</table>

$^a$To measure the frequency of cells with chromosome bridge, 200 telophase cells were counted. The number in a parenthesis shows the number of cells with a chromatin-bridge per 200 telophase cells examined.

$^b$To measure the frequency of cells with micronuclei, 10,000 cells were counted. The number in a parenthesis shows the number of cells with micronuclei per 10,000 cells examined.

$^c$Differences between groups were tested for significance using a conditional test between two Poisson distribution groups described by Kendall and Stuart$^{16}$. Differences were significant at $p < 0.01$ against the non-irradiated control group of each cell.
Fig. 3. The appearance of aneuploid cells with increasing population doubling number in non-irradiated- and repeatedly irradiated-human embryo cells with a low dose rate of $^{60}$Co $\gamma$-rays. Closed circles; non-irradiated cells; Open circles, irradiated cells.

Table 3. Incidence of aneuploid cells in repeatedly irradiated human embryo cells with low dose of $\gamma$-rays and non-irradiated cells$^{a}$

<table>
<thead>
<tr>
<th>Cell</th>
<th>Total population doubling number until senescence</th>
<th>Population doubling number at time of test</th>
<th>Accumulated dose (Gy) until time of test</th>
<th>% of aneuploid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE17</td>
<td>41.3</td>
<td>38.1</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>53.1</td>
<td>48.4</td>
<td>0.93</td>
<td>67 (2.68)$^{b}$</td>
</tr>
<tr>
<td>HE35</td>
<td>47.1</td>
<td>44.9</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>53.9</td>
<td>49.9</td>
<td>0.71</td>
<td>57 (2.85)</td>
</tr>
<tr>
<td>HE50</td>
<td>34.0</td>
<td>31.0</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>45.9</td>
<td>43.3</td>
<td>0.99</td>
<td>58 (2.00)</td>
</tr>
</tbody>
</table>

$^{a}$Data show the average of two independent experiments.

$^{b}$The number in a parenthesis shows the ratio of percent of aneuploid cells in irradiated cell population for that of non-irradiated cell population.

ments and deletions. Figure 3 shows the frequencies of aneuploid cells in the three different HE cell populations. The frequency of aneuploid cell was higher in irradiated cell population than in non-irradiated cell population at equal PDN level. At the late passages (30–50 PDN), only 20–29% of the non-irradiated cells were aneuploid, while 57–67% of the irradiated cells were aneuploid (Table 3). Figure 4 shows the distribution of chromosome number in non-irradiated and irradiated cells. Result shows that increase in chromosome number was rare. These suggest that multiple irradiations with low doses of $\gamma$-rays may cause abnormalities in the division device of cells.
Fig. 4. The distribution of chromosome number at various passages in non-irradiated- and repeatedly irradiated-human embryo cells with a low dose rate of $^{60}$Co $\gamma$-rays.

DISCUSSION

Treatment with carcinogens increased the frequency of escape from senescence in fibroblasts and transformed them. Therefore, infinite proliferative life-span is the greatest characteristic of cancer cell. However, a big difference in the frequency of escape from senescence is present between rodent cells and human cells. Generally, rodent cells easily escape from senescence and are immortalized by radiations\textsuperscript{9-12}, but human cells are not\textsuperscript{13-19}. This means that we cannot use rodent cells instead of human cells in carcinogenesis experiment. Therefore, we continued looking for the condition that human cell was easily immortalized.

We have found that repeated irradiation of low dose of $\gamma$-rays (7.5 cGy per week) extended the life-span of the human cell \textit{in vitro}\textsuperscript{31}. In that study, we used 50 cGy/min as an exposure dose rate, which is a comparatively higher dose rate than dose rate of natural radiation. In the present study, therefore, we tried to use lower dose rate (about 0.0012cGy per min) than that. Of course, this dose rate is still quite high for considering the effects of low-level natural radiation on humans.

In the present study, we observed the prolongation of life-span of three HE cell strains by $\gamma$-rays, but not the immortalization, as reported\textsuperscript{11,18}. A similar phenomenon has been also observed with the cell that was transfected SV40 large T-antigen domain\textsuperscript{20}. SV40 T-antigen-expressing human cells generally have an extension of life-span over mortality stage 1 until mortality stage 2 begins. SV40 T antigen is a large multifunctional protein. It has helicase activity and ATPase activity, and it has binding activities to DNA, ATP, DNA \textit{pol} \textit{a}, Rb protein, pl07 and p53. The mechanism of the action is apparently simple: this viral protein bind to the protein products of
two key tumor suppressor genes (Rb and p53) of the host cell, putting them out of action and so permitting the cells to replicate it's DNA and divide\textsuperscript{21}. Therefore, the higher frequency of escape from mortality stage 1 in irradiated cells is best explained by postulating a reactivation of DNA synthesis. It is well known that low dose irradiation activated several genes to be related to cell growth, induced DNA replication, and cell division following it\textsuperscript{22-25}. Mortality stage 2 is an independent mechanism that is responsible for the failure of cell division during crisis. The inactivation of mortality stage 2 is a rare event, probably of mutational origin in human cells, independent of or only indirectly related to the expression of T antigen.

T-antigen-transfected fibroblasts were uniformly highly aneuploid both before and after crisis\textsuperscript{20}. We also found a tendency for the chromosome number to decrease in repeatedly irradiated cells. We have previously reported that the chromosome number in irradiated cells decreased slightly. About 40-60% of cells lost several chromosomes, and 70-80% of them lost a number 1 chromosome\textsuperscript{3}. Although we were not able to specify which chromosome disappeared in this study, we found that the number of cells having a decreased chromosome number also increased in both non-irradiated and irradiated cells with the passage of time. However, our results show that frequency of numerical chromosome change in multiply irradiated cell is higher than that in non-irradiated cells.

If the abnormality arises during cell division, a decrease in chromosome number should be found in one of two sister cells, and an increase in chromosome number should be found in the other cell. Therefore, increase of chromosome number should be done at the almost equal frequency as decrease of chromosome number. In our experiments, including the present study, however, an increase in chromosome number was rare in the cells repeatedly irradiated with a low dose rate of $\gamma$-rays and X-rays. The reason for this phenomenon is not clear. The most appealing hypothesis is that chromosome end-fusion is being generated through the telomere structure. A dicentric chromosome will be produced by the fusion of 2 chromosomes. Most dicentric chromosomes may cause lethal damage. Therefore, when dicentric is produced in the cell, cell must brake and rejoin it to survive. During this fusion-breakage process, any cell that loses a large quantity of genetic information should die. We examined changes in telomerase activity through out the life of human cells by telomere repeat amplification protocol assay\textsuperscript{18}. Although 39% of HE cell strains (16 out of 41) showed the activity of the telomerase during early passages, telomerase activity in the HE cells completely lost by passage 4. Telomerase activity never revived after undergoing multiple X-irradiation before senescence\textsuperscript{18}. This means that telomere becomes unstable and does not act on stabilization of chromosome at late passages. In fact, the frequency of chromosome bridge increased significantly in the progeny of the irradiated cells. It seems that the multiple irradiation at a low dose rate of $\gamma$-rays causes abnormalities in the division apparatus. If so, the probability that there is a non-equal separation of genetic material should be high. In fact, the frequency of cells with micronuclei increases 3-5 times of normal levels in the irradiated cells. Low dose radiation clearly induced chromosome instability in human cells. We previously found that these abnormalities appeared in cells in colony of normal human cells surviving after 6 Gy of X-rays\textsuperscript{26}. Therefore, this phenomenon may be not specific for repeated irradiation with low dose. In the present study, total accumulated dose of $\gamma$-rays was below 1.5 Gy. However, the frequencies of abnormalities observed (average 7.1% for chromo-
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...some bridge and 1.02% for micronuclei for 1.5 Gy) were similar to those of the previous experiment (average 8.9% for chromosome bridge and 0.86% for micronuclei for 6 Gy)\(^\text{26}\). Therefore, it may be presumed that repeated radiation with low dose X-rays is easy to produce chromosome instability than acute radiation with high dose.

We have not yet proved that chromosome bridge was correlated with dicentric chromosomes. However, a kind of recombination process could be suggested to be involved in the expression of the chromosome bridge. So far, a few studies have suggested that a recombination process may be involved in the expression of delayed effects\(^\text{27–29}\). Other studies have suggested that delayed effects are revealed by recombination between interstitial telomere-like sequences\(^\text{30–32}\). Previous studies have indicated that multiple rearrangements were seen because of delayed chromosome instability, suggesting that activation of a physiological process may be involved\(^\text{33–34}\). Thus, chromosome instability was thought to be induced by the variety of stress response processes subsequently activated by the unscheduled breakage of potentially unstable chromosome region. Potentially unstable chromosome region is a non-lethal damage and may be introduced during the rejoining of DNA strand breaks caused by ionizing radiation\(^\text{\text{35}}\). Although the molecular nature of potentially unstable chromosome region remains to be determined, a previous report has indicated that fragile sites are associated with bridge-breakage-fusion cycle\(^\text{36}\). Therefore, further studies are required to determine whether the repair process of the DNA damage caused by low dose of ionizing radiation produces fragile sites that may result in a potentially unstable chromosome region, and whether those artificial sequences are involved in chromosome instability.

In conclusion, repeated irradiation with low dose of \(\gamma\)-rays may induce chromosomal instability in human embryo cells and may cause extension of life-span of human cells. Repeated irradiation system may be useful to study carcinogenesis mechanism of human cells.

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