

No Induction of p53 Phosphorylation and Few Focus Formation of Phosphorylated H2AX Suggest Efficient Repair of DNA Damage During Chronic Low-dose-rate Irradiation in Human Cells

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Low-dose-rate radiation/p53/Phosphorylated H2AX protein/DNA repair/Signal transduction.

Human fibroblast cells obtained from a normal individual and immortalized by introduction of the *hTERT* gene were irradiated with 0 to 5 Gy of acute high-dose-rate radiation (1.8 Gy/min) or chronic low-dose-rate radiation (0.3 mGy/min) in the G₀ phase, and p53 activation was studied. After high-dose-rate irradiation, a dose-dependent induction of Ser15 phosphorylation was observed, whereas after low-dose-rate irradiation almost none was observed. Then we analyzed the focus formation of phosphorylated histone H2AX protein, which is closely correlated with the induction of double-strand breaks. High-dose-rate radiation induced a significant number of foci in a dose-dependent manner, whereas, low-dose-rate radiation could induce only a few foci even at the highest dose. These results strongly suggest that DNA damage induced by low-dose-rate radiation such as a double-strand break is efficiently repaired during chronic irradiation.

INTRODUCTION

Ionizing radiation (IR) induces a variety of damage to cellular DNA, such as double-strand breaks (DSBs), single-strand breaks, and base damage, as well as inducing various cellular responses. In this decade, the response of human cells to acute exposure to high-dose-rate (HDR) radiation has been thoroughly studied and its molecular pathways are currently being elucidated. Immediately after irradiation, an increased expression of so-called stress-response genes has been observed, and proteins involved in the checkpoint function are also activated by phosphorylation and/or increased expression.^{1,2)}

It has been proposed that the p53 gene is a key factor in the radiation-induced checkpoint and also in the adaptive response that is one of the typical cellular responses to low-dose radiation.³⁾ A recent work on human cancer cells suggested that irradiation of small dose of low-dose-rate (LDR) radiation can induce p53 protein.⁴⁾ Another *in vivo* study with mice also indicated that very low-dose radiation

induced p53 protein in liver and spleen cells.⁵⁾ The response of p53 to IR is involved not only in the cellular checkpoint function and the adaptive response but also in the induction of apoptosis, which is very important for the defense system against radiation induced carcinogenesis.⁶⁾ However, it is not yet clear how human diploid cells respond when they are irradiated with LDR. It is important to elucidate the p53-response to LDR in human cells to determine its effect on our health since many possible exposures of human populations to radiation are expected as LDR exposure such as medical examinations, occupational exposure, frequent intercontinental flights, and manned space flights. However, so far most studies of human cells have been performed using human cancer cells and SV40-immortalized cells, with an attendant disruption of signal transduction systems including the p53 gene, since in the course of carcinogenesis the p53 gene is the most frequent target of genetic changes in various cancers and the large T antigen of SV40 also abrogates p53 protein.⁷⁾

In this study we used human diploid cells immortalized by introduction of the human telomerase (*hTERT*) gene, since we already showed that such cells exhibit a normal response to HDR in cellular survival and the induction of p53 protein.⁸⁾ We analyzed p53 phosphorylation by Western blotting after chronic exposure to LDR and determined the number of DSBs by analysis of the focus formation of the phosphorylated histone H2AX protein (γ H2AX), which has already been shown to be closely correlated with DSBs.⁹⁾

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MATERIALS AND METHODS

Cells and cell culture

A human diploid cell line (SuSa/T-n) derived from a normal individual and immortalized by the introduction of the *hTERT* gene was previously described.⁸⁾ When irradiated with HDR, this cell line exhibited the same survivals as the original diploid cells. Activation of p53 protein by HDR was also the same as in the original cells. The cells were cultured in DMEM supplemented with 10% FBS in 5% CO₂-air at 37°C.

The cells in all our experiments were irradiated at a confluent and quiescent phase (G₀ phase). A quiescent cell culture for Western-blotting analysis was prepared by inoculating 5×10^5 cells into a T25 flask and culturing with 10% FBS-DMEM for 3 days, after which the medium was changed to 3% FBS-DMEM, and the cells were further cultured for another 3 days. Then the medium was changed to fresh 3% FBS-DMEM, and the cells were irradiated on the following day with LDR or HDR. For immunohistological studies, 10^5 cells were inoculated into a SlideFlask (NUNC Brand Products, Roskilde, Denmark), and a quiescent culture was prepared in the same way as that in the T25 flask.

HDR and LDR irradiation

For acute HDR, an X-ray machine for experimental irradiation (MBR1520R, Hitachi Medico, Tokyo) was used and the dose-rate was set at 1.8 Gy/min. For chronic LDR, we used ¹³⁷Cs γ -source (1850 GBq, Sangyo Kagaku Co. Ltd., Tokyo) at Radiation Biology Center, Kyoto University at a dose-rate of 0.3mGy/min. During chronic irradiation, the total dose required 3 days for 1.1 Gy, 6 days for 2.2 Gy, and 13 days for 5 Gy, with the cells maintained at 37°C in a CO₂-incubator set in front of the γ -source.

Western blotting

After HDR, the cells were kept at 37°C for 4 h before protein isolation since our previous study showed that after acute irradiation the Ser15 phosphorylation of p53 protein increased to its highest level at 3–4 h after irradiation.⁸⁾ Cells irradiated with LDR were treated for protein isolation just after the completion of irradiation to avoid unexpected protein degradation and de-phosphorylation. Protein samples were prepared as follows: briefly, cells were washed with PBS, and 200 μ l of RIPA solution (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS) was then added to each T25 flask to extract the protein. Western blotting was then performed as previously described. The primary antibodies used were: mouse monoclonal anti-p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-Ser15-phosphorylated-p53 (Cell Signaling Technology, Beverly, MA), and anti-beta-actin (Abcam, Cambridge, UK). After incubation with a peroxi-

dase conjugated secondary antibody, bands were visualized by chemiluminescence (WLC Reagent Plus, PerkinElmer Life Sciences) and exposure to X-ray film. For quantitative analysis, band intensity of repeated Western blotting was determined by densitometry with the LAS 1000 CH image analyzer (FUJI PHOTO FILM Co., Tokyo) and the mean value was obtained.

γ H2AX focus assay

The cells irradiated with LDR were kept at room temperature for 3 h on the return from Kyoto to Nagoya since it was difficult to fix cells at the Kyoto site. Then, they were fixed with paraformaldehyde in PBS for 5 min and then permeabilized in 100% methanol at –20°C for 5 min after washing with PBS. The cells irradiated with HDR were also kept at room temperature for 3 h to ensure the same conditions as the cells irradiated with LDR, and fixed in the same way. For observation of γ H2AX foci, the cells were incubated with anti- γ H2AX monoclonal antibody,¹⁰⁾ and then with anti-mouse Alexa 488 secondary antibody (Molecular Probes, Eugene, OR). To counterstain DNA, 0.5 μ g/ml 4'6-diamidino-phenylindole-dihydrochloride (DAPI) was used. The numbers of γ H2AX foci in each nucleus were counted under a fluorescent microscope. We scored more than 100 cells for each dose point.

RESULTS

Western blot analysis

Figure 1 shows the results of Western blotting of the total p53 protein and that of Ser15-phosphorylated p53 protein in growing and confluent G₀ cells without irradiation or irradiated with 5 Gy of HDR. In the G₀-phase cells, the expression level of p53 protein was high without irradiation, and even after HDR only a minor additional increase was observed. However, in the growing cells p53 was hardly detected without irradiation, whereas after HDR a significant increase was

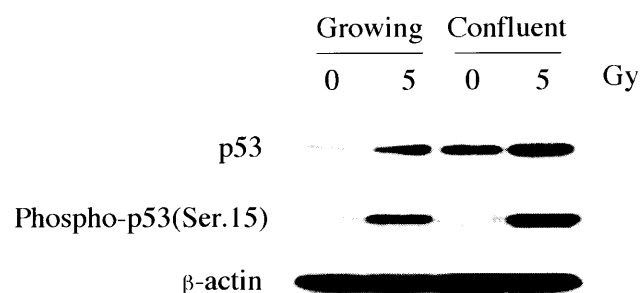


Fig. 1. Western-blot analysis of p53 and Ser15-phosphorylated p53 protein in growing and confluent cells. Protein samples were extracted from both growing and confluent cells after irradiation with or without 5 Gy of HDR, and used for Western blotting. β -actin was used as a control so that identical amounts of protein samples were loaded.

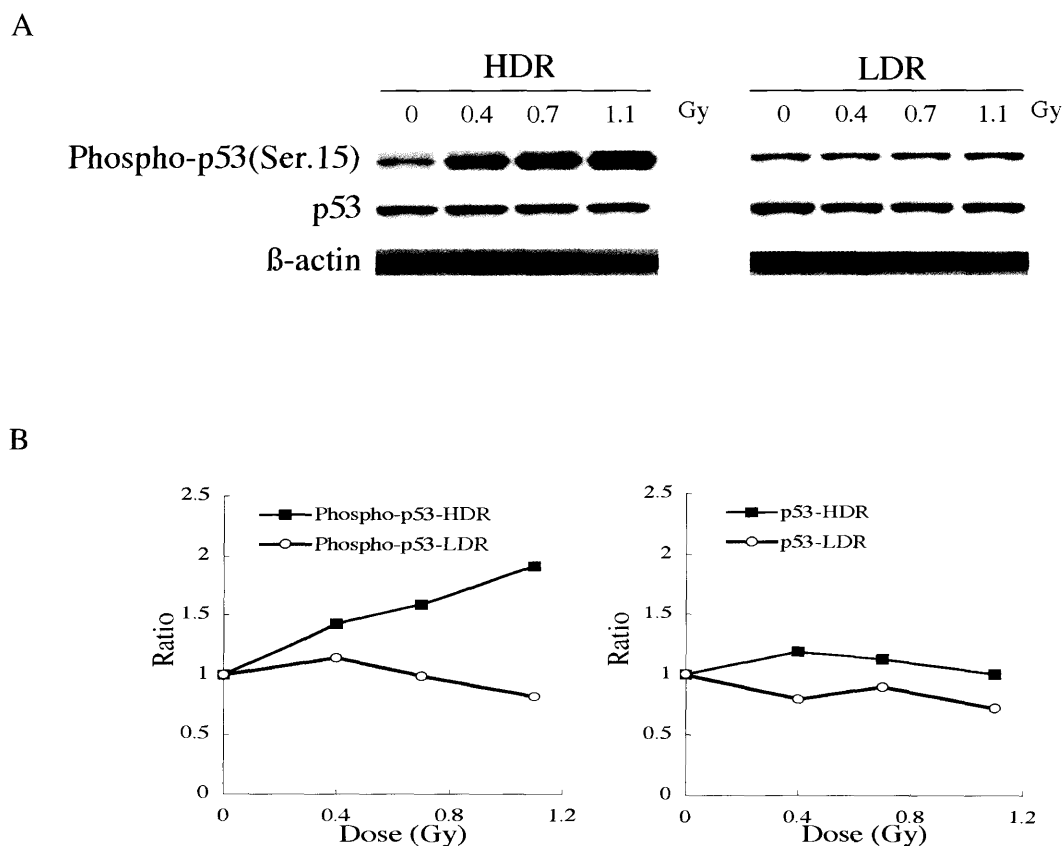


Fig. 2. Western blotting of Ser15-phosphorylated p53 and p53 protein after increasing doses of HDR or LDR. A: Confluent G_0 cells were irradiated with various doses of HDR or LDR, and protein was extracted for Western-blot analysis. B: Intensity of the bands corresponding to Ser 15-phosphorylated-p53 and p53 protein in the Western blot shown in A was determined by densitometry. Mean values of three independent experiments were shown as ratios of the amounts in irradiated cells to those in not-irradiated cells.

observed. In contrast, a significant induction of the Ser15-phosphorylation of p53 protein was observed after HDR in both growing and confluent G_0 cells.

Figure 2A shows an example of Western blotting of total p53 protein and Ser15-phosphorylation of p53 protein in the G_0 -phase cells after various dose of HDR or LDR. A summary of repeated quantitative analyses of the amount of total p53 protein and that of the Ser15-phosphorylation of p53 protein is shown in Fig. 2B. After HDR, a dose-dependent increase in Ser15-phosphorylation was observed, whereas after LDR no significant change was seen. As for total p53 protein, after both HDR and LDR a slight decrease instead of an increase was observed, but the change was not significant. This might have been due to the cells being arrested in the G_0 phase.

Analysis of γ H2AX foci

Figure 3 shows representative focus formations after HDR and LDR. We scored clear and bright dots on nuclei stained with anti- γ H2AX antibody as foci. The numbers of foci on each nucleus were counted and the mean numbers of foci

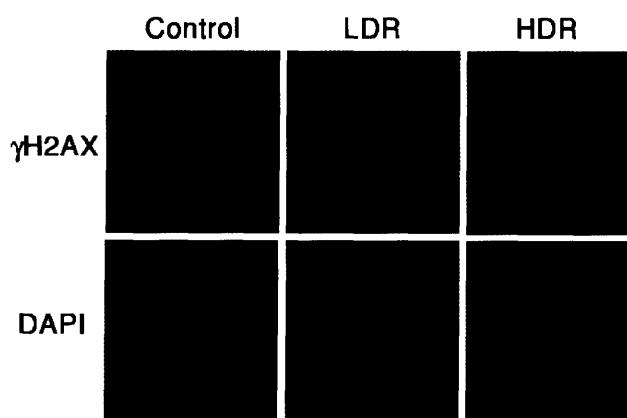


Fig. 3. Examples of γ H2AX foci observed after irradiation with 5 Gy of LDR or HDR. Quiescent cells in the SlideFlask were irradiated with HDR or LDR and stained with a specific antibody to γ H2AX protein. DNA was counterstained with DAPI.

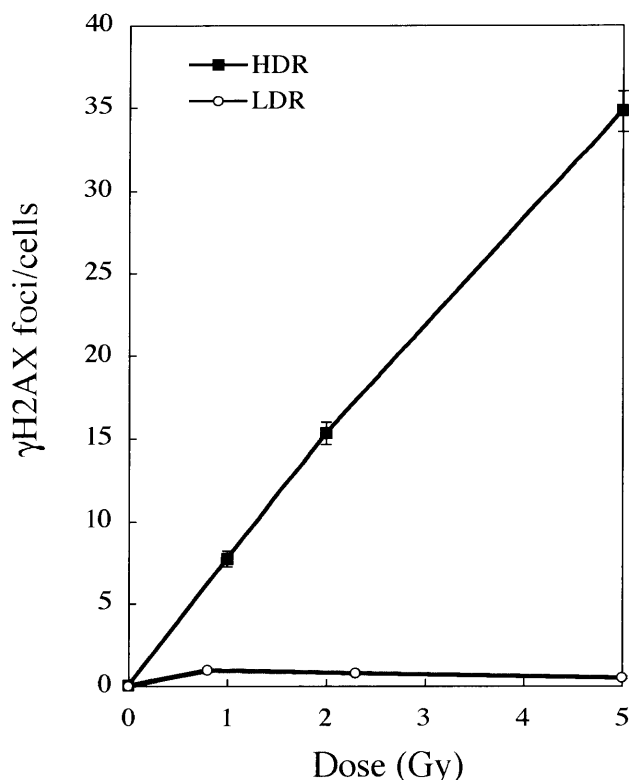


Fig. 4. Dose response of γ H2AX focus formations after irradiation with LDR or HDR. γ H2AX foci were stained as shown in Fig. 3, and numbers of foci on each nucleus were counted under a fluorescent microscope. For each dose, more than 100 nuclei were scored and mean values with standard errors were shown.

induced by each dose of radiation are shown in Fig. 4. After HDR, the number of foci per nucleus increased in proportion to the total dose, while after LDR only a very small increase was observed at lower doses. No further increase was detected at higher doses.

DISCUSSION

In this study, we first analyzed the activation of p53 protein by LDR and HDR. The amount of total p53 protein almost reached saturated level without irradiation since the cell cycle was arrested at the G₀ phase (Figs. 2), which is consistent with the previous report that showed that amount of p53 protein was increased in confluent and nutrition-deprived human fibroblasts¹¹⁾. However, after HDR, the Ser15-phosphorylation of p53 protein increased dose-dependently. This result is consistent with the finding that the Ser15 of p53 protein is phosphorylated by ATM and ATR both of which are activated by DNA damage induced by IR and UV.¹²⁾ This also suggests that p53 activation by increasing its half-life and by the phosphorylation of Ser15 functioned as independent mechanisms, both of which were simultaneously observed when growing cells were irradiated

with HDR (Fig. 1). In contrast to HDR, LDR induced almost no Ser15 phosphorylation even after the identical total dose was irradiated. Since in the case of LDR the cells were fixed immediately after irradiation, it is possible that the time needed for the maximum activation of p53 was insufficient. However, since during LDR the cells were irradiated for more than 24 h even at the lowest dose, this might not be the case. When survival was determined by colony-formation assay, after 5 Gy of HDR, the survival was 0.01 whereas after the same dose of LDR it was 0.3 or higher (details to be published elsewhere by Nakamura *et al.*). The fact that LDR could induce almost no Ser15 phosphorylation of p53 protein and exhibited very low cellular toxicity suggests that the DNA damage induced by LDR might be efficiently repaired during irradiation.

To confirm that during LDR induced DNA damage is efficiently repaired, we analyzed γ H2AX focus formation. It is well known that histone H2AX protein is rapidly phosphorylated when DSBs are induced and forms foci at the DSB sites.¹³⁾ Furthermore, the numbers of γ H2AX foci were shown to be closely related with those of DSBs in a nucleus at the time of cell fixation.⁹⁾ In accordance with these previous reports, after HDR irradiation the mean number of foci observed on each nucleus increased in proportion to irradiated doses up to 5 Gy (Fig. 4). However, after LDR, only a very small increase in foci at low doses and no further increase at higher doses were observed. This is completely consistent with the results of Western blotting, where the increase in the Ser15 phosphorylation of p53 protein was almost negligible after LDR. Previous time-course studies on γ H2AX focus formation reported that γ H2AX foci in normal human cells were observed immediately after irradiation, and that the number of foci was biphasically decreased with a half life of 2 h during post-irradiation incubation.⁹⁾ It is possible that some foci might have disappeared during the 3 h while the cells were kept at room temperature before fixation. However, in this study a significant number of foci were still observed in a dose-dependent manner after HDR although both HDR and LDR irradiated cells were fixed 3 h after irradiation, suggesting that only a few foci were actually induced by LDR.

Our results showing only a few γ H2AX focus induction and almost no induction of the Ser15 phosphorylation of p53 protein by chronic LDR strongly suggest that DSBs induced by LDR are efficiently repaired during irradiation. A recent study by Vilenchik and Knudson¹⁴⁾ suggested that endogenous DSBs are formed in replicating human cells at a rate equivalent to that of DSBs induced by IR at a dose rate of 0.47 cGy/min, which is more than 10 times the dose rate of LDR used in this experiment (0.3 mGy/min). This also supports our contention that most DSBs induced by LDR might be efficiently repaired during irradiation.

In eukaryote cells, two types of DSB repair are known.¹⁵⁾ One is homologous recombination (HR) repair that is spe-

cific to the S-G₂ phase of the cell cycle,¹⁶⁾ and the other is non-homologous-end-joining (NHEJ) repair that is active through all cell cycle phases.¹⁷⁾ Since in this experiment cells were kept in the G₀ phase, most of the repair might have been performed by the NHEJ mechanism. However, after HDR dose-dependent p53 and H2AX phosphorylation possibly mediated by active ATM was observed even in G₀ cells. Since phosphorylation of MRE11-RAD50-NBS1 complex by active ATM is considered to be required for efficient HR repair,¹⁸⁾ we have to determine whether NHEJ repair is mainly working in the G₀ cells under our condition. It would be also important to determine whether DSB repair during LDR irradiation is error-prone or error-free. We are now conducting further studies to clarify these issues.

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