Radiation-Induced Progressive Decreasing in the Expression of Reverse Transcriptase Gene of hEST2 and Telomerase Activity

Hanneng Zhu1, Sidong Xiong * and Wenyi Chen
Shanghai Institute of Radiation Medicine
Department of Immunology*
Shanghai Medical University
No. 2094, Xie Tu Road,
Shanghai, P. R. China 200032

Telomerase is a ribonucleoprotein complex that adds heximeric repeats called telomeres to the growing ends of chromosomal DNA. Telomerase activity is present in a vast majority of tumors but is repressed in most normal tissues. Human telomerase catalytic subunit gene (hEST2) reverse transcriptase (RT) segment was cloned by PCR according to the sequence published in GeneBank. PCR was used to investigate the expression of the hEST2 RT segment in diverse tumors as well as in various normal tissues. Results indicated that hEST2 RT segment was detectable in tumor cells lines but not in normal cells and tissues.

In order to identify the relationship between telomerase and the biological effect of radiation injury, HeLa cells, KB cells and A431 cells were employed to measure the change in telomerase activity after 60Co-ray irradiation at RNA level and protein level. Quantitative PCR determined that expression of hEST2 RT segment that encodes seven motifs of the human telomerasodynamics decreased with increasing dosage of radiation. In addition, a PCR-based telomeric repeat amplification protocol was used to assay telomerase activity after exposure to radiation. The results strongly support the experiments we had made: Telomerase activity decreases with increasing dosage of radiation. We conclude that detection of the hEST2 RT segment by Northern blotting is a new method for detecting telomerase activity. Furthermore, radiation can cause a dose-dependent decrease in telomerase activity. The effect of radiation on telomerase is one possible reason for the death of cancer cells after irradiation.

KEY WORDS: telomerase hEST2 reverse transcriptase segment irradiation

INTRODUCTION

Cellular radiation has long been a research focus in the field of radiation biology and oncology because it relates directly to the consequences of radiotherapy10. The factor most generally believed to influence cellular function after ionizing radiation is DNA repair capacity, though it has also been reported that cellular damage can differ after exposure to various dosages12. It has been identified that DNA damage and loss of proliferative capacity are the common form of radiation-induced cell death in many kinds of cells13. The accumulation of various cytogenetic charges and DNA damage, due to either exposure to DNA-damaging agents or to reduction and errors in DNA synthesis, has been correlated with DNA repair capacity decreasing14. Among the different types of radiation-associated genetic instabilities described to date15, telomeric shortening represents a structural change in chromosomes15. The telomere, which caps the chromosome ends and protects them from degradation and end-to-end fusion, is the sensitivity target of radiation damage. Shortened telomeres in transformed cells16 suggest that decreases in telomeric length may contribute to genomic instability and cellular transformation.

The DNA component of telomeres consists of tandem repeats of guanine-rich sequences that are essential to telomere function. These repeats are replicated by conventional DNA polymerases and by a specialized enzyme, telomerase, which is essential to complete replication of telomeric DNA. Telomerase surmounts the problem of telomere incomplete end replication17. In human somatic cells, telomerase activity cannot be detected and reappear in immortalized cell lines and in about 85% of human tumors18. Most recently, three human telomerase catalytic subunit genes have been identified that are associated with telomerase activity- hEST212, hTERT and hTRT13. Comparison of protein gene structure features in telomerase catalytic subunits with shows that, there are seven sequence motifs conserved in RT, with the RNA template. They consist of a core enzyme complex and play an important role in telomerase activity, but the contributions that domains outside of the RT motifs make to telomerase function have yet to be defined in detail.

In this report, we try to understand the critical role telomerase plays in cellular damage after exposure to γ-irradiation. We examined the change in expression of the RT domain. Our results indicate that the expression of telomerase activity and the RT gene of hEST2 decrease after radiation; telomerase is perhaps the important component of DNA damage;and the detection of the hEST2 RT segment by RT-PCR is a new way for detecting...
telomerase activity.

**MATERIALS AND METHODS**

**Cell Culture and Irradiation**

All the cell lines (HeLa, KB and A431) were grown in an alpha minimal essential medium (MEM), supplemented with 10% fetal calf serum and antibiotics at 37°C in an atmosphere containing 5% CO₂. Stock cultures were maintained with a routine subculture at about 80% confluence after seeding at an initial density of 1-4x10⁵ per 25 cm² flask or 1-4x10⁶ cells per 80 cm² flask. For all the studies reported here, the cells were used for experiments when growing exponentially. For Northern Blotting, TRAP (telomeric repeat amplification protocol) and RT-PCR assay, cells were irradiated in suspension; the irradiation was performed with a cobalt-60 irradiator at dosages of 2, 4 and 8 Gy/min.

**Telomerase Assay**

Subconfluent cultures were used to prepare the detergent CHAPS (14). Telomerase enzyme activity was measured using a PCR-based TRAP kit from Gene Company. Each reaction product was amplified with the presence of an internal TRAP assay standard (36 bp). The TRAP reaction products were separated by serial dilution of the protein extracts. Each set of TRAP assay included control reaction tubes without any extract, and with extracts treated with RNase A (200 μg). To quantitate the levels of telomerase activity, the average densitometric optical density of the first six TRAP bands after a primer band was presented as a ratio to the internal TRAP assay standard band.

**Cloning**

Total cellular RNA was isolated from HeLa cells by the method of guanidine isothiocyanate extraction. RT of RNA with degenerate oligonucleotides was done using AMV RT (GibCO). Reactions were performed at 50°C according to the protocol. The precipitated primers of PCR were designed according to the cDNA sequence of hEST2. GeneBank accession number AF 018167. (1677-3002bp,1326bp). The Primers were (forward-5’CGGAATTCCTGGCCAAGTTCCTGCAC-3’) and (reverse-5’-CAGAACGCCGACTTCACACTTAAGGC-3’).

PCR reaction contained 10 pmol of each of the two primers, 100 μM of each of the four dNTPs, 1x Taq DNA polymerase reaction buffer and one unit Taq DNA polymerase. Thermal cycling was carried out with paraffin overly as follows: 35 cycles of 94°C for 45 seconds, annealing temperature for 45 seconds, and 72°C for 90 seconds with an initial denaturation step of 2 min for 94°C, then 72°C for 10 min. The yield a 1.3 kb fragment in the RT domain of telomerase hEST2. DNA fragments were subcloned into PCDNA3 vector, and nucleotide sequences were determined by the dideoynucleotide chain-termination method using an annotated sequencing system. A 1.3-kb DNA sequence was isolated and cloned into E. coli DH5α.

**Quantitative RT-PCR Analysis**

A quantitative analysis of the 1.3-kb gene in HeLa cell lines was performed by using a noncompetitive RT-PCR method in the presence of two pairs of primers for telomerase RT fragment and GAPDH. Equal amounts of RNA were transcripted into cDNA; the cDNA was amplified by PCR in two separate reactions with telomerase RT fragment and GAPDH primers. GAPDH primers were Sense: 5’AGATCCACAACGGATACATT3’ and antisense: 5’TCCCTCAAGATGTGCAAGCCA-3’. The processing steps of the PCR cycle were described as above. To standardize the assay conditions, we performed a time course analysis of the generation of the GAPDH PCR product in the presence of an amount of cDNA corresponding to 1 ng of RNA. This experiment indicated that the PCR product remained undetectable until cycle 25. When the reaction became detectable and exponential until it reached a plateau of cycle 35. For GAPDH, the analysis of the PCR products was performed at cycle 31; and for the telomerase RT fragment, the analysis of PCR products was performed at cycle 35. The amount of fluorescence present in each PCR product was determined by laser spectrometry.

**Northern Blot Analysis**

Cytoplasm RNA was prepared from subconfluent cultures of cells lysed in 0.5% NP40 and centrifuged at 2000 x g for 10 min to eliminate nuclei. Samples of cytoplasmic RNA (20 μg) were electrophoresed on a 1% formaldehyde agarose gel and blotted onto nylon membranes. The 1.3-kb fragment of telomerase RT and GAPDH cDNA were gel purified and uniformly labeled with [α-32P] dATP using the Random Primed DNA Labeling Kit (Boehringer Mannheim) according to the manufacturer’s instructions. Blots were sequentially hybridized at 65°C for 18 hour in the presence of 32P-labeled cDNA probe. Hybridization blots were
sequentially washed in SSC at 60 °C before being autoradiographed at -80 °C.
RESULTS

Fig. 1  Enthidium bromide-stained agarose gel of telomerase reverse transcriptase fragment
From left to right, Lane 1: size markers, numbers on the left indicate in bp. Lane 2: product of 1.3 kb telomerase reverse transcriptase fragment amplified by PCR from total RNA. Lane 3: the product of 1.3 kb telomerase reverse transcriptase fragment by PCR which use extracted 1.3 kb fragment as template.
FIG. 2. Shows an ethidium bromide-stained gel. The expression of 1.3-kb telomerase RT fragment from HeLa, KB and K431 cell lines decreased after exposure to the increasing dosage of radiation. Panels A, B and C show the 1.3-kb fragment amplified from HeLa, KB and K431 cell lines of RNA. Panels A, B: Lane 1 is control; lanes 2, 4, and 5 show the different fluorescence density of 1.3-kb bands detected by PCR after exposed to 2, 4 and 8 Gy irradiation. Lane 3 is the size marker (Lambda DNA/EcoR I +Hind III Marker). Panel C: Lane 1 is the size marker (Lambda DNA/EcoR I +HindIII Marker), Lane 2 is control; Lanes 3, 4 and 5 show the PCR products after 2, 4, and 8 Gy irradiation. Below the pictures are GAPDH control.
FIG. 3. Northern blot analysis of 1.3 kb telomerase RT fragment expression in telomerase–positive HeLa, KB and K541 cell lines (lines 2, 1 and 6); but no expression in the telomerase–negative tissues: rat heart, lung and kidney (lines 3, 4 and 5).
FIG. 4. Telomerase activity analysis of HeLa, KB and K431 cell lines (lines 3, 5 and 7); and rat heart, lung and kidney (lines 2, 4 and 6). The negative control (line 1) was heated at 85°C. Line 8 is positive control. Results indicated that HeLa, KB and K431 are telomerase positive but the normal rat tissues are telomerase negative.
FIG. 5. Panels A, B and C represent the telomerase activity analysis of HeLa, KB and K431 cell lines after exposure to radiation of 2, 4 and 8 Gy. The results show that the telomerase activity decreased with the increasing dosage of radiation. lines 1, 10 and 13 means cells exposure to 8 Gy; lines 2, 9 and 14 means cells exposure to 4 Gy; lines 3, 8 and 15 means exposure to 2 Gy. lines 4, 7 and 12 means negative control; lines 5, 6 and 11 means positive control.

Telomerase Test After Irradiation

We initially examined the expression of 1.3-kb telomerase RT fragment using the quantitative RT-PCR analysis in HeLa, KB and K431 cell lines after exposure to radiation of 2, 4 and 8 Gy. The data (Figs. 2, 5) revealed that the expression of telomerase RT domain decreased with increasing dosage of radiation. Consequently, we examined telomerase activity by the TRAP method in HeLa, KB and K431 cell lines after exposure to radiation. The results were similar to those of PCR, definite dose-dependent telomerase activity decreasing by the effect of radiation.

The Telomerase RT Fragment

Telomerase RT fragment has been cloned (fig.1), the similar results of telomerase activity analysis (Fig. 4) and Northern blot assay (Fig. 3) of telomerase RT fragment indicated the important role the RT domain plays in the structure and function of telomerase. The domain has been identified as a specialized area, different from sequences of most somatic cells or tissues.
DISCUSSION

Telomerase has attracted considerable interest as a possible target of therapeutic intervention for cancer. Its apparent lack of measurable activity in many (but not all) normal tissues suggests that telomerase inhibitors might have a place in cancer therapy. However, despite a strong causal link between telomerase activity and cancer, a definitive proof that inhibition of telomerase activity by radiation effectively inhibits tumor growth has yet to be established.

In human solid tumors, chromosomal instability and jumping rearrangements are generally observed in premalignant or low-grade malignancies. Chromosomal instability induced by telomere shortening may be a major step between a normal cell and a cell with aneuploidy as is observed in many cancer cells. A large body of evidence suggests a causal relationship between chromosome aberrations that originate from specific telomeric attritions and tumor development. This raises the question of the relevance of telomerase activity and chromosome instability, and the role telomerase plays in tumor cell survival and immortalization with genomic abnormality.

Radiation damage is a main cause of chromosomal instability; severe radiation damage induces chromosome aberration that results in cell death. Various similarities between DNA damage-induced checkpoint arrests and senescence have been reported. Senescence is a permanent arrest, where as most radiation-chromosome aberration that results in cell death. Various similarities between DNA damage-induced checkpoint genomic abnormality.

We think telomerase plays an important role in this kind of repair. It is an enzyme complex capable of maintaining telomeres at a constant length so that the cells do not senesce but are capable of unlimited division. RNA template and telomerase RT enables it to extend or to hold constant the length of telomere over multiple cell division, which has been demonstrated to go hand-in-hand with unlimited growth potential and the development of malignancy.

Our experiment identified that telomerase activity decreased with increasing dosage of radiation. Several reasons can be used to explain this phenomenon. First, radiation activation of p53, and p53 with Rb are the checkpoints of the cell immortalization process. The activation of p53 caused programmed cell death and cycle arrest, which decreased the telomerase activity. Second, the existence of telomerase activity may be the reason that many DSB cells eventually re-entered the cell cycle, due to a “healing” result from the addition of a telomere onto the broken end of a chromosome.

Catalytic protein subunits of telomerase contain RT motifs. Using the S. cerevisiae telomerase, Est2p and from the euploid p123 protein, Meyerson identified a human homolog, hEST2. Like the yeast and ciliate telomerase protein, it is a member of the RT family of enzymes. Seven conserved sequence motifs, which define the polymerase domains of these enzymes, are shared among the otherwise highly divergent RT family. Within the RT motifs are several amino acids that are invariant in the telomerase but divergent between telomerase and nontelomerase RTs. In the sequence motifs conserved in RTs, telomerase-specific motif T and motifs 1, 2, and A through E, telomerase activity was more strongly correlated with the RT motifs. In our experiments, we cloned the RT fragment including the seven motifs and telomerase specific motif T. The RT-PCR and Northern blotting data indicated that it expressed in diverse tumor cell lines but not in normal cells and tissues. Quantitative PCR determined the expression of hEST2 RT segment, which encodes motifs of human telomerase, decreased with increasing dosage of radiation. The results described above correlated with that made by TRAP assay, which suggest that the seven motifs and motif T appear to play a pivotal role in telomerase activity. It seems reasonable to propose that testing the expression of motifs domain is similar to the measurement of telomerase activity, and mutating the RT fragment is perhaps the way to break tumor cell proliferation. Understanding this process and its regulating factors will be critical for those who want to apply such findings to research on cancer and aging.
REFERENCES