Telomerase- and angiogenesis-related gene responses to irradiation in human umbilical vein endothelial cells

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Abstract. In this study, we investigated the effects of ionizing radiation (IR) on human umbilical vein endothelial cells (HUVECs) in the context of senescence. HUVECs at passage number (PN)1, PN2 and PN3 were exposed to irradiation (2 Gy). The growth rate of the HUVECS was measured by proliferation assay and senescence-associated β-galactosidase assay was used to measure the number of senescent cells. Telomerase activity and the expression of telomerase- and angiogenesis-related genes were measured by telomerase assay and real-time PCR, respectively. The number of senescent cells was significantly increased in the irradiated HUVECs at all PNs. Compared to the controls, telomerase activity, the expression of human telomerase reverse transcriptase (*hTERT*) and *c-Myc* in the irradiated HUVECs were downregulated during serial passage. The downregulation of vascular endothelial growth factor (*VEGF*) was observed in the irradiated HUVECs as the PN increased. The data presented in this study may aid in the understanding of the mechanisms behind IR-induced EC senescence and telomerase- and angiogenesis-related gene response.

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Abbreviations: FGF, fibroblast growth factor; CTGF, connective tissue growth factor; EC, endothelial cell; *hTERT*, human telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; IGFBP4, insulin-like growth factor-binding protein4; IR, ionizing radiation; MCAM, melanoma cell adhesion molecule; PBS, phosphate-buffered saline; PN, passage number; *VEGF*, vascular endothelial growth factor; vWF, von Willebrand factor

Key words: telomerase, endothelial cells, radiation, angiogenesis, genes

Introduction

The important role of microvasculature in tumor growth has been well established (1). Tumors are known to be angiogenesis-dependent, and thus, the occurrence of endothelial cell (EC) apoptosis in the tumor microvasculature is a critical homeostatic factor regulating the rate of tumor growth. As a result, the effectiveness of tumor therapy may be determined by the responsiveness of the tumor microvascular endothelium, as well as tumor cells themselves to treatment. The activated vascular endothelium is an attractive therapeutic target as it is easily accessible for drugs, is genetically stable (less likely to develop resistance), homogeneous, and cells are in a proliferative state primarily within tumor tissue (2). Garcia-Barros *et al* reported that microvascular damages regulated tumor cell response to radiation (1).

Telomeres consist of short tandemly repeated DNA sequences. In humans, this sequence is TTAGGG, and the average telomere length is 5-15 kb (3). When the telomere length is short, it signals the arrest of cell proliferation, senescence and apoptosis (4). Telomere sequences are mainly synthesized by a cellular reverse transcriptase, telomerase, which is an RNA-dependent DNA polymerase that adds telomeric DNA onto telomeres (3). In humans, telomerase has a minimum of two essential components: a functional RNA component (*h*TER) that serves as a template for telomeric DNA synthesis and a catalytic protein component with reverse transcriptase activity (hTERT) that adds the telomeric repeats onto the end of the chromosome (5).

Telomerase is highly expressed in the advanced stages of the majority of cancers. Previous studies have shown that targeting telomerase with antisense oligonucleotides against *h*TERT, as well as pharmacological and genetic approaches, may be a promising cancer therapeutic strategy (6). Vascular ECs have been observed to express *hTERT* mRNA by *in situ* hybridization in human astrocytoma (7). There was a significant correlation observed between the level of *hTERT* mRNA expression and the proliferation rate of the ECs within the tumor vasculature. In addition, there was a significant correlation observed between the *hTERT* mRNA expression level

and the histological grade of the tumor. Falchetti *et al* reported that the inhibition of telomerase in human umbilical vein ECs (HUVECs) completely suppressed the angiogenic behavior of these cells in tumor xenografts (8). The hTERT inhibitor, BIBR1532, has been shown to induce vascular smooth muscle cell senescence (9). These findings suggest a contribution of telomerase activity to angiogenesis in ECs *in vitro* and *in vivo*.

Tumor angiogenesis involves a number of angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin, basic fibroblast growth factor (FGF), and platelet-derived growth factor. VEGF exerts a variety of effects on vascular ECs that together promote the formation of new blood vessels, stimulate ECs to migrate and divide, and profoundly alter their pattern of gene expression (10,11). VEGF has been reported to delay the onset of senescence in microvascular ECs and to reverse the senescence process (12). VEGF has also been shown to exert a radio-protective effect and to promote post-radiation survival in the tumor endothelium (2). Li *et al* showed that VEGF receptor (VEGFR)2 blockade using monoclonal antibody in ECs attenuated the proliferation, reduced migration and disrupted the differentiation of cells (2). Tie-1 and Tie-2 are tyrosine kinases with immunoglobulin-like and EGF-like domains (13). FGF upregulates the telomerase activity of HUVECs (14). Tie-2 is required for EC maintenance and proliferation. The angiopoietins and the Tie-2 receptor are considered key regulators of tumor-induced angiogenesis, cancer growth and metastasis (15).

It has been suggested that the tumor endothelium is quantitatively different from the endothelium derived from normal tissue. However, the normal and tumor endothelium have many similarities, sharing many EC-specific markers. St Croix *et al* reported 15 pan-endothelial markers that are expressed at substantially higher levels in both normal and tumor-associated endothelium compared with other tissues (16). Pan-endothelial markers are involved in the regulation of tumor angiogenesis (17,18). Among them, collagen type IV, α 2 (COL4α2) inhibits EC migration and proliferation and induces EC apoptosis (19). Collagen type XVIII, α 1 (COL18α1), a highly selective inhibitor of angiogenesis, increases EC apoptosis and decreases the tumor cell expression of several pro-angiogenic and invasive molecules (20). Collagen type VI, α 1 (COL6α1) is known to govern cell anchorage to the extracellular matrix and is downregulated in multiple myeloma ECs (21). Insulin-like growth factor-binding protein (IGFBP)4, IGFBP7, connective tissue growth factor (CTGF), interferon-induced transmembrane protein 1 (9-27), von Willebrand factor (vWF), and melanoma cell adhesion molecule (MCAM) have also been identified as pan-endothelial markers (16).

Ionizing radiation (IR) exerts powerful antitumor effects as it induces cytotoxicity via DNA damage. IR targets both tumors and ECs. The radiosensitivity of the tumor microvasculature and microvascular damage significantly contributes to tumor response to radiation (11). Thus, targeting an intrinsic treatment threshold in tumor vasculature ECs sensitizes the tumor cells to IR. Considering that IR directly induces DNA double-strand breaks, it is possible that cellular senescence is activated under these conditions (22). EC senescence may be an important factor for determining angiogenic activity following IR. Igarashi *et al* reported that the majority of growing ECs (80-90%) exhibited the senescence phenotype 3-5 days following exposure to 8 Gy of IR (23). It has been reported that the DNA damage response elicited by IR-induced DNA double-strand breaks is associated with telomere-initiated cellular senescence (24).

However, the mechanisms of action of IR in tumor angiogenesis are largely unknown (23). We hypothesized that the effect of IR-induced EC senescence may be associated with changes in telomerase- and angiogenesis-related gene expression. To examine this hypothesis, we investigated the effects of IR on telomerase- and angiogenesis-related gene expression in HUVECs *in vitro*.

Materials and methods

Cell lines. HUVECs were obtained from ATCC (Manassas, VA, USA). HUVECs were grown in Ham's F12K medium (Gibco, Invitrogen, Grand Island, NY, USA) with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 10% heat-inactivated fetal bovine serum (Omega Scientific, Inc., Tarzana, CA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml endothelial growth supplement (BD Biosciences, Bedford, MA, USA). Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air in a 37˚C incubator.

At confluence (70-80%), cells were harvested by treatment with 0.05% Trypsin-0.02% ethylenediaminetetraacetic acid (EDTA). Trypsin was inactivated by the addition of 1.25 mg of soybean trypsin inhibitor, and the cells were routinely subcultured at a constant 1:5 split ratio. The passage number (PN) was defined as the number of times cells have been subcultured into a new vessel. HUVECs were used between the first and third PNs.

HUVECs at PN1, PN2 and PN3 were irradiated at room temperature at 2 Gy/min with a PRIMART linear accelerator (Siemens AG, Erlangen, Germany). Irradiated HUVECs were cultured for 8 days and were then harvested for the evaluation of gene expression (Fig. 1).

Measurement of growth rate. The growth rate of the HUVECs was examined by proliferation assay. Cells were seeded at 20% confluence ($5x10^3$ cells) in a 24-well plate, and cell numbers were counted using methylene blue staining every 24 h for 8 days. The growth curve was drawn using the mean cell number of duplicated determinations, and the growth rate was calculated as follows: growth rate = ln (N2/N1)/(t2 - t1); where N1 and N2 were the cell numbers at time 1 (t1) and time 2 (t2), respectively.

Senescence-associated β-galactosidase assay. Senescence-associated (SA) β-galactosidase-positive cells were detected using the method described below. Briefly, cell monolayers were washed twice with phosphate-buffered saline (PBS) and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min. The cells were then washed twice with PBS, and staining solution [1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (X-Gal) in dimethylformamide (20 mg/ml, stock), 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$] was added. The cells were then incubated at 37˚C for 16 h and washed with PBS, and the number of stained cells was counted.

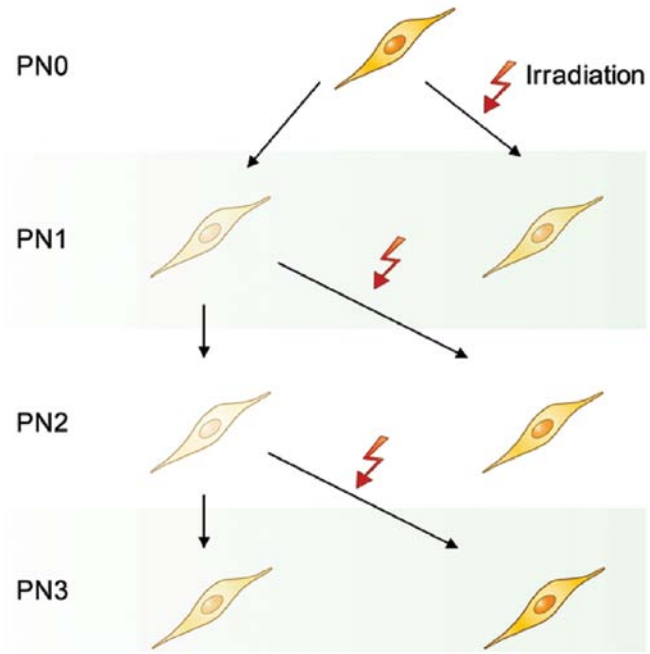

Figure 1. Human umbilical vein endothelial cells (HUVECs) were irradiated at 2 Gy/min. Irradiated HUVECs were cultured for 8 days and then harvested for the evaluation of gene expression. Cells were routinely subcultured at a constant 1:5 split ratio. PN, passage number.

Telomerase assay. Briefly, the telomeric repeat amplification protocol (TRAP) was performed according to the method described by Kim *et al* (25), with some modifications. Cells were washed in ice-cold wash buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol], and then with 100 μl of ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.5% CHAPS and 10% glycerol] in Kontes tubes. The homogenate was placed on ice for 30 min and centrifuged at 14,000 x g for 30 min at 4˚C. Supernatants were transferred into frozen vials and stored at -80˚C. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). Telomerase activity was assayed by the TRAP method. A total of 6 μg of sample was mixed in 50 μl of reaction mixture [forward telomerase substrate (TS) primer (5'-AATCCGTCGAGCAGAGTT-3') (0.1 μg), 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween-20, 0.1 mg bovine serum albumin and 50 μM dNTP including ^{32}P-labeled dCTP], and incubated at 20˚C for 30 min for the telomerase-mediated extension of TS primers. After heating the mixture at 94˚C for 3 min to inactivate telomerase, 1 μg of CX primer [5'-(CCCTTA)3CCCTAA-3'] and 2 U of Taq DNA polymerase were added, and the mixture was then subjected to 30 cycles at 94˚C for 30 sec, 50˚C for 30 sec and 72˚C for 90 sec. Polymerase chain reaction (PCR) products were analyzed by electrophoresis on 12% polyacrylamide non-denaturing gels. The gels were dried and exposed overnight on X-ray films. The criterion for a positive TRAP assay was a hexanucelotide ladder of 3 or more bands. A total of 293 human embryonic kidney cell extracts were used as the positive control. The standard 6 μg of protein extract was serially diluted 10- and 100-fold to remove possible false-negative results from the Taq polymerase inhibitor and to create a relative comparison system.

Real-time PCR. Total RNA was collected from the cells using TRIzol reagent. RNA integrity was initially checked on a 1% agarose gel by confirming the presence of the 18S and 28S ribosomal RNA bands. Approximately 5 μg of total RNA was used to create cDNA using a First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. For quantitative real-time PCR, primers and the QuantiTect SYBR-Green PCR kit (Qiagen, Santa Clarita, CA, USA) were used with the Rotor-Gene 2072D real-time PCR machine (Corbett Research, Sydney, Australia). Briefly, in a total reaction mixture volume of 20 μl composed of 1X QuantiTect SYBR-Green PCR Master Mix containing HotStarTaq DNA polymerase, QuantiTect SYBR-Green PCR buffer, dNTP mix including dUTP, SYBR Green, ROX (passive reference dye), 5 mM $MgCl_2$, 0.5 μM primers and 0.5 μg of cDNA, PCR was performed as follows: 15 min at 95˚C and then 45 cycles of 15 sec at 94˚C, 15 sec at 60˚C, and 20 sec at 72˚C. The primers used are listed in Table I. The relative expression level of each gene was calculated by dividing the gene expression of the irradiated HUVECs by that of the control HUVECs at the same PN.

Statistical analysis. An unpaired two-tailed Student's t-test was used for the evaluation of the senescence rate; a P-value <0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS software for Windows (version 12.0, SPSS, Chicago, IL, USA).

Results

Changes in biological phenotypes following IR. Following IR, the growth rate was delayed. The mean growth rate of the control cells was 0.156/day at PN1, 0.132/day at PN2 and 0.098/day at PN3. The mean growth rate of the irradiated cells was 0.086/day at PN1, 0.042/day at PN2 and 0.042/day at PN3. The growth inhibition rates following IR treatment were 44.8, 62.2 and 57.1 % at PN1, PN2 and PN3, respectively.

Appearance of senescence following IR. The number of IR-induced senescent cells was significantly increased in the irradiated HUVECs at all PNs (mean ± SD; PN1, 7.12±1.1 vs. 32.4±4.4%, $P<0.001$; PN2, 11.7±4.3 vs. 30.9±6.2%, $P<0.05$; PN3, 18.6±3.1 vs. 43.1±5.5%, $P<0.01$) (n=3 for each passage) (Fig. 2).

Telomerase activity following IR. In the control cells, there was no difference observed in telomerase activity as the PN increased. However, following IR treatment, there was a 20% decrease in telomerase activity in the irradiated cells compared to the control cells at PN1, a 20% reduction at PN2 and a 25% reduction at PN3.

Changes in telomerase-related gene expression following IR. Compared to the control cells at the corresponding PNs, the downregulation of *hTERT* and *hTER* was observed in the irradiated HUVECs (Figs. 3 and 6A). The *hTERT* expression level continuously decreased at all PNs. The expression of *Mad1* decreased at PN1 and 2, but increased by approximately

Table I. Primers used for real-time PCR.

Gene	Sense sequence (5'-3')	Antisense sequence (5'-3')
hTER	CTAACCCTAACTGAGAAGGGCGTAG	GAAGGCGGCAGGCCGAGGCTTTTCC
hTERT	CGGAAGAGTGCTCTGGAGCAA	GGATGAAGCGGACTCTGGA
hTEP	TCAAGCCAAACCTGAATCTGAG	CCCGAGTGAATCTTTCTACGC
c-Myc	AAGTCCTGCGCCTCGCAA	GCCTGTGGCCTCCAGCAGA
Mad1	TTCAGACTTGGACTGTGTCA	GAAGGAAGTCCAGAAGGTTT
VEGF	GTGGACATCTTCCAGGAGTA	TCTGCATTCACATTTGTTGT
VEGFR-1	GGCTCTGTGGAAAGTTCAGC	AATCACTTGGAAGAGGGGCT
VEGFR-2	CCCACCCCCAGAAATAAAAT	ACATTTGCCGCTTGGATAAC
VEGFR-3	GCTGAAGCAGAGAGAGAGAA	GTCACACTCCTTGTCCACTT
Tie-1	GTCCTTTGGAGTCCTTCTTT	AAGTTCTCAAACAGCGACAT
Tie-2	CAAAGATGATCACAGGGACT	GAAGGAAGTCCAGAAGGTTT
COL18α1	CTCCCTGCTCTACACAGAAC	CTCTGGAACTCCTCACAGTC
COL4α2	GACATCGGGGACACTATAAA	ACCTTCTGTTCCCTTCTCTC
COL6α1	ATGCCATGGACTTTATCAAC	GAGTTGCCATCTGAGAAGAG
CTGF	CCTCAATTTCTGAACACCAT	AACAATCTGTTTTGACGGAC
IGFBP4	CACGAGGACCTCTACATCAT	GTCCACACACCAGCACTT
IGFBP7	GGGTCACTATGGAGTTCAAA	TGTAATTTTTGCTGATGCTG
9-27	TTACTGGTATTCGGCTCTGT	CACTGTAGACAGGTGTGTGG
MCAM	CTGTAAATACCTGGCTCCTG	CACAGGAGACTTTGAAGAGG
vWF	GAACGGGTATGAGTGTGAGT	CAAGGTGACTTTCTTTCCTG
β-actin	GGGAATTCAAAACTGGAACGGTGAAGG	GGAAGCTTATCAAAGTCCTCGGCCACA

hTERT, human telomerase reverse transcriptase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; COL4α2, collagen type IV, α 2; COL18α1, collagen type XVIII, α 1; COL6α1, collagen type VI, α 1; IGFBP, insulin-like growth factor-binding protein; CTGF, connective tissue growth factor; MCAM, melanoma cell adhesion molecule; vWF, von Willebrand factor.

3-fold in the irradiated cells compared to the control cells at PN3 (Figs. 3 and 6A). *c-Myc* was continuously downregulated at all PNs.

Changes in angiogenesis-related gene expression following IR. Compared to the control cells at the corresponding PNs, the downregulation of *VEGFR1*, *VEGFR2*, *VEGFR3*, *Tie-1* and *Tie-2* was observed in the irradiated HUVECs (Figs. 4 and 6B). *VEGF* expression decreased as the PN increased compared to the control cells. Among the pan-endothelial markers, *COL4α2*, *COL18α1* and *COL6α1* were downregulated in the irradiated HUVECs at all PNs (Figs. 5 and 6C). The levels of *IGFBP4*, *IGFBP7*, *CTGF*, *MCAM*, *9-27* and *vWF* were also downregulated in the irradiated HUVECs (Figs. 5 and 6C).

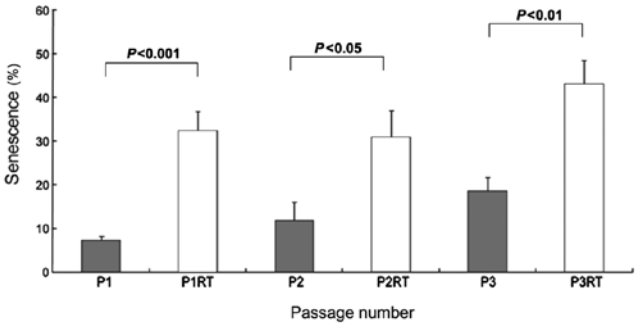

Figure 2. Effects of irradiation on human umbilical vein endothelial cell (HUVEC) senescence. Irradiation-induced senescence of HUVECs at all PNs. HUVECs were irradiated at room temperature at 2 Gy/min. Irradiated HUVECs were cultured for 8 days, and then cellular senescence was evaluated by senescence-associated (SA)-β-galactosidase assay. Data shown are the means ± SD from 3 independent experiments. RT indicates that the cells were treated with irradiation. PN, passage number; P1, PN1; P2, PN2; P3, PN3.

Discussion

This study demonstrates that IR is a potent inducer of EC senescence, as well as the downregulation of telomerase-related genes. Telomere and hTERT are associated with cellular senescence. It has been reported that telomeres in senescent cells directly play a role in DNA damage response, and that uncapped telomeres are associated with DNA damage response proteins (24). Takano *et al* reported that hTERT induced a delay in senescence, and that hTERT-overexpressed ECs appeared more resistant to stress (26). Certain studies have demonstrated that telomerase inhibitors increase the sensitivity of cancer cells to IR. Nakamura *et al* showed that

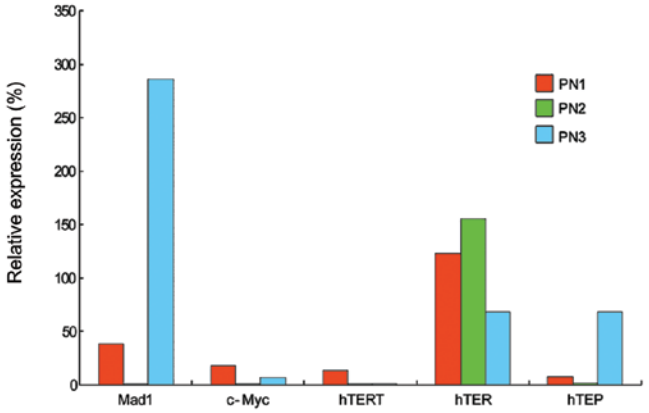

Figure 3. Changes in relative gene expression of *Mad1*, *c-Myc*, *hTERT*, *hTER* and *hTEP*. Human umbilical vein endothelial cells (HUVECs) were irradiated at room temperature at 2 Gy/min. Irradiated HUVECs were cultured for 8 days and then harvested for the evaluation of gene expression. Real-time PCR was carried out once for each telomerase-related gene. Relative gene expression was the ratio of expression of irradiated HUVECs and that of the control HUVECs at the same PN. Zero (0) indicates that the gene was not expressed or the expression was below the range of detection. PN, passage number; *hTERT;* human telomerase reverse transcriptase.

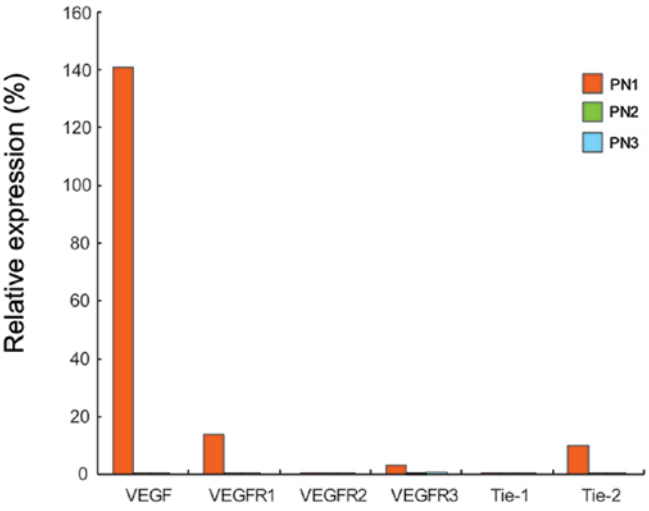

Figure 4. Changes in relative gene expression of *VEGF*, *VEGFR1*, *VEGFR2*, *VEGFR3*, *Tie-1* and *Tie-2*. Human umbilical vein endothelial cells (HUVECs) were irradiated at room temperature at 2 Gy/min. Irradiated HUVECs were cultured for 8 days and then harvested for the evaluation of gene expression. Real-time PCR was carried out once for each angiogenesis-related gene. Relative gene expression was the ratio of expression of irradiated HUVECs and that of the control HUVECs at the same PN. Zero (0) indicates that the gene was not expressed or the expression was beneath the range of detection. PN, passage number. VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

HeLa cells lacking *hTERT* treated with small interfering RNAs had a decreased telomerase activity and a significantly increased sensitivity to radiation compared with control cells (6). Wu *et al* reported that treatment with imetelstat, a telomerase antagonist, reduced the telomerase activity of esophageal cells by more than 70% compared to the controls. In addition, imetelstat increased the number and size of 53BP1 foci following IR (27).

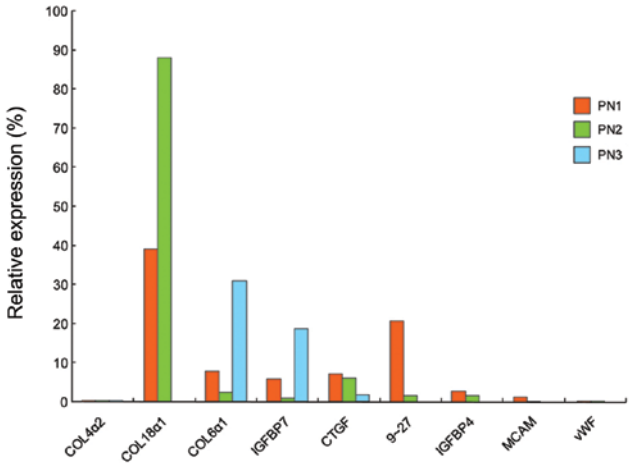

Figure 5. Changes in relative gene expression in *COL4α2*, *COL18α1*, *COL6α1*, *IGFBP7*, *CTGF*, *9-27*, *IGFBP4*, *MCAM* and *vWF*. HUVECs were irradiated at room temperature at 2 Gy/min. Irradiated HUVECs were cultured for 8 days and then harvested for the evaluation of gene expression. Real-time PCR was carried out once for each angiogenesis-related gene. Relative gene expression was the ratio of expression of irradiated HUVECs and that of the control HUVECs at the same PNs. *COL18α1*, *9-27*, *IGFBP4*, *MCAM* and *vWF* gene expression in irradiated HUVECs were not evaluated at PN3. PN, passage number; *COL4α2*, collagen type IV, α 2; *COL18α1*, collagen type XVIII, α 1; *COL6α1*, collagen type VI, α 1; *IGFBP*, insulin-like growth factor-binding protein; *CTGF*, connective tissue growth factor; *MCAM*, melanoma cell adhesion molecule; *vWF*, von Willebrand factor.

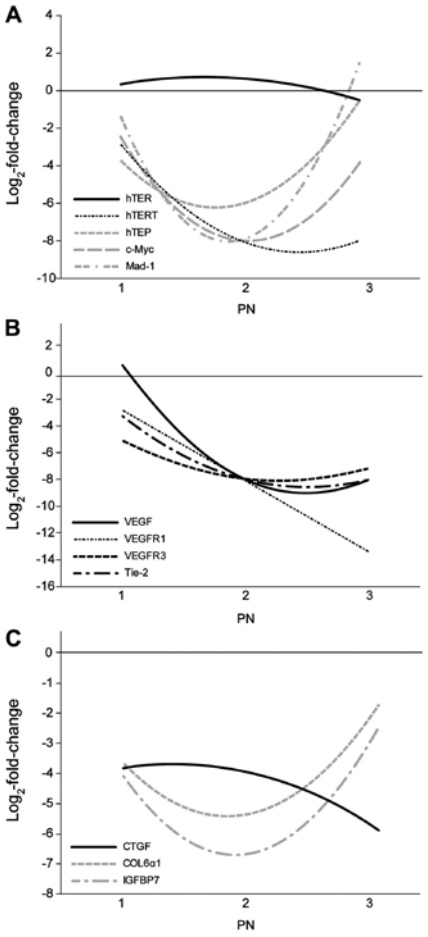

Figure 6. Expression fold change in telomerase- and angiogenesis-related genes was calculated as the $\log_2$ ratio of the fold change between irradiated human umbilical vein endothelial cells (HUVECs) and control HUVECs at the same PNs. Only the fold change of *Mad-1* increased over zero. PN, passage number.

In our study, the number of senescent cells increased in the irradiated HUVECs, while the telomerase activity and *hTERT* expression were decreased. *c-Myc* expression was also downregulated following IR. By contrast, the expression of *Mad1* in the irradiated cells increased by approximately 3-fold compared to the control cells at PN3. The regulation of hTERT expression is a major control mechanism of telomerase activity (3). The *hTERT* promoter contains several binding sites for transcription factors, including c-Myc and Mad1 (28). Mad1 is a transcriptional repressor that represses the c-Myc-mediated transactivation by competing for the ubiquitous binding partner, Max, preventing it from binding to c-Myc. The *hTERT* promoter contains 2 E-box consensus sites. One is located close to the translational initiation codon at position -29 to -34 (proximal E-box), and the other is located at position -238 to -243 with regard to the ATG (distal E-box). c-Myc and Mad1 exert their transcriptional effects by binding to the same site in the *hTERT* promoter (3). Though we have no direct evidence that IR directly regulates *c-Myc* and *Mad1* expression, these data suggest that the upregulation of *Mad1* and the downregulation of *c-Myc* in irradiated HUVECs deactivate *hTERT*.

In the present study, *VEGF,* as well as *c-Myc* were downregulated in the irradiated HUVECs during serial passage. c-Myc has been shown to increase VEGF production in several cell types (29). Myc-overexpressing B cells have been shown to increase VEGF production during the early stages of lymphomagenesis in Eμ-*c-Myc* mice (29). The *VEGF* promoter contains a consensus Myc-binding site. In addition, VEGF induces *hTERT* expression and telomerase activity in human ECs (30). We suggested that the downregulation of *c-Myc* in irradiated HUVECs was associated with the downregulation of *VEGF*, which contributed to the IR-induced *hTERT* downregulation. However, there is little evidence that c-Myc directly induces *VEGF* mRNA transcription (29). Further studies are required to evaluate the correlation among c-Myc, hTERT and VEGF expression in irradiated HUVECs.

The expression of endothelial markers, such as VEGFR2, vWF and MCAM, is known to increase during endothelial progenitor cell differentiation toward ECs (31). Considering that IR decreased the expression of these markers in our study, IR may negatively modulate the differentiation of endothelial progenitor cells toward ECs. IR may hinder the ability of endothelial progenitor cells to adhere, migrate and form a capillary-like structure (31). This may be one of the mechanisms of action of IR in tumor angiogenesis.

In conclusion, IR can induce human microvascular EC senescence at doses relevant to clinical radiotherapy. During the serial passage of irradiated HUVECs, the expression of *hTERT*, *c-Myc* and *VEGF* was downregulated. The data presented in this study may aid in the understanding of the mechanisms behind IR-induced EC senescence and telomerase- and angiogenesis-related gene response.

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