**hTERT-Immortalized Cells Useful for Analyzing Effects of Low-Dose-Rate Radiation on Human Cells**

Hideaki NAKAMURA*

*Corresponding author: Phone: +81-52-762-6111 (ext. 7012), Fax: +81-52-763-5233, E-mail: hnakamu@aichi-cc.jp

Central Laboratory and Radiation Biology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan. doi:10.1269/jrr.07088

**Introduction**

It may be important to know the effects of low-dose-rate radiation on human cells in order to assess the effects of radiation from such as nuclear power plants and space flights. Epidemiological studies of nuclear industry workers have not yielded definitive evidence that exposure to occupational low-dose-rate radiation increases cancer mortality. However, the HPRT mutation fraction in clean-up workers in Chernobyl was estimated to be higher than the mutation fraction in control subjects. Therefore, determining the effects of low-dose-rate radiation on human cells is a key issue in radiation protection. While several studies on mammalian cells reported the effects of low-dose-rate radiation, knowledge on the genetic effects of low-dose-rate radiation, especially on human cells, is far from complete.

In analyzing the genetic effects of low-dose-rate radiation on human cells, the adaptive response to radiation may be an important factor. It is also becoming clear that this response is mediated by cellular signal transduction mechanisms, which are mainly involved with the TP53 gene and others. This suggests that the use of SV40-immortalized cells or cancer-derived cells may not be appropriate for analyzing the effect of low-dose-rate radiation on human cells since these model cell lines frequently acquire TP53 inactivation or mutation. Moreover, these cells often exhibit abnormality in cell cycle control, functional checkpoints, anchorage-dependent contact inhibition, and growth factor-dependent proliferation. However, since normal human primary cells have a limited life span and reach senescence after a certain number of cell divisions, they may also not be suitable for this purpose.

It has been reported that the introduction and forced expression of the human telomere reverse transcriptase (hTERT) gene can extend the life span of human primary fibroblast cells without any change in the fundamental characteristics of the cells. In addition, hTERT-transfected cells were reportedly not tumorigenic when transplanted in mice. Moreover, in our laboratory, our hTERT-immortalized cells still exhibited normal activity in the TP53 pathway as primary fibroblast cells, and also indicated apparent growth arrest after reaching a confluent state. These find-
ings indicate that these cells may be suitable materials to analyze the effects of low-dose-rate radiation on human cells. Here, we review the study in our laboratory on the effects of low-dose-rate radiation on hTERT-immortalized cells.

CHARACTERIZATION OF HUMAN SKIN FIBROBLAST TRANSFECTED WITH THE hTERT GENE

We transfected human fibroblast cells obtained from normal individual (SuSa) and ataxia telangiectasia (AT) patients (AT1OS, AT1KY, AT2KY and AT5KY) with the hTERT gene using retrovirus vector system. The cells introduced with the hTERT gene were identified by adding ‘T-n’ behind their original names (e.g., SuSa/T-n), and cells transfected with vector alone were identified by adding ‘neo’. After transfection, while telomerase activity was not detected in the vector alone-transfected cells, all of the cells transfected with the hTERT gene exhibited significant telomerase activity. In addition, the telomere lengths were longer in the hTERT-transfected cells than in the original cells.

After transfection of the retrovirus vector (hTERT gene inserted or vector alone), stable transfectants were selected by G418. G418-resistant cells were then continuously subcultured more than 2 years. Although the cells transfected with vector alone reached completed senescence before the population doubling number (PDN) 50, all the cells transfected with hTERT gene have continued to grow beyond PDN 300 with no indication of senescence like a flat shape and reduced growth rate (Fig. 1). Especially, SuSa/T-n and AT1OS/T-n cells continued to grow beyond PDN 500 (data not shown). Therefore, the expression of hTERT can immortalize skin fibroblasts derived from normal individual and AT patients.

Our immortalized cells exhibited apparent growth arrest after reaching a confluent state, and karyotypes of immortalized cells kept a diploid range with a modal number of 46. In addition, hTERT-immortalized cells from normal individuals retain arrest of cell-cycle checkpoints and TP53 response after radiation. Moreover, immortalized cells maintained their original radiosensitivity. Therefore, it suggests that our immortalized cells showed original characteristics and radiosensitivity except for immortalization, and they might be useful analyzing various effects of radiation on human cells.

EFFECTS OF LOW-DOSE-RATE RADIATION ON hTERT-IMMORTALIZED HUMAN CELLS

We analyzed the genetic effects of low-dose-rate radiation on hTERT-immortalized normal (SuSa/T-n) and AT cells (AT1OS/T-n, AT2KY/T-n and AT5KY/T-n). These cells were cultured to a confluent state and arrested at G0/G1 phase for irradiation. High-dose-rate irradiation was delivered at 2 Gy/min using a model MBR1520R X-ray machine (150 kV, 20 mA, 2-mm aluminum filter). Low-dose-rate irradiation was delivered at a dose rate of 0.3 mGy/min using a 137Cs γ-ray source at the Radiation Biology Center, Kyoto University. Cells were irradiated continuously at 37°C and 5% CO₂ in an incubator set in front of the 137Cs source (maximum 13 days), and cells were maintained in a low-serum environment.

Fig. 1. Growth curves of control and hTERT-introduced cells. All hTERT-introduced cells have grown far beyond the points at which control cells stopped growing. AT1OS/neo cells were lost in transfection of the neo-vector because of contamination.

medium (3% FBS) in a confluent state without a medium change. We confirmed that the unirradiated immortal cells, after being maintained under this condition for up to 13 days, exhibited the same plating efficiency, micronucleus induction, mutation induction and γ-H2AX focus formation as the cells without holding.

**Survival and Micronucleus Induction in hTERT-Immortalized Normal Cells**

The survival curves in SuSa/T-n cells obtained after high-dose-rate and low-dose-rate irradiation were significantly different (Fig. 2A). While the survival after 5 Gy of high-dose-rate radiation was approximately 0.01, survival after 5 Gy of low-dose-rate radiation was 0.3. The \( D_0 \)'s after irradiation at high- and low-dose rate were 1.14 and 6.11 Gy, respectively. The dose-rate-modifying factor (DRMF), calculated from the \( D_0 \)'s of the low-dose-rate and high-dose-rate survival curves, was 5.4. These results are consistent with previous reports that showed a high survival of normal fibroblast cells exposed to low-dose-rate radiation. In addition, it was reported that the DRMF for low-dose-rate radiation (0.023 or 0.153 Gy/h) relative to high-dose-rate radiation (0.70 Gy/min) ranged from 4.3 to 6.3 among six human fibroblast cell strains.\(^{28}\) Our results are also in this range of DRMF.

The micronucleus induction was approximately 74% after 5-Gy high-dose-rate irradiation compared to only 16% after low-dose-rate irradiation at the same dose (Fig. 2B). The increase in the micronucleus induction after low-dose-rate irradiation was much smaller than that after high-dose-rate irradiation.

**Mutation Induction in hTERT-Immortalized Normal Cells**

After irradiation at high- or low-dose rate, we selected HPRT-deficient mutants with \( 10^{-5} \) M 6-thioguanine (6-TG). The mutation induction at HPRT locus in SuSa/T-n cells observed after 5-Gy low-dose-rate irradiation was 7.6-fold higher than the spontaneous mutation induction observed in cells cultured continuously for 13 days without irradiation (Fig. 3). However, the mutation induction after low-dose-rate irradiation decreased to approximately one-eighth of that after high-dose-rate irradiation. This was consistent with the results of survival analysis. These results also indicated a significant dose-rate effect in mutation induction. Physical reactions such as DNA damage induced by radiation are reported to be independent of dose rate,\(^{11}\) suggesting that low-dose-rate radiation-induced damage might be repaired efficiently by cellular repair systems.

The dose response curves for mutation induction were fitted to linear-quadratic model for the high-dose-rate radiation-induced mutation, and the linear relationship for the low-dose-rate irradiation. High-dose-rate radiation induces more simultaneous damage, which might cause the mutation

![Fig. 2](http://jrr.jstage.jst.go.jp)
induction to increase with dose in a quadratic manner. On the other hand, with low-dose-rate radiation, it is supposed that the induced mutant fractions increase linearly due to the sparse damage induced at any given time and the efficient repair of such damage.

**Mutation Pattern at HPRT Locus in hTERT-Immortalized Normal Cells**

Total deletion was not detected in 6-TG-resistant clones that developed from unirradiated cells (Table 1). In the case of high-dose-rate irradiation, 61% of 6-TG-resistant clones lost all exons, and 1.7% of that exhibited a partial deletion. In contrast, after low-dose-rate irradiation, only 5.3% of 6-TG-resistant clones lost all exons, and 14.6% of that exhibited a partial deletion. This indicates that most mutations induced by low-dose-rate radiation exhibited either deletions of small-size or point mutations. Both the size of the deletions and fraction of deletions among the induced mutants are smaller for low-dose-rate radiation than for high-dose-rate radiation. It was reported that the proportion of total deletions of the entire HPRT locus was dependent on the radiation dose. Therefore, it is possible that the proportion of total deletions at the HPRT locus is dependent on both the dose and the dose rate. Our data suggest that low-dose-rate radiation-induced damage was repaired efficiently and correctly with a system that was relatively error-free compared to that of repairing damage caused by high-dose-rate irradiation.

**Survival and Micronucleus Induction in hTERT-Immortalized AT Cells**

While SuSa/T-n cells showed a greater resistance after low-dose-rate irradiation than after high-dose-rate irradiation, AT cells irradiated at a low-dose rate showed virtually

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### Table 1. Mutation Spectra in Spontaneous and Radiation-Induced HPRT Mutants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of mutants</th>
<th>Number of total deletion mutants (%)</th>
<th>Number of partial deletion mutants (%)</th>
<th>Number of other mutants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>69</td>
<td>0(0)</td>
<td>6(8.7)</td>
<td>63(91.3)</td>
</tr>
<tr>
<td>LDR 5 Gy</td>
<td>87</td>
<td>4(4.6)</td>
<td>12(13.8)</td>
<td>71(81.6)</td>
</tr>
<tr>
<td>After back-ground subtraction</td>
<td>4*(5.3)</td>
<td>11*(14.6)</td>
<td></td>
<td>61*(80.1)</td>
</tr>
<tr>
<td>HDR 5 Gy</td>
<td>61</td>
<td>36(59.0)</td>
<td>1(1.6)</td>
<td>24(39.3)</td>
</tr>
<tr>
<td>After back-ground subtraction</td>
<td>36*(61.0)</td>
<td>1*(1.7)</td>
<td></td>
<td>22*(37.3)</td>
</tr>
</tbody>
</table>

Note. Each mutant was confirmed by multiplex PCR exon analysis. Total deletion mutants refer to mutants that lack all HPRT exons. Partial deletion mutants consist of either intragenic or end deletion. Other mutations refer to mutants that show no exon deletion. *These values are obtained by subtraction of expected background number for each type of mutation.
the same survival as those irradiated at a high-dose rate (Fig. 2A). The average $D_0$ in all AT cell lines after high- and low-dose-rate irradiation was 0.39 and 0.44 Gy, respectively. Our results were consistent with the report that the primary AT cells showed no dose-rate effect.²⁸

Although micronucleus induction by low-dose-rate radiation in SuSa/T-n cells exhibited a large reduction that was consistent with the results of their survival and mutation assay, micronucleus induction after low-dose-rate irradiation in all AT cell lines showed no reduction compared with those after high-dose-rate irradiation (Fig. 2B). Thus, the increase in micronucleus induction in all AT cell lines after low-dose-rate irradiation was much greater than that in SuSa/T-n cells. The micronucleus assay has been used as a measure of the capacity of cells to repair DNA double-strand break (DSB) and as a marker for induction of chromosomal aberrations.¹⁴ Therefore, these results also suggest that AT cells are defective in DNA repair mechanisms and/or that they would exhibit an increased frequency of misrejoining.

**γ-H2AX Focus Formation in hTERT-Immortalized AT Cells**

Recent studies demonstrated a close correlation between the number of γ-H2AX foci in a nucleus and the number to DSBs expected after irradiation.³⁰ To analyze repair of DSBs induced by irradiation, we used γ-H2AX focus formation assay. Dose-dependent γ-H2AX focus formation was observed in both AT and SuSa/T-n cells after high-dose-rate irradiation (Fig. 4). In contrast, only a few γ-H2AX foci were observed in SuSa/T-n cells irradiated at a low-dose rate. However, a significant and dose-dependent number of γ-H2AX foci per cell were observed in all AT cell lines even after low-dose-rate irradiation, while the numbers of γ-H2AX foci induced in all AT cell lines after low-dose-rate irradiation were lower than those after high-dose-rate irradiation. These results indicate that DNA damage such as DSBs persisted in AT cells even after low-dose-rate irradiation because AT cells may be defective in repair of some DNA damage such as DSB.

**Phosphorylation of Ataxia Telangiectasia Mutated (ATM) Protein in hTERT-Immortalized Normal Cells**

It was reported that activation of ATM by phosphorylation of serine 1981 was a primary response to the induction of DSBs after irradiation.³¹ Then, activated ATM proteins are involved in signal transduction by phosphorylation of the downstream proteins. Thus, we investigated ATM phosphorylation to examine the activation of ATM by low-dose-rate irradiation (Fig. 5). A significant and dose-dependent increase in positive cells was observed after a very low dose of high-

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**Fig. 4.** Dose-response curves for γH2AX focus induction in hTERT-immortalized normal and AT cells after high- (HDR: closed symbol, 2 Gy/min) or low-dose-rate (LDR: open symbol, 0.3 mGy/min) radiation. For each dose, more than 100 nuclei were scored, and mean values with standard errors were shown.

**Fig. 5.** Dose-response curves for the rate of cells with phosphorylated ATM after high- (HDR: closed symbol, 2 Gy/min) or low-dose-rate (LDR: open symbol, 0.3 mGy/min) radiation in hTERT-immortalized normal cells. For each dose, mean values with standard deviations obtained with 3 experiments were shown.

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dose-rate radiation. Namely, phosphorylated ATM was observed in all cells even after 0.6 Gy of high-dose-rate irradiation. In the case of low-dose-rate irradiation, a few positive cells were detectable after 0.3 Gy, and the percentage of positive cells reached a plateau after 0.6 Gy. These results indicated that a significant amount of ATM was activated in normal cells even after low-dose-rate irradiation. Therefore, activated ATM after low-dose-rate irradiation may play an important role in the response of cells to DNA damage induced by low-dose-rate radiation.

In our present experiments, cells were irradiated in the G0/G1 phase after they became a non-proliferative conditions. If hypersensitivity to radiation in AT cells is due to only a defect in the cell cycle checkpoints, AT cells irradiated at a low-dose rate should be more resistant than AT cells irradiated at a high-dose rate, as normal cells are. However, survival and micronucleus induction in all AT cells irradiated at a low-dose rate were the same as those in cells irradiated at a high-dose rate, indicating that the radiosensitivity of AT cells may not be produced by only a defect in the cell cycle checkpoint. It was reported that Artemis is a downstream component of the ATM signaling pathway required uniquely for the DSB repair function such as non-homologous end-joining (NHEJ). Therefore, it is suggested that AT cells are hypersensitive to low-dose rate radiation because of some defect in NHEJ repair pathway.

**PERPECTIVE**

We characterized the effects of low-dose-rate radiation on human cells as follows: [1] the genetic effects of low-dose-rate radiation on hTERT-immortalized normal cells were quantitatively and qualitatively less severe than the effects of high-dose-rate radiation; and [2] hTERT-immortalized AT cells might be partly defective in repair of DSBs and were severely affected by low-dose-rate radiation. According to these results, our established cell lines must be appropriate for analyzing the effects of radiation on human cells compared to other immortal human cells such as SV40-immortalized or cancer-derived cells.

For the genetic modification of cells such as gene transfer and gene inactivation, primary cells are inappropriate because they readily reach senescence in long-term experiments. However, with the present hTERT-immortalized cells, any kind of genetic engineering method may be applicable. In addition, these hTERT-immortalized cells may also be used with shuttle vector systems developed to analyze radiation-induced mutations more precisely at the molecular level. Therefore, the technique of immortalization by hTERT introduction and hTERT-immortalized cells are useful for future experiments analyzing various effects of radiation on human cells.

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