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X-irradiation alters the telomerase activity and the telomere length distribution of cultured human vascular endothelial cells

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ABSTRACT

To assess the effect of X-irradiation on somatic cell, we analyzed the telomeric changes of cultured human umbilical venous endothelial cells (HUVECs) induced shortly after low-dose X-irradiation. The effect of X-irradiation on HUVECs was assessed by the analysis of the changes in telomere length, telomerase activity, and the expression of telomere-associated proteins after 2 to 8Gy X-irradiation. The cell growth activity decreased, whereas the telomerase activity of the surviving cells decreased only at low X-ray doses. The expression levels of telomere-associated components, TRF1 and TRF2, increased in the surviving cells. As the X-ray dose level increased, senescent cells increased. However, the mean telomere length of the surviving cells became longer, long telomeres increased, and short telomeres decreased. These observations suggested that X-irradiated HUVECs bore telomeric features similar to those of young cells, and the cells bearing short telomeres, i.e., aged cells, were selected out. The surviving cells that had gone through low dose X-irradiation might represent a radiation-resistant feature of telomere conditions. The telomeric changes at low dose X-ray disappeared at a high dose. Higher dose of X-irradiation might induce a cellular protective reaction against X-ray-induced cell damage through the restoration of telomerase activity and up-regulated telomere-associated proteins.

KEYWORDS

Telomere;
Telomerase;
Vascular endothelial cell;
X-irradiation;
Cell survival.

INTRODUCTION

A telomere is a structure consisting of thousands of repeats and accessory peptide factors located at the termini of human chromosomes[1,2]. Telomeres become shortened gradually because of the incomplete DNA duplication at the chromosome ends at each cell cycle. Such telomere shortening has been observed in peripheral blood nuclear cells with aging[3-5]. The elderly have shorter telomeres in their somatic cells. Telomere shortening is accelerated by physical and mental stress with disease conditions[6-13].

X-ray exposure has been reported to induce cytopathological effects. X-irradiation to induce telomere-associated cell senescence among different kinds of transformed immortal cells derived from fibroblasts,

Regular Paper

lens epithelial cells, endothelial cells, chondrocytes or keratinocytes[14-18]. All these previous reports concerning to telomere conditions affected by X-irradiation have described about cancer cells or immortalized cells. In addition, vascular endothelium has not been well analyzed in terms of X-ray-induced genotoxic effects including telomeric changes.

The aim of the present study was to elucidate how the different doses of X-irradiation affect the telomeric features including the telomere length, and the function and the expression of telomere-associated components (TERT, TERC, TRF1 and TRF2) of non-transformed vascular endothelial cell *in vitro*.

MATERIALS AND METHODS

Cell culture

Human umbilical venous endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). They were cultured in endothelial cell growth medium (Clonetics Corp.). The cells were cultured at 37□ and 5% CO_2 in a gelatin-coated flask (Iwaki Glass, 2 Chiba, Japan), and routine subcultivation was done every 2 days with a split ratio of 1:4, and used at the third passage. Cells were counted at this stage. They were X-irradiated 2Gy/min. On day 3 after the irradiation, the cells were collected and subjected to further analyses. Cells were counted using a hemocytometer. Population doublings (PDs) were calculated using the formula: PD=[log (expansion)/log 2], where expansion was the number of cells harvested divided by the initial number of cells seeded.

X-irradiation

X-rays were delivered from a soft X-ray generator (SOF-TEX M-150WE, Japan) operating at 100kVp and 3.5mA. The cells in the cell culture dishes were placed on an irradiation stage 30cm from the radiation source. The corresponding dose rate was 2Gy/min.

Senescence-associated β-galactosidase (SA-β -Gal) expression

The cells were washed in PBS, fixed for 10min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, rinsed in PBS, and incubated at 37 □ (no CO_2) with fresh SA-β-Gal staining solution. The staining solution was made up as follows: 1mg X-gal, per ml solution, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$, 150mM NaCl, and 40mM citric acid and sodium phosphate at pH 6.0. This solution was left on the cells for 12 h to achieve the maximum staining. Hundred cells were scored from each well (plate) using a light microscope.

Telomere detection

Telomere detection was performed as previously described[4]. Restriction enzyme *Msp*I was used. *Msp*I recognizes and cuts tetranucleotide CCGG. Briefly, blood cell DNA was extracted from samples using and the DNA (0.1μg) were digested at 37ºC with 1U *Msp*I for 2h. The digests (10μl) were resolved by agarose gel-electrophoresis, and transferred by Southern blotting to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The blotted DNA fragments were hybridized to a hyper-sensitive probe of 500bp long (TTAGGG)n labeled with digoxigenin. The membrane was then incubated with anti-digoxigenin-AP-specific antibody. The telomere probe was visualized by CSPD (disodium 3-(4-methoxy-spiro {1,2-dio-xetane-3,2'-(5'-chloro) tri-cyclo [3.3.1.1] decan} -4-yl) phenyl phosphate) (provided with the kit). The membrane was then exposed to Fuji XR film with an intensifying screen. The smears of the autoradiogram were captured on an Image Master, and the telomere length was then assessed quantitatively. Every sample was measured in triplicate.

Terminal restriction fragment (TRF) length analysis

Telomere length distribution was analyzed by comparing the telomere length using a telomere percentage analysis with three intervals of length as defined by a molecular weight standard, *Hin*d III-digested λ phage DNA, as previously described[4]. The intensity (photostimulated luminescence: PSL) was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: >9.4, 9.4≥≥4.4 and >4.4kb. The percent of PSL in each molecular weight range was measured (%PSL=intensity of a defined region-background×100/ total lane intensity-background). The percentage of PSL in each molecular weight range was measured (%PSL = intensity of a defined region – background x 100/

total lane intensity - background). The mean TRF was estimated using the formula S(ODi - background)/ S(ODi - background/Li)[19], where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

RT-PCR analysis

Total RNA samples were extracted using RNAzol B (Teltest). mRNAs for human TERT, telomerase RNA component (TERC), and TRF1 and TRF2 were determined by RT-PCR using a DIG detection system (Roche Applied Science). Each human cDNA was produced by RT-PCR according to each human-derived sequence, as follows:

TERC forward primer: 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3',

TERC reverse primer: 5'-GTTTGCTCTAGAATGAACGGTGGAAG-3',

β-actin forward primer: 5'-CCTTCCTGGGCATGGAGTCCT-3' and

β-actin reverse primer: 5'-GGAGCAATGATCTTGATCTTC-3' were used according to the published human TERT-cDNA sequences[20,21]. The TERC mRNA level was normalized to the β-actin mRNA level in the same sample. The PCR products were directly synthesized from 2µg of total RNA isolated from each sample using the Superscript one-step RT-PCR system with Platinum *Taq* (Invitrogen) and gene-specific primers according to the recommendations provided by the supplier. The PCR products were analyzed by agarose gel electrophoresis (1.3%) followed by staining with ethidium bromide and scanning with Gel-Doc (Bio-Rad). For semiquantitative PCR, β-actin was used as an internal control to evaluate total RNA input, as previously described by our group[22].

Western blot and other analyses

Cells from a dish were homogenized with 100µl lysis buffer (100mM Tris pH 6.8, 4% SDS, 20% glycerol containing the protease inhibitor M phenylmethanesulfonyl fluoride, 0.1mM, leupeptin, 0.1µl, and aprotinin, 0.1µl). Gel electrophoresis was used to separate 10µg protein on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (162-0112, Bio-Rad Labo-ratories, Hercules, California) blocked with 5% dry milk or blocking solution for Western blot (Roche). Membranes were blocked and incubated with antibodies against telomerase reverse transcriptase (TERT) (Rockland), TRF1 (Imgenex), TRF2 (Cell Signaling), or β-actin (Santa Cruz Biotechnology). Detection was performed with secondary horseradish peroxidase-conjugated antibodies (Chemicon) and the ECL detection system as previously described[22].

Telomerase activity

Telomerase activity was examined by means of a modified telomerase repeat amplification protocol (TRAP) method[23] with TeloChaser (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, the substrate oligonucleotide is added to 0.5mg protein extract. If telomerase is present and active, telomeric repeats (GGTTAG) are added to the 3' end of the oligonucleotide. After amplification, the PCR products were resolved on a 12% polyacrylamide gel, stained with ethidium bromide, and detected using a FLA 5000 system (Fuji Film, Tokyo, Japan). The intensities of the bands were quantified with Image J (NIH). According to the manufacturer's instructions, the telomerase activities were calculated and presented as TPG (Total Product Generated).

Statistical analysis

Assays were repeated three times and analyzed statistically. Intergroup comparisons were performed using an independent samples *t*-test and one-way ANOVA. Paired samples were compared using the paired *t*-test. Significance was defined as p-values of <0.05. Group data are expressed as mean $\pm$ standard deviation. Statistical analyses were performed using the SPSS 10.0 software package (SPSS, Chicago, IL).

RESULTS

The alteration of population doubling (PD) and the induction of cell senescence after X-irradiation

The PD of the HUVECs was assessed on day 3 of culture after X-irradiation. The PD appeared to decrease proportionally with the increasing dose of X-ray (Figure 1a). The decreased PD indicated that cell growth was suppressed by X-irradiation in a dose dependent manner. Senescence-associated β-galactosi-

Regular Paper

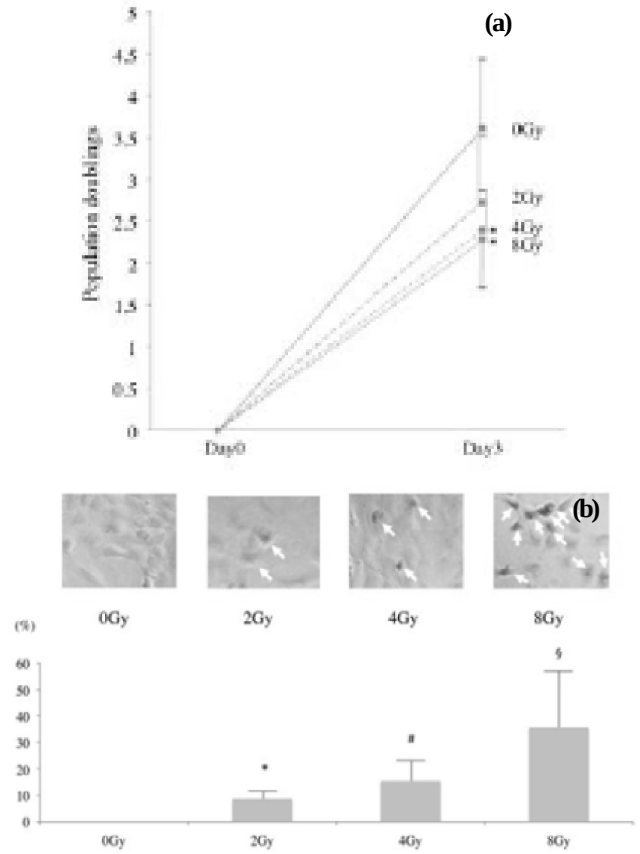

Figure 1 : The population doubling and the ratio of senescence-associated β-galactosidase (SA-β-Gal) staining of HUVECs cultured after X-irradiation; (a) The PD after 3 days in culture. (b) The upper panels show the SA-β-Gal-stained cells (white arrows) after different doses of X-irradiation. The lower panel shows the percentages of the cells that were stained. *p<0.05 vs. 0Gy. #p<0.05 vs. 2Gy. §p<0.05 vs. 4Gy.

dase (SA-β-Gal) expression became higher as X-ray dose increased (Figure 1b).

The alteration of the telomere lengths after X-irradiation

The mean TRF of the cultured HUVECs on day 3 was measured to see how much X-irradiation affected the telomeric DNA. Unexpectedly, the mean TRF level became longer within the lower doses of X-ray, 2 and 4Gy (Figure 2a). In the telomere length distribution, the longer telomeres were increasing and the shorter telomeres were decreasing after 2Gy- or 4Gy-X-ray irradiation (Figure 2b). This tendency disappeared in the case of 8Gy (Figure 2b).

The alteration of the telomerase activity after X-irradiation

The telomerase activity of HUVECs after 2Gy and 4Gy X-irradiation significantly decreased, however, did not changed after 8Gy irradiation (Figure 3).

Expression of telomere-associated RNA and proteins

The observation of telomere length change and the elevated telomerase activity in the cells led us to examine whether the expression of RNA and proteins associated with maintaining the telomere structure. The expression level of TERC did not change significantly after X-irradiation (Figure 4). The TERT protein expression tended to increase only after 4Gy irradiation, and the expression level after 8Gy irradiation was similar to

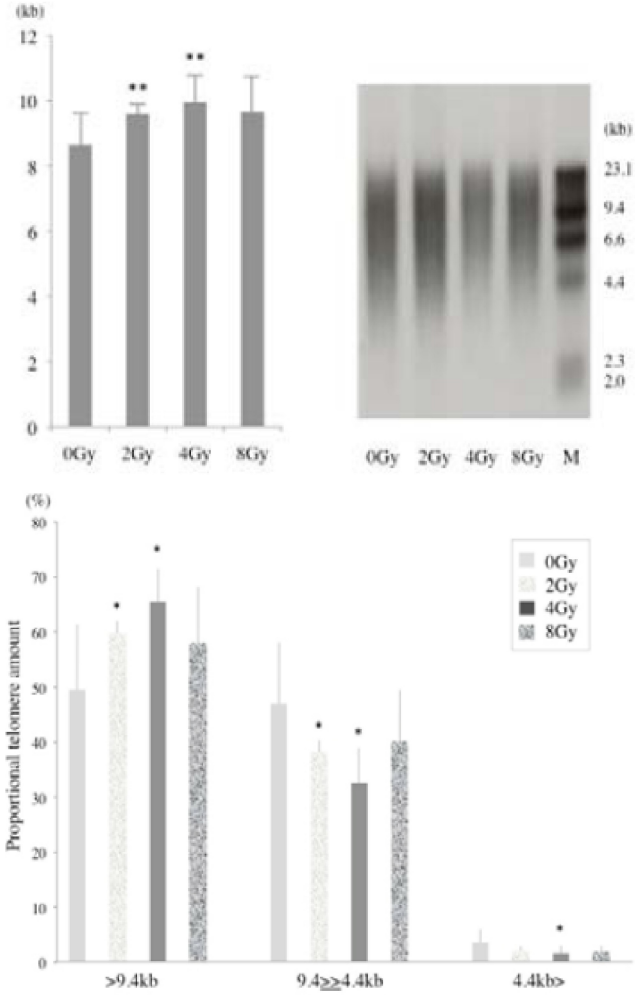

Figure 2 : The mean telomere length and the telomere length distribution of HUVECs after X-irradiation; (a) The mean telomere lengths and a representative genomic Southern blot result with telomere DNA probe are shown. (b) The telomere length distribution. The horizontal bars represent the standard deviation. The *Msp*I-terminal restriction fragment lengths are presented as the mean values ± standard deviation. The horizontal bars represent the standard deviation.

Regular Paper

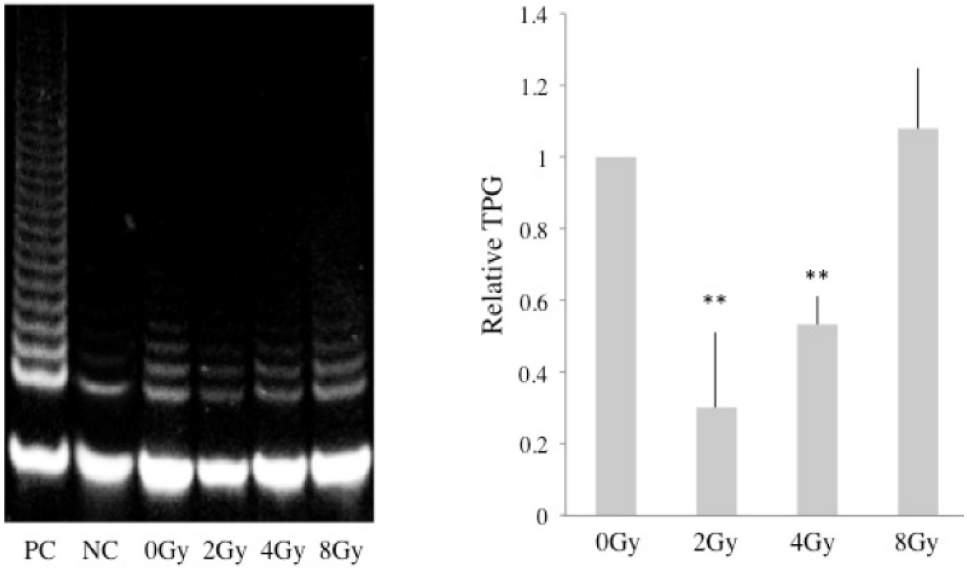

Figure 3 : The telomerase activity of endothelial cells after X-irradiation; The left panel shows the telomerase activity at different doses of X-irradiation as the TPG (Total Product Generated) levels. The TPG is presented as a proportional ratio of a ladder density of a sample to that at 0Gy. The left panel shows a photograph of representative TRAP assay result for HUVECs after X-irradiation. The materials used for the positive control (pc) and negative control (nc) were provided with the kit. *p<0.05 vs. 0Gy

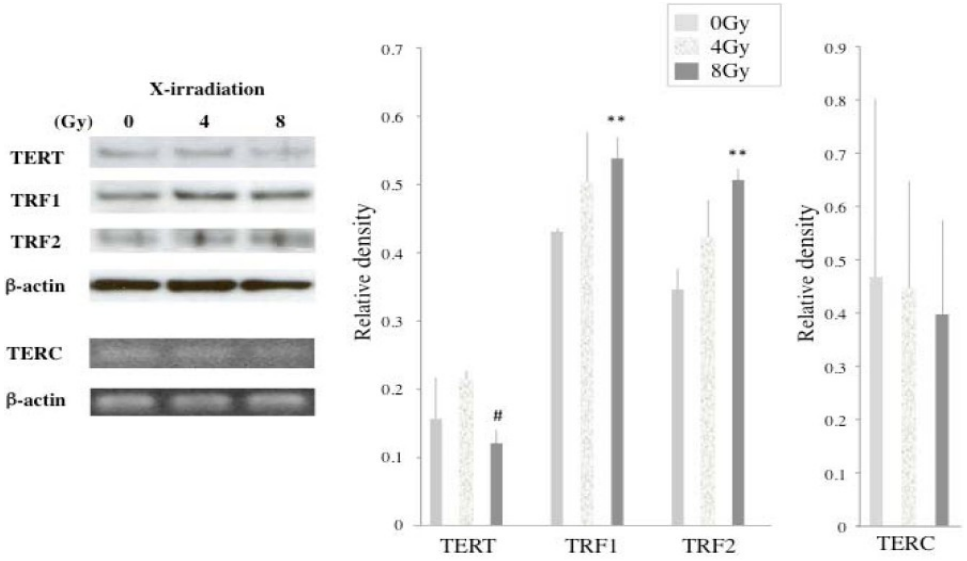

Figure 4 : The expression level for telomerase RNA component (TERC) and the expression levels of telomere-associated proteins, TERT, TRF1 and TRF2. Representative (left) and summarized (right) results for the mRNA expression (semiquantitative RT-PCR) of TERC and the western blot analysis of telomere-associated proteins (TERT, TRF1 and TRF2) after X-irradiation are shown. The relative expression levels were determined compared to that of β-actin (set at 1). Horizontal bars represent standard deviations. n=6. ** p<0.01 vs. 0Gy, #p<0.05 vs. 4Gy

the original level at 0Gy. On the other hand, the protein expressions of TRF1 and TRF2 increased significantly after 8Gy irradiation.

DISCUSSION

In the present report, telomerase activity did not coincide to the level of RNA or protein expression of telomerase components, either. Unexpectedly restored telomerase activity under X-irradiation was observed, and it was accompanied by both of the increased levels of TRF1 and TRF2. This implies the possibility that the levels of accessory components for telomerase activation, including not only TRF1 and TRF2 possibly but

Regular Paper

also others, are increased under X-irradiation. Such an up-regulation of telomerase activity at X-irradiation without an increase of RNA (TERC) or protein component (TERT) of telomerase has been reported. Telomerase activity of leukemia cell lines has been enhanced under X-irradiation independently of TERC and TERT transcription, and the inhibitors of transcription or protein synthesis have erased the X-ray induced-up-regulation of telomerase activity, implying that a certain positive regulator enhances telomerase activity by X-irradiation[24]. Our case might be a similar case.

The radiation-associated alterations of telomere length and telomerase activity have been described mainly in cells with high telomerase activity such as including cancer cells, tissue stem cells or cells transduced with a TERT expression vector[25-29]. In the present study, a non-transformed somatic cell, HUVEC, was used to analyze telomere-associated alterations induced by X-irradiation. Aging-associated genomic changes contain X-ray-induced mutations brought by natural environmental radiation. Vascular endothelium can be regarded as a representative tissue where aging-associated somatic change is prominent, i.e., atherosclerosis. We chose HUVECs to assess if cellular damage induced by ionizing radiation can be similar to aging-associated cellular changes in terms of telomeric changes.

In the present study cell growth was suppressed, and cell senescence was induced, however, the telomere length was seemingly elongated after X-irradiation. The X-ray-associated changes in the telomere length distribution were not similar to those observed in peripheral blood leukocytes of aged people, whose mean TRF shortens, longer telomeres decrease and shorter telomeres increase in comparison with young people[4]. From this view, the survived cells seemingly bore rather younger patterns of the telomere length distribution than non-irradiated control cells. Moreover, the mean telomere length did not coincide to the telomerase activity, and telomere elongation was observed even with telomerase activity lowered by X-irradiation. Together with the reduction of the proportional amount of short telomeres, the apparent X-ray-associated-elongation of telomere length was not likely induced by the activation of telomerase but by loss of cells bearing short telomeres. Cells bearing short telomeres were old cells having experienced many cycles of mitosis, which were more fragile to X-irradiation than young cells bearing long telomeres.

The present results suggested that the endothelial cells bearing long telomeres could survive after X-irradiation, and the cells bearing short telomeres, which could be regarded as old cells, were fragile to X-ray exposure. However, the X-ray resistance of the cells with long telomeres was lost at 8Gy irradiation, as the pattern of the telomere length distribution of 8Gy appeared to return to a pattern similar to that of 0Gy. Telomerase activity was suppressed after X-irradiation at lower doses, 2Gy and 4Gy, and recovered to a control level at 8Gy. This fluctuation of telomerase activity coincided neither with the TERT RNA amount, nor with the TERC RNA amount, nor with the TERT protein level. It seems that a lower dose of X-ray suppressed the cell growth and lowered the telomerase activity of most cells, and at a higher dose only cells with a potential to induce higher telomerase activity could survive. The TERC expression level was not changed after any dose of X-irradiation. The TERT protein level was kept at a similar level to a control level after 8Gy irradiation. The TERT protein level at 8Gy was significantly lower than that at 4Gy, whereas the telomerase activity was higher at 8Gy than at 4Gy. After X-irradiation, the telomerase activity seemed not to be controlled quantitatively by the TERC RNA level or the TERT protein level. We analyzed some telomere-associated proteins to pursue the hints for the paradoxically elevated telomere-elongating activity. The amount of telomere-associated proteins, TRF1 and TRF2, were elevated at 8Gy irradiation. The increase of these co-factors might lead to the elevation of telomerase activity even with a decreased amount of TERT protein at 8Gy. The increases of TRF1 and TRF2 might be induced by cellular protective mechanism in jeopardized cells, so-called hormesis effects. TRF1 has been reported to negatively control the telomerase-associated telomere length maintenance[30]. Under telomere-erosive conditions, however, TRF 1 can contribute to telomere stability[31,32]. TRF2 is associated with stabilizing the telomere structure[33,34]. The TRF1 and TRF2 expression levels were maintained within the normal or elevated to a higher range after X-irradiation. While the X-ray-irradiation seemed to impair the telomere length maintenance, the expression of TRF1 and TRF2 may contribute to

telomerase activation possibly with other telomere- or telomerase-associated factor(s) rather for cell survival than elongating telomeres. Our observation of the paradoxical enhancement of the telomerase activity may therefore be a pivotal step for the understanding of cell protection under genotoxic condition with X-irradiation. Somatic cells with highly elevated telomerase activity can survive through X-ray exposure, but may lead to tumorigenesis at a later stage[35,36]. The cellular mechanism(s) responsible for the protective enhancement in the telomerase activity of somatic cells injured by X-ray irradiation will need to be elucidated in further studies. Alterations in the behaviors of the telomere structure-associated components in vascular endothelial cells exposed to X-ray also warrant further investigation.

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Regular Paper

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