Ionizing Radiation Up-regulates Telomerase Activity in Cancer Cell Lines by Post-translational Mechanism via Ras/Phosphatidylinositol 3-Kinase/Akt Pathway

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Abstract

Purpose: Telomerase is considered currently as a hallmark of cancer, and its inhibition is expected to become an important anticancer modality. In contrast to abundant data concerning the effect of cytotoxic drugs on telomerase activity (TA), there is scant information on the effect of radiation on telomerase. The mechanism of telomerase regulation by irradiation has never been evaluated in detail. In the present study, we investigated the effect of radiation on TA and its regulation in cancer cells.

Experimental Design: The effect of various radiation doses on TA in several malignant and non-malignant cell lines was evaluated. All malignant cells exhibited similar telomerase response to radiation and its regulation was assessed at transcriptional and post-translational levels in K562 cells. Next step was the evaluation of the upstream signaling pathways leading to changes in TA using kinetics and specific inhibitors.

Results: Radiation up-regulated TA in dose-dependent manner only in cancer cells. Telomerase was activated by phosphorylation by Akt and by cytoplasmic-nuclear shift. Transcriptional processes were not involved in TA. This telomerase regulation is mediated by Ras/phosphatidylinositol 3-kinase/Akt pathway. The canonical membrane effectors of irradiation (epidermal growth factor receptor, insulin-like growth factor-I receptor, and Ca2+ influx) were not involved in this process.

Conclusions: Radiation up-regulates telomerase activity specifically in cancer cells. This study adds to accumulating evidence pointing to post-translational level as important mode of telomerase regulation. Telomerase activation due to radiation may be detrimental in treatment of cancer. Data described in this study may add to future interventions aiming at inhibition of telomerase activation during irradiation.
telomerase inhibition and telomere shortening have been shown to sensitize cancer cells to chemotherapy. In addition, TA exerts an antiapoptotic effect and its inhibition may promote apoptosis in malignant cells. In keeping with this concept, it is important to evaluate the effects of various anticancer treatments on TA. The effect of cytotoxic drugs has been extensively studied. Others and we showed that various cytotoxic drugs down-regulate TA (9, 10).

The effect of radiation on telomerase is much less clear. Radiation is common modality in the treatment of cancer and is used in ≥50% of all cancer patients (11). Radiation-induced cell death is usually attributed to DNA damage to tumor cells, which induces cell apoptosis and/or necrosis. Telomerase can play a role in the healing of chromosomes or chromatid breaks produced by this damage (12–15) and, as such, might enhance the radioresistance of cells. Telomerase inhibition in experimental mouse models enhances telomere shortening and increases their radiosensitivity (16). Data regarding the effect of radiation on TA are conflicting. Several reports described up-regulation, whereas others showed the opposite (12, 13, 17). These discrepancies in TA can be attributed to different methodologies such as different cell lines, varying doses of radiation, and different time points assessing its effect. In addition, there are few data concerning the regulation of telomerase following irradiation of malignant cells.

In view of the importance of telomerase in cancer treatment, we studied the effect of radiation on regulation of TA in cancer cells. Results of our study show that radiation in conventional therapeutic doses increases TA in malignant cells, whereas it does not affect it in normal cells. This up-regulation is mediated by post-translational phosphorylation of telomerase caused by Ras/phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The canonical membrane effectors of irradiation are probably not involved in this process.

Materials and Methods

Cell lines. SK-N-MC (Ewing sarcoma) cell line was kindly provided by Dr. Gad Lavie (Sheba Medical Center), MCF-7 (breast cancer) and K562 (blastic phase of chronic myelogenous leukemia) cell lines were kindly donated by Dr. M. Shaklai (Felsenstein Medical Research Center, Rabin Medical Center). 1146 cells (cell line established from lymphocytes donated by a healthy volunteer) were given by the molecular oncology laboratory (Felsenstein Medical Research Center). In addition, mononuclear cells obtained from 10 mL blood from a healthy volunteer served as another source of normal cells. All cell lines were maintained in RPMI 1640 supplemented with 10% to 15% heat-inactivated FCS, glutamine (2 mmol/L), and penicillin/streptomycin (1%; Biological Industries). Proliferation and TA assays were done in all cell lines. K562 line was chosen for detailed analysis of various mechanisms related to telomerase regulation.

Radiation conditions. The cells were grown for 24 h before irradiation in the following conditions: adherent cells (1 × 10^6/mL SK-N-MC and MCF-7) were seeded in quadruplicate in 24-well plates. Nonadherent cells (2 × 10^6/mL K562 and 1146) were seeded in quadruplicate in 96-well plates or 50 mL flasks according to the requirements of the relevant analysis. All cells were irradiated using photon accelerator (Varian 600c) energy 6×, rate 200 MU/min. A 1.5 cm² bolus has been used to maximize the dose. Radiation doses ranged from 2 to 8 Gy in a 40 × 40 cm² field. Accelerator conditions were strictly fixed throughout all experiments.

Proliferation assay. Proliferation of adherent cells was determined by the sulforhodamine B assay (18). Briefly, cells were fixed with 10% TCA (Sigma) and stained with 0.4 (w/v) sulforhodamine B (Sigma) dissolved in 1% acetic acid. Unbound dye was removed by four washes with acetic acid (1%), and the dye-stained protein was extracted with unbuffered Tris (10 mmol/L). The absorbance of protein samples was determined in a 96-well microtiter ELISA plate reader (550 nm).

The proliferation of nonadherent cells (K562 and 1146) was determined using WST-1 assay that measures the colorimetric formation of formazan dye from tetrazolium salt cleavage by mitochondrial enzymes. Briefly, 48 h after irradiation, 20 μL cell proliferation reagent WST-1 was added to 200 μL cell suspension in a 96-well microtiter plate. After incubation of 2 h, the color intensity was determined by ELISA reader (550 nm).

Telomeric repeat amplification protocol assay. Measurement of TA was done by the PCR-based telomeric repeat amplification protocol (TRAP) assay, using the TRAPex telomerase detection kit (InterGene), according to the manufacturer’s instructions and as described previously (19). Briefly, isolated cells were incubated in ice-cold CHAPS lysis buffer for 30 min at 4°C (1 × 10^6 cells/100 μL) and were subsequently centrifuged at 13,000 rpm for 30 min at 4°C. The protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad Laboratories). Protein extract (0.2 μg) was subjected to TRAP analysis. Each reaction was done in 50 μL reaction mixture containing 1× TRAP buffer, deoxynucleotidetriphosphatemix,TSprimer,TRAPprimermix,andTaqpolymerase.Reactionsweredoneat30°Cfor30minandthenPCRamplifiedby30cyclesof94°C,59°C,and72°Cfor30s each, and separated by electrophoresis on 12.5% polyacrylamide gels, in a Mighty Small II gel apparatus (Hoffer Scientific Instruments). Gels were stained with SYBR Green nucleic acid gel stain (Amresco). Quantification was done using the Quantity-One software for Bio-Rad Image analysis systems (Bio-Rad Laboratories). TA was calculated according to the following formula: TPG (U) = (X - B) / C(r - B)/Cr × 100, where TPG is the total product generated, X is sample signal, B is background signal intensity, C is the 36-bp internal PCR control, and r is the TR8 reverse quantification control. Relative TA was calculated by dividing TA in certain point to baseline TA. Negative control consisted of 1× CHAPS lysis buffer instead of cell lystate. All results represent an average of four to six independent TRAP assays.

RNA purification and reverse transcription-PCR analysis of hTERT, PTEN expression, and hTERT alternative splicing. Expression of the relevant genes was evaluated by a semiquantitative multiplex reverse transcription (RT-PCR) technique. Total RNA was extracted from cells using the Purescript RNA isolation kit (GenTa Systems) according to the manufacturer’s instructions. RNA (1 μg) was then reverse transcribed into single-stranded DNA with SuperScript TMII RNAse
Reverse Transcriptase (Life Technologies). Each RT-PCR was carried out with the genes’ specific primers and β-actin primers as an internal control. RT-PCR products were separated on 2% agarose gels and the relative intensity of the amplified products was calculated compared with the housekeeping gene, β-actin, using the Quantity-One software for Bio-Rad Image analysis systems (Bio-Rad Laboratories).

hTERT mRNA was amplified by using the following primers: forward primer 5’-CGGAAGAGTTGCTGGAACAA-3’ (corresponding to position 1785-1804, GenBank accession no. AFO 18167) and reverse primer 5’-AGGCTGGACGAGCTACTGGTGACG-3’ (corresponding to position 2531-2507). Amplification was done with 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s.

Primers for hTERT alternative splicing were: forward primer 5’-GCC-TGAGCTTACTTGCTCA-3’ (corresponding to position 2109-2130, GenBank accession no. AF128893/4) and reverse primer 5’-AGGCTGGACGAGCTACTGGTGACG-3’ (corresponding to position 2531-2507). Amplification was done with 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 40 s.

The PTEN primers sequences were: forward primer 5’-GGGAGTAACGG-3’ (corresponding to position 1523, GenBank accession no. NM_000314) and reverse primer 5’-ACTIN primer sequences were forward primer 5’-GACCA-1785-1804, GenBank accession no. AFO 18167) and reverse primer 5’-AGGCTGGACGAGCTACTGGTGACG-3’ (corresponding to position 2531-2507). Amplification was done with 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 40 s.

The β-actin primer sequences were forward primer 5’-GCC-TGAGCTTACTTGCTCA-3’ and reverse primer 5’-AGGCTGGACGAGCTACTGGTGACG-3’. Amplification conditions were identical for each specific gene as above. All PCRs were conducted in duplicates and repeated at least three times.

Western blotting. Levels of phosphorylated Akt (pAkt) were measured in the cells after starvation and irradiation as follows: cells were grown in RPMI 1640 deprived of serum for 24 h. To induce phosphorylation of Akt, cells were irradiated as described above. Cells were then harvested, washed by PBS, and lysed using CHAPS lysis buffer (TRAPese kit). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories). Identical protein amounts of all samples were subjected to PAGE. The expression of total Akt (tAkt) or pAkt was detected by specific monoclonal antibody (Cell Signaling) in 1:1,000 dilution followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (The Jackson Laboratory). Visualization of both proteins was done by the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) according to the supplied protocol. Quantification of the signals was done by using the Quantity-One software for Bio-Rad Image analysis systems (Bio-Rad Laboratories). The levels of pAkt expression were calculated relatively to the total level of the Akt expression.

Cell compartmentalization assay. Nuclear and cytoplasmic fractionation was done as described by Bakhanshivi and Hizzy (20). In brief, cells were incubated in the presence of ice-cold hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.5 mmol/L DTT, and 0.2 mmol/L phenylmethylsulfonyl fluoride] and 0.6% NP-40 for 15 min followed by centrifugation at 15,000 × g for 2 min at 4°C. The supernatant was collected and considered as the cytoplasmic fraction. The pellet (the nuclear fraction) was incubated with ice-cold CHAPS lysis buffer for 30 min. TA was determined in each fraction by the TRAP assay and expressed as total activity and as the cytoplasmic-nuclear ratio activity.

Exposure to various inhibitors. The following inhibitors were used to identify specific molecular pathways evaluated in this study (the numbers given as final concentration): 2.5 μmol/L 124020, an Akt inhibitor (Calbiochem/Merck Biosciences), was added 1 h before irradiation; 0.075 μmol/L wortmannin, a PI3K family inhibitor (Upstate), was added 30 min before irradiation; 1 μmol/L

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Fig. 1. Effect of irradiation on TA and proliferation index. A, representative example of TA in K562 cells measured by TRAP assay at different doses of radiation. Numbers, radiation doses (in Gy). IC, negative control; R8, standard PCR control; IC, internal control. Each dose is shown in duplicates. B, quantification of TA and proliferation index in response to different radiation doses in three cell lines. Results of three independent experiments. PI, proliferation index; RTA, relative TA (see Materials and Methods). C, kinetics of TA at different time points (hours) from irradiation. TRAP assay describing TA (representative experiment). Time from irradiation is indicated above. Each time point is shown in duplicates. D, kinetics of TA at different time points in irradiated K562 and 1146 cells and peripheral blood lymphocytes (PBL). Results of three independent experiments. Rx, irradiation; TP, telomerase products shown as ladder in the gel.
farnesylthiosalicylic acid (FTS), a specific Ras inhibitor (synthesized and kindly provided by Prof. Y. Kloog, Tel Aviv University), was added 1 h before irradiation; 0.05 \( \mu \)mol/L AG1478, epidermal growth factor receptor inhibitor (synthesized and kindly supplied by Dr. Aviv Gazit, Hebrew University), was added 1 h before irradiation; 30 \( \mu \)mol/L BAPTA, calcium influx inhibitor (Sigma), was added 1 h before irradiation; 10 nmol/L AG1024, insulin-like growth factor-I receptor inhibitor (synthesized and kindly donated by Dr. Aviv Gazit, Hebrew University), was added 1 h before irradiation. TA was measured after the exposure of the cells to each inhibitor. pAkt/tAkt ratio was measured after exposure to wortmannin and FTS.

**Results**

**Radiation enhances TA in malignant cell lines**

TA was measured in SK-N-MC, MCF-7, and K562 cell 24 h after irradiation. Increase in TA was observed in all cell lines and was dose-dependent up to 4 Gy. Maximal TA was 2.3-, 1.7-, and 2.5-fold of baseline in K562, SK-N-MC, and MCF-7 cell lines, respectively. At higher doses up to 8 Gy, the activity returned to baseline (Fig. 1A and B). In contrast to the increase in TA, irradiation caused dose-dependent reduction in cell proliferation (Fig. 1B). To verify whether radiation-induced telomerase up-regulation is restricted to malignant cells, normal lymphoid cell line and lymphocytes obtained from a healthy volunteer were exposed to the same doses of radiation. In contrast to malignant cells, radiation at a dose of 4 Gy did not induce any change in TA in 1146 normal lymphocytic cell line. Similarly, the negligible TA of normal lymphocytes remained unchanged after irradiation (Fig. 1C and D). K562 cells were chosen to further explore the mechanism by which radiation affects the regulation of TA.

**Mechanism of Telomerase Up-regulation by Irradiation**

**Fig. 2.** Transcriptional regulation of hTERT in irradiated K562 cells. A, hTERT expression was evaluated by RT-PCR using primers homologous to the gene total transcript measured 24 h after different doses of irradiation. Numbers, radiation doses. \( \beta \)-Actin, a control housekeeping gene. Quantification of hTERT expression expressed as hTERT/\( \beta \)-actin. Representative of three independent experiments. B, alternatively spliced variants were amplified using primers homologous to the four splice variant transcripts of the gene: \(-a\), \(-b\), \(-a+b\), and full transcripts. Representative experiment of alternative splicing of hTERT at different time intervals with or without irradiation. Right, sizes of various splice forms; M, molecular size marker; ft, full transcript. C, quantification of the various splice forms at different time intervals with or without irradiation. Results of three independent experiments.
irradiation, reaching a maximum at 24 h (2.4-fold increase) and remained elevated, albeit to a lesser extent at 48 h (2-fold; Fig. 1C and D).

**Telomerase regulation mechanism following irradiation**

To elucidate the molecular changes responsible for irradiation-dependent telomerase up-regulation, the known mechanisms of telomerase regulation were examined.

**Radiation-dependent TA is not transcriptionally mediated.** The expression of the hTERT gene and its alternative spliced forms was determined before and 24 h after the exposure of the cells to 2 to 6 Gy (Fig. 2). Irradiation did not increase hTERT expression as determined by RT-PCR. Moreover, in cells exposed to 6 Gy, hTERT expression was decreased 24 h after irradiation (Fig. 2A). Analysis of hTERT alternative splice forms [-α, -β, -(α + β)] did not reveal any changes following irradiation at the above doses (Fig. 2B and C). These data indicate that irradiation-induced TA is not transcriptionally regulated.

**Radiation-dependent TA is post-translationally mediated by pAkt.** Telomerase post-translational modifications switch the enzyme from an inactive form (dephosphorylated) to an active one (phosphorylated). Phosphorylation of its tyrosine residues is done by Akt (protein kinase B; ref. 21).

We followed the levels of pAkt and compared them with tAkt following irradiation. pAkt/tAkt ratio increased already 30 min after irradiation compared with nonirradiated cells (20% versus 10%, respectively; Fig. 3A). This ratio increased further 120 min after irradiation (33% versus 8%, respectively). After 24 h, it remained high (22%), but the baseline pAkt increased probably due to prolonged starvation stress of the cells. To further verify the direct involvement of Akt in the up-regulation of telomerase in these settings, we inhibited its activity by a specific inhibitor, 124020, which does not affect PI3K, its upstream regulator. The increase of TA after irradiation was less pronounced in cells that were exposed to 124020 compared with control irradiated cells 6 and 24 h after irradiation (1.25-, 1.4-, 1.2-, and 2.4-fold, respectively; Fig. 3B and C). Therefore, the up-regulation of TA after irradiation is mediated by phosphorylation of Akt.

**Compartmentalization of telomerase after irradiation.** Phosphorylated telomerase is translocated from the cytoplasm into the nucleus before binding to its telomeric substrate (22).
To determine possible changes in telomerase compartmentalization post-irradiation, we fractionated the irradiated cells and assessed TA in the nuclear and cytoplasmic fractions.

Before irradiation, the nuclear TA was more prominent than the cytoplasmic one (Fig. 4). At 6 h post-irradiation, there was no change in nuclear TA. However, after 24 h, irradiated cells showed an increase in the nuclear TA compared with baseline TA (5.9 and 3, respectively). The increase in nuclear activity was accompanied by a decrease in the cytoplasmic activity of the enzyme (0.8 and 1, respectively). Accordingly, the ratio of TA in the nuclear-cytoplasmic compartments was also increased in irradiated cells (7.4 and 3, respectively).

These results suggest that irradiation promotes the translocation of telomerase from the cytoplasm into the nucleus.

**Signaling components upstream of Akt**

After the identification of Akt as the main regulator of telomerase activation in irradiated cells, its upstream signaling pathways were further characterized.

**Akt/PI3K mediates the irradiation-dependent telomerase up-regulation.** The main activator of Akt is PI3K. To assess its role in radiation-dependent TA, we added wortmannin, a specific PI3K inhibitor, before irradiation and measured TA at its peak: 24 h post-irradiation. Inhibition of PI3K antagonized the 1.9-fold irradiation-induced activation of telomerase. As shown in Fig. 5B, TA in irradiated cells treated with wortmannin was slightly increased compared with its level 24 h before irradiation (1.5-fold).

A kinetic analysis of the pAkt/tAkt ratio in cells treated with wortmannin revealed that 2 h post-irradiation the levels of pAkt increased to a lesser extent compared with the non-wortmannin-treated cells (22% and 25%, respectively; Fig. 5A). However, the difference between the PI3K-inhibited and the noninhibited cells was markedly increased 24 h post-irradiation; the increase in pAkt in noninhibited cells was ~50% versus only 25% in the PI3K-inhibited cells.

Taken together, these results suggest that the up-regulation of TA in irradiated cells is mediated by the PI3K/Akt signaling pathway.

**Ras mediates the irradiation-dependent PI3K/pAkt activation and the telomerase up-regulation.** Several signaling pathways that activate the PI3K/Akt pathway are activated by irradiation. The involvement of Ras in these pathways is well documented (23). To decipher which pathway is relevant in the setting of ionic irradiation, we examined the involvement of Ras in the up-regulation of TA by using a specific inhibitor of Ras, FTS. The levels of pAkt and tAkt were measured 2 and 24 h post-irradiation following treatment with FTS. Inhibition of Ras down-regulated the increase in TA post-irradiation (Fig. 5B) to below baseline levels. Accordingly, even nonirradiated cells exhibited a slight decrease in the activity of the enzyme on Ras inhibition. This result shows the involvement of Ras in the

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**Fig. 4.** TA in subcellular fractions of K562 cells in response to irradiation. A, TA in cytoplasm (C) and nucleus (N) at different time intervals after radiation of 4 Gy. TRAP assay (representative experiment). B, quantification of TA in different intervals with or without radiation. Results are average of three independent experiments. N/C, nuclear-cytoplasmic ratio.
PI3K/Akt signal transduction, regulating the increase in TA after irradiation. In addition, it may attribute another regulatory role to Ras, related directly to telomerase.

PTEN is not involved in the up-regulation of the PI3K/Akt signaling cascade after irradiation. PTEN may dephosphorylate Akt in the cell and thus inhibit its activity. We examined its expression in our experimental system by RT-PCR. As shown in Fig. 5C, irradiation did not decrease PTEN expression; in fact, some increase was noted. We therefore concluded that PTEN is not involved in the irradiation triggered signaling pathway that up-regulates TA.

Canonical sensors of irradiation trigger are not involved in the up-regulation of telomerase following irradiation. Radiation exerts its intracellular effects on Ras/PI3K/Akt pathway through several membrane-associated mediators. These membrane-associated pathways were assessed by the inhibition approach. Epidermal growth factor receptor inhibitor AG1478, insulin-like growth factor-I receptor inhibitor AG538, and Ca²⁺ chelator BAPTA were applied separately on the cells before irradiation. TA was assessed 24 h afterwards. As shown in Fig. 5D, none of them affected the up-regulation of telomerase in the irradiated cells. We therefore conclude that these sensors do not mediate the up-regulation of TA in these cells.

Discussion

Our study showed a consistent up-regulation of TA in response to ionizing radiation in all examined cancer cell lines in both solid and hematologic tumor cells. Most of the studies addressing this issue are consistent with our results showing up-regulation of TA after irradiation (14, 15, 24, 25). Several reports, however, describe down-regulation of TA (12, 26), sometimes after very high irradiation dosages (20-160 Gy), which probably nonspecifically destroyed cellular proteins (13, 27). In another study, mouse sarcoma cells were used and the differences may stem from different telomere biology between human and murine cells (28).

An interesting and potentially important finding of our study is the different behavior of malignant and nonmalignant cells in response to irradiation. Exposure to the same doses of irradiation did not result in any change of TA in human lymphoblastoid cell line (which possesses TA) or in primary culture of normal lymphocytes in which TA is minimal. These results suggest that the up-regulation of TA after irradiation may be specific to cancer cells. Similar results were reported by Finnon et al. (24), albeit in mouse cells, and by Leteurtre et al. in AML-driven cell line (29). In contrast, an up-regulation of TA after irradiation was reported in peripheral blood mononuclear cells after mitogen stimulation and in hematopoietic cell lines (30). Interestingly, the group of Xing et al. found that lymphocytes from normal controls did not activate telomerase after ex vivo irradiation, whereas lymphocytes isolated from patients with bladder and lung cancers exhibited telomerase activation (17). The apparent difference in response to irradiation of normal and malignant cells may reflect different signaling pathways, such as differential activation of...
the PI3K/Akt pathway in response to irradiation between tumor and primary endothelial cells (31). It is of interest that this pathway was shown in our study to be responsible for telomerase activation after irradiation.

The issue of differential response to irradiation between malignant and nonmalignant cells is intriguing in both basic cellular and clinical aspects and needs to be further analyzed.

It is of note that TA increased even at the presence of decreased proliferation of the cells, showing that these two phenomena are not necessarily linked. The decrease in TA at higher doses probably reflects an accumulating damage of radiation to the cells exemplified by the extreme decrease in their viability.

The present study is the first to suggest a detailed mechanism for telomerase activation following irradiation. It has been assumed that the principal mode of telomerase regulation in most biological settings is transcriptional (22). Indeed, the few studies that looked at the mechanism of irradiation-induced telomerase regulation assumed transcriptional modifications (26). In our study, the transcriptional analysis showed that hTERT expression was not changed in response to irradiation. The stability of hTERT transcript was preserved up to dose of 8 Gy, beyond which reduction in the transcript was observed together with decline in proliferation probably due to general damage induced by irradiation. Consistent with these results, two other studies have shown similar results in AML mice model and in HeLa cells (24, 27).

One study reported an increase in hTERT expression after irradiation but at different time points (15) relatively to the increase in TA.

Changes in the alternative splicing of hTERT transcript were reported to cause up-regulation of telomerase in cancer cells (32). hTERT is alternatively transcribed into 6 to 8 splice variants whose relative abundance results in different activity of the enzyme (5). We analyzed four variants including deletions in α and/or β fragments. No differences of hTERT alternative splice variants ratio were detected after irradiation, suggesting that the up-regulation of TA is not transcriptionally mediated.

The other regulatory level of telomerase is related to the enzyme post-translational modifications. Akt phosphorylates telomerase in two phosphorylation sites (21, 33), and we have shown previously the regulatory effect of Akt on TA in another experimental system (9). pAkt after irradiation was increased compared with the total amount of Akt. This is in line with other studies showing up-regulation of Akt phosphorylation post-irradiation albeit at other time points (34, 35). To verify the direct involvement of pAkt in the effect of irradiation on TA, we used a specific Akt inhibitor (124020), which decreased the irradiation-dependent up-regulation of TA. Another pathway operative in telomerase regulation is compartmentalization of the enzyme. To be accessible to the telomere, telomerase needs to be confined to the nucleus (36). Post-translational modifications of the enzyme and members of the 14-3-3 family of proteins regulate the compartmentalization of telomerase (5, 22). Subcellular fractionation analyses revealed that up-regulated TA was translocated from the cytoplasm to the nucleus after irradiation. These results support the proposed post-translational modification as the main regulatory pathway of TA post-irradiation.

Following the identification of the regulatory level of telomerase in present setting, we tried to decipher the upstream modulators of that regulation. The involvement of the key upstream activator of Akt, PI3K, was examined by using wortmannin, PI3K inhibitor. Exposure to wortmannin before irradiation inhibited the up-regulation of telomerase and that of pAkt, suggesting the contribution of PI3K/Akt pathway to the up-regulation of TA after irradiation. We further explored whether PTEN, the tumor suppressor phosphatase with tensin homology, is involved in the signaling pathway related to telomerase activation (37). PTEN can affect TA by dual mechanism. Wild-type PTEN down-regulates the PI3K/Akt pathway by dephosphorylation of PIP3 and its loss results in constitutive activation of this survival pathway. This Akt activation will lead, as explained earlier, to telomerase activation by phosphorylation. In addition, PTEN decreases hTERT mRNA levels and thus down-regulates telomerase transcriptionally, whereas inhibition of PTEN increases hTERT expression (37). In our experimental system, the expression of PTEN did not decrease after irradiation, suggesting that PTEN is not involved in the signaling cascade leading to the up-regulation of TA in this setting. After defining the involvement of PI3K/Akt in the up-regulation of telomerase post-irradiation, we proceeded by analyzing the upstream regulators of PI3K that are instrumental after irradiation. PI3K can be activated by irradiation via several membranal receptors or by Ras activation (23). Exposure of the cells to Ras antagonist FTS decreased the levels of pAkt and markedly down-regulated TA after irradiation. The inhibitory effect of FTS on telomerase after irradiation to lower levels compared with its basal level in nonirradiated cells implied on other possible effects of Ras on TA, which are not necessarily related to irradiation. One report found a different connection between Ras and telomerase, according to which Ras activated the expression of hTERT via the mitogen-activated protein kinase pathway in breast cancer cells (38). This transcriptional process was initiated by activation of membrane epidermal growth factor receptor (39). The canonical pathway by which radiation activates the Ras and/or PI3K/Akt pathways is mediated by activation of membrane receptors (40–42) such as insulin-like growth factor-I receptor and epidermal growth factor receptor (43). Increased influx of intracellular calcium triggered by irradiation is another pathway of possible Ras activation (41, 44). Inhibition of these three pathways did not affect the up-regulation of telomerase after irradiation. These results suggest that the canonical pathways of radiation-induced intracellular signaling are not operational in the up-regulation of telomerase and Ras activation is a result of another yet undefined upstream pathway.

Our study points to Ras oncprotein as the first mediator of the up-regulation of TA in response to irradiation. Although this pathway is known to be activated by irradiation, the canonical signaling mediated by membrane receptors was not operational in our setting. Probably, DNA damage induced by irradiation promoted Ras activation. The exact pathway leading to Ras activation by irradiation and thus up-regulating TA is not clear yet and needs to be explored in future studies. Several articles provide data that indirectly support the concept of irradiation-DNA damage and telomerase activation axis. Klapper et al. showed an up-regulation of TA after exposure to etoposide, a DNA-damaging drug (45). The mechanism of this phenomenon was not elucidated, but a transcriptional regulation manner was suggested. Another possible pathway connecting DNA damage and telomerase activation has been
suggested by Cataldi et al. (46). They showed that nuclear factor-κB activation after irradiation promoted the expression of the hTERT gene, unlike our results.

Although irradiation has been appreciated as a cellular death-inducing agent, this therapeutic modality has the potential to enhance proliferation, at least in the surviving fraction of cells, thus promoting long-term resistance to multiple cytotoxic stresses. DNA damage induced by irradiation (e.g., DNA double-strand breaks) may be repaired by cellular mechanisms such as nonhomologous end joining (47). The proper function of these DNA repair mechanisms affects cell sensitivity to irradiation. The results of our study are in concert with this general approach. Up-regulation of TA may represent a physiologic protective mechanism against DNA damage, contributing to DNA repair by chromosomal healing (48) and enhanced survival. This hypothesis is supported by studies such as of Akiyama et al., showing that overexpression of telomerase protects from apoptosis triggered by double-stranded DNA damage (49).

The results of our study add to increasingly accumulating information pointing to post-transcriptional mechanism as an important modality in regulation of telomerase. Down-regulation or inactivation of telomerase in cancer cells is an important goal of anticancer therapy. Irradiation-induced up-regulation of telomerase may lead to increase in telomere length or resistance to therapeutic modalities in malignant cells. This reasoning is supported by the report of Neuhold et al. showing elongation of telomeres after irradiation in human lymphoblasts (50). If the results of our study will be verified in clinical conditions, the logical approach will consist of incorporation of continuous inhibition of telomerase during radiation therapy. Knowledge of cellular pathways implicated in radiation-induced telomerase up-regulation is essential in the development of interventions aimed at prevention of this phenomenon. Akt has been already recognized as an attractive target for anticancer treatment. Inhibition of Rap1/P13K/Akt pathway was shown to increase the radiosensitivity of various carcinoma cell lines. If inhibition of these signaling pathways will prevent radiation-induced telomerase activation, it may have important clinical implications.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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