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Suppression of Alternative Telomere Lengthening in Cancer Cells with Reverse Transcriptase Inhibitors

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Abstract—Telomerase is a ribonucleoprotein enzyme that elongates telomeres and therefore maintains chromosomal stability in germ lines, as well as in the majority of cancer cells during cell doubling. However, up to 30% of human tumors of different types do not express telomerase but instead use an alternative lengthening of telomeres (ALT). Here we show that human tumor-derived ALT cell lines express a *LINE*-1 (*L*1) retrotransposon. This indicates its participation in telomere maintenance, possibly, by a slippage mechanism during telomeric DNA synthesis. Moreover, the suppression of *L*1-encoded reverse transcriptase activity by antisense strategy or treatment of ALT cells with reverse transcriptase inhibitor 3'-azido-2',3'-dideoxythymidine (AZT) induces progressive telomere shortening, arrest in G2 phase of the cell cycle, and, eventually, cancer cell death. This finding suggests a unique opportunity to cure cancer in a number of cases.

Keywords: telomeres, telomerase, alternative lengthening of telomeres, reverse transcriptase inhibitors
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INTRODUCTION

An asymmetry in the synthesis of leading and lagging DNA strands leads to the end-replication problem of linear genomes [1]. To overcome this problem, eukaryotic chromosomes have specialized end structures, telomeres, consisting of TTAGGG repeats [2]. A gradual loss of DNA from the ends of telomeres during cell doubling was associated with control of the proliferative potential of somatic cells [14]. In contrast, generative lines [29] and the majority of cancer cells [17] express a special enzyme (telomerase) that elongates telomeres, thereby maintaining chromosome stability [10]. Telomerase inhibition limits the growth of human cancer cells [13]. However, not all cancer cells express telomerase. It was shown that telomerase negative cells have very long and heterogeneous telomeres along with immortalization, presumably, due to one or more mechanisms of alternative lengthening of telomeres (ALT) [24]. The presence of ALT was reported in 30% of human tumors of different types, tumor-derived cell lines and human cell lines immortalized in vitro [4–6, 24], and up to 50% in some tumor subtypes [12]. The nature of the ALT mechanism is still unclear, but the possible involvement of homologous recombination between telomeres was reported [9]. At the same time, different DNA polymerases, including HIV reverse transcriptase, are able to elongate telomere DNA sequences in vitro by slippage DNA synthesis [21], which leads to the generation of products exceeding template DNA by size. Since a protein encoded by the open reading frame-2 (ORF2) of human *L*1 retrotransposon is a true reverse transcriptase (*L*1-RT) [7, 8] and expression of the *L*1 retrotransposon and corresponding activity of the reverse transcriptase in some human tumors was reported [3, 19, 28], it can be assumed that, after completion of retrotransposition of its own RNA, *L*1-RT [16] can perform slippage during telomeric DNA synthesis and provide for maintenance of extremely long telomeres in ALT cells.

MATERIALS AND METHODS

Cell lines. All cell lines used in the study were obtained from American Type Culture Collection (Rockville, MD). Osteosarcomas (Saos-2 and U-2 OS) and cell lines of liver tumor (HEC-1) and cervical cancer (HeLa) were among the sources of the cells. The cells were cultured according to ATCC recommendations. Cultural media were supplemented with 0.2 μM 3'-azido-2',3'-dideoxythymidine (AZT, Sigma) for cell treatment [27].

Dot blotting. Total cellular RNA was isolated with the use of a RNA-STA 60 solution (Tel-Test, Inc.). The reaction was carried out with 30 μg of total RNA and HRP North2South (Pierce) labeled pBS-$L1_{RP}$-EGFP plasmid [22] as a specific probe according to the manufacturers protocol.

Bromodeoxyuridine (BrdU) incorporation. Cell staining for BdU incorporation was performed on cells

incubated with 10 mM BrdU (Sigma) for 2.5 h, stained with BU-33 monoclonal anti-BrdU antibodies (Sigma) and FITC-labeled goat anti-mouse IgG (H + L) (Fab') fragments (Molecular Probes), contrastained with 50 μg/mL propidium iodide (PI, Sigma), and analyzed by flow cytofluorimetry as described [26].

Telomere length measurement by flow cytometry. The cells were stained with telomere-specific FITC conjugated $(C_3TA_2)_3$ PNA (Applied Biosystems) probe and contrastained with 0.06 μg/mL PI as described [20].

Inhibition of *L*1 reverse transcriptase by antisense strategy. To obtain *L*1-specific reverse transcriptase targeted antisense construct, PCR was constructed with RT-F (5'-ATG ACA GGA TCA ACT TCA CAC-3'), RT-R (5'-TCC TGC TTT CTC TTG TAG GCA-3') primers and pBS-$L1_{RP}$-EGFP plasmid as a template. PCR product (929 bp) was cloned into pTargetT vector (Promega). Recombinant constructs containing the insert in sense and antisense orientation were purified with a Plasmid Midi Kit (Qaigen), digested with Xmn I (Promega), and transfected into U-2 OS cells with a Lipofectamine (Gibco) according to the manufacturers instructions. After 40 days of selection on media containing 0.5 mg/mL of G418 (Gibco), the cells were harvested, stained with PNA and PI, and analyzed by flow cytometry [25].

RESULTS AND DISCUSSION

To detect *L*1-specific RNA in two cell lines (osteosarcomas U-2 OS and Saos-2) that were reported [24] to maintain telomeres by an ALT mechanism, total mRNA was analyzed by dot blotting and a *L*1-retrotransposon specific probe. Telomerase-positive cell lines (HEC-1 and HeLa) were used for comparison [17]. Both ALT cell lines gave a positive reaction in this test. As expected, HEC-1 cells were completely negative. Analysis of HeLa cells showed only traces of *L*1 transcripts, as previously reported [19].

Further, to test the hypothesis, ALT cell lines were treated with therapeutic concentrations of AZT in order to determine if slippage telomeric DNA synthesis could be inhibited by AZT-TP, followed by induced telomere shortening. Telomere length in AZT-treated and untreated cell lines was measured by flow cytometry with a telomere-specific probe in the form of peptide nucleic acid (PNA) [15, 25]. The cells were stained with PI to determine the cell-cycle distribution [25]. Both ALT cell lines demonstrated telomere shortening (on average by 50%), massive apoptosis, and G2 arrest 14 days after treatment with AZT. To confirm the specificity of AZT-induced telomere shortening for ALT cells, the HeLa cell line, which is known to be telomerase-positive, was treated with AZT under the same conditions. AZT at the chosen concentration had no effect on telomere length or cell-cycle distribution in HeLa cells.

To demonstrate telomere shortening and changes in DNA synthesis intensity in dynamics, U-2 OS cells were treated with AZT for different time intervals and simultaneously analyzed by flow cytometry. The rate of DNA synthesis was determined by BrdU incorporation. The results showed progressive telomere shortening (on average up to 50%) and decreased DNA synthesis (on average by 40%). It is important to note that changes in the cell-cycle distribution, DNA synthesis, and telomere length were rapid and could be detected 10 days after AZT treatment.

At the same time PI staining demonstrated a higher DNA content in AZT treated cells (on average by 25%) at the latest stages of the treatment (21 and 40 days) as compared with untreated cells. A rational explanation of this fact is that short telomeres cause fusion of chromosomes end-to-end.

To confirm the fact that ALT is conducted only by *L*1 reverse transcriptase, U-2 OS cells were transfected with expressing vectors containing part of human *L*1-ORF2 in sense and antisense orientation. The cells were harvested and analyzed by flow cytometry after 40 days of selection with G418. The cells carrying the antisense construct, as expected, demonstrated massive apoptosis, G2 arrest, and telomere shortening. In contrast, the cells expressing the sense construct showed no difference in telomere length or cell cycle.

CONCLUSIONS

These data are in good agreement with other reported cases of ALT mechanism suppression [23] or telomerase inhibition [11]. Induction of apoptosis in ALT cells treated with AZT seems to be p53 independent, since U-2 OS and Saos-2 represent p53+/+ and p53−/− cancer cell lines [8]. Since tumors with suppressed elongation of telomeres lose proliferative potential [14] and AZT is already in clinical use, these findings provide a unique opportunity to treat up to 30% of cancer cases. Some other nucleoside reverse transcriptase inhibitors (e.g. 2',3'-dideoxyinosine (ddI) or 2',3'-didehydro-3'-deoxythymidine (d4T)) already in clinical practice could be also used.

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