Antisense Sp1 oligodeoxynucleotide decreases telomerase activity by inhibiting hTERT mRNA expression in Jurkat T cells

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ABSTRACT

AIM: To study the effects of transcriptional factor Sp1 antisense oligodeoxynucleotide (ODN) on telomerase activity and human telomerase reverse transcriptase (hTERT) expression. METHODS: Antisense oligodeoxynucleotide (ODN) was designed to inhibit Sp1 expression and transferred to Jurkat T cells by lipofectamin. Telomerase PCR-ELISA was used to detect telomerase activity. RT-PCR analysis was used to assess the mRNA expression of Sp1 and hTERT, and Western blot was used to analyze the levels of Sp1 protein. RESULTS: Treatment of Jurkat T cells with Sp1 antisense ODN (1 µmol/L) dramatically reduced Sp1 mRNA and protein levels. The inhibition rate was 44.8 % (P<0.05) and 57 % (P<0.01), respectively. Following the transcriptional factor Sp1 functionally altering, hTERT mRNA expression were suppressed with a 43.7 % inhibition rate (P<0.01). A dose-dependent inhibition of telomerase activity by antisense Sp1 ODN was also discovered. From 0.25 to 2.0 µmol/L, telomerase activity was reduced from 27.1 % to 64.6 %. CONCLUSION: Antisense Sp1 ODN decreases telomerase activity by inhibiting hTERT mRNA expression in Jurkat T cells.

INTRODUCTION

Telomeres, the ends of eukaryotic chromosomes, are composed of repeat sequences (TTAGGG in human). They become progressive shortening with each replication cycle due to the inability of DNA polymerase to fully replicate extreme 5' terminus of the lagging strand in somatic cells. When they reach a critical point, the cellular senescence is triggered. But in immortal cells and tumor cells, the telomeres are maintained by acti-
mented reduction in telomere length with continued treatment, and whether the mechanism of action was specific to telomerase and telomere erosion would have been expected[3]. Unlike hTR expressed in both telomerase-positive and telomerase-negative tissues, hTERT subunit is only expressed in immortal, telomerase-positive cell lines[4,5]. hTERT has been generally acknowledged as a rate-limiting determinant of the telomerase[6], and hTERT would be a specific target to inhibit telomerase activity. However, little is known about hTERT inhibitors.

Transcriptional regulation of the hTERT gene plays a key role in the activation of telomerase[7]. Inhibiting hTERT expression would be a useful step in killing tumor cells. Recently, the promoter region of the hTERT gene was cloned, and the proximal 181 bp region of the promoter which contains E-boxes and GC-boxes specifically binding with Myc and Sp1 respectively, was identified essential for hTERT transactivation[7]. Using hTERT promoter reporter plasmids, Satoru and his colleagues[8] verified that Sp1 cooperated with c-Myc (cellular oncogene protein of myelocytoma) to activate transcription of hTERT gene, but when Sp1 sites were mutated, the effects of Myc on transactivation were marginal. Recent studies have demonstrated that many oncogene-correlated proteins which mediate upregulation or downregulation of hTERT expression, such as E6[9], p53[10,11], and telomerase activators, such as tricostatin[12] (a histone deacetylase inhibitor), affect telomerase activity and hTERT expression through Sp1 but not c-Myc. These indicate that Sp1 plays a central role in hTERT transcription and may be a key target for scanning telomerase inhibitors. In the present study, using highly specific antisense oligodeoxynucleotides that target the mRNA encoding Sp1, we attempted to observe the direct effect of Sp1 on telomerase activity and hTERT expression in cell level.

**MATERIALS AND METHODS**

**Cells and reagents** The Jurkat T cell line was obtained from American Type Culture Collection and maintained in a humidified atmosphere of 5% CO₂ in 95% air at 37°C in RPMI-1640 containing 10% fetal bovine serum. Telomerase PCR-ELISA kit, One Tube RT-PCR kit and DNase-free RNase was purchased from Roche; Lipofectamin, TRizol, and RPMI-1640 medium were obtained from Gibco; Mouse monoclonal antibody of Sp1 was from Santa Cruz Biotechnology, Inc; Goat anti-mouse immunoglobulins coupled to horseradish-peroxidase (HRP) were purchased from Sino-American Biotechnology Co.

**ODN synthesis and treatment of cells** The sequences of ODN used are as follows: Sp1 antisense, 5'-CACCACAGCTGTCAATTTCATCCATGG-3'; Sp1 sense, 5'-CCATGGATGAAATGACAGCTGTGGTG-3' (11-36 of human Sp1 mRNA sequences). Sense ODN was used as control. All oligonucleotides modified with phosphorothioate were commercially synthesized by Sagon Ltd, Canada. Various concentrations of ODN in the presence of lipofectamine were added to cell suspensions in six-well (35 mm) tissue culture plates at a density of 2-3x10⁵ cells·L⁻¹, and incubated at room temperature for 30 min with serum-free RPMI-1640 medium prior to addition of complete growth medium (with 10% serum) and cells were incubated at 37°C in a CO₂ incubator for a total of 36 h.

**Preparation of cell extracts and telomeric repeat amplification protocol (TRAP)** Cell extracts were prepared according to Telomerase PCR-ELISA kit product profile. Briefly, cells were collected and washed at 3000xg for 10 min at 4°C with PBS and resuspended in pre-cooled lysis reagent at a concentration of 2.5x10⁶ cells/L. After 30 min incubation on ice, the lysate were centrifuged at 16 000xg for 20 min at 4°C. The supernatant was stored at -80°C after shock freezing in aliquots in liquid nitrogen for the future using unless otherwise the TRAP reaction was immediately performed[13] as described below. Cell extract 3 µL was added to a mixture (final volume of 50 µL) containing dNTP, biotin-labeled TS primer, Taq DNA Polymerase and CX primer. After 30 min primer elongation at 25°C and 5 min telomerase inactivation at 94°C, PCR amplification was cycled 30 times: 94°C for 30 s, 50°C for 30 s, 72°C for 90 s. The PCR amplification was quantified by ELISA assay for the PCR products following the manufacturer’s instruction. Briefly, 5 µL amplification product which had been denatured at room temperature for 10 min with 20 µL denaturation reagent was hybridized with a digoxigenin-labeled probe specific for human telomeric repeats. The probe bound to the strand with the labeled biotin at the 5' end. The hybrid was immobilized to a streptavidin-coated microtiter plate via the biotin-labeled primer at 37°C on a shaker for 2 h, and washed 3 times. The reaction product was detected with 100 µL anti-digoxigenin-peroxidase and 100 µL peroxidase substrate TMB.
Color intensities were measured with a model 450 microplate reader (BIORAD) at 450 nm.

**RNA isolation and RT-PCR** Total RNA was extracted from the antisense ODN, sense ODN or medi- treated cells using TRIzol and RT-PCR was performed with the Titan One Tube RT-PCR kit for detecting Sp1 mRNA and hTERT mRNA expression. The primers (synthesized by Shenggong Bioengineering Co Ltd, Shanghai, China) were (LT5) 5'-CGGAAGAGTGTCTGGACCA-3' and (LT6) 5'-GGATGAAGCGAGGAGTCTGGA-3' for hTERT[5] which generated a PCR fragment of 126 bp, 5'-ACAGGTGAVTTGACCTCAC-3' and 5'-GTTGGTTGCACTGTGATG-3' for Sp1[14] (370 bp), 5'-TCTCTGACTTCAACAGCGACCC-3' and 5'-TCTCTTCTCTTTGTGCTTTGC-3' for hGAPDH (208 bp), 5'-CCAAGCCACCGAGGAGCTGAC-3' and 5'-AGGTACATGTGGTGTCGCGCAGAC-3' for hGAPDH (208 bp). PCR consisted of one cycle at 50 ºC for 30 min, 94 ºC for 2 min, and subsequently 30 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 68 ºC for 45 s by using a thermal cycler (TC-100, MJ Research). Amplification products were subjected to electrophoresis through 2 % agarose gels stained with ethidium bromide and were quantified by Gel Base/Gel Blot/Gel Excel/Gel Sequence analysis software (UVP).

**Western blot analysis** Whole cell extract from control cells or those transfected with Sp1 ODN were harvested and lysed in TRIzol reagent, and proteins were isolated according to the manufacturer ’s instruction. The protein samples were equally mixed with 2×loading buffer (Tris-HCl 100 mmol/L, pH6.8, dithiothreitol 200 mmol/L, 4 % SDS, 20 % glycerol, and 0.2 % bromophenol blue), boiled for 3 min. The denatured proteins were fractionated on 7.5 % SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using a Bio-Rad mini transfer apparatus. The membrane was blocked with 1 % skim milk powder in 0.02 mol/L PBST (PBS plus 0.005 % Tween 20) at room temperature for 1-2 h, and incubated with mouse anti-human Sp1 monoclonal antibody 1 mg/L in PBST plus 1 % skim milk powder. Following two washes in PBST, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (1:200) for 20 min at 37 ºC. The immune complexes were visualized by the ECL chemiluminescence method (Amerham Corp) according to the manufacturer ’s instruction and protein bands were quantified by BIO-PROFIL/BIO-CAPT/BIO-ID++ image analysis software (Vilber lourmat).

**Statistical analysis** Data were expressed as mean ±SD and statistically compared by one-way ANOVA with Dunnett’s test, $P<0.05$ was taken as statistically significant.

**RESULTS**

**Inhibition of Sp1 expression by antisense oligonucleotide** Antisense Sp1 in this research was a phosphorothioate antisense ODN designed to hybridize with 3'-translated sequences contained within human Sp1 mRNA. To determine whether this antisense ODN inhibited target gene expression in leukemia cells, Jurkat T cells were treated with oligonucleotide 1 µmol/L, Sp1 mRNA and protein expression was examined by RT-PCR and Western blot analysis. Treatment of Jurkat T with the Sp1 antisense inhibitor resulted in dramatically reducing Sp1 mRNA levels (Fig 1A). Semi-quantified results showed the inhibit rate was 44.8 % ($P<0.05$, Fig 1B). As a control, the sense ODN did not exhibit any effect on Sp1 mRNA.
Western blot analysis confirmed that reduction of Sp1 protein levels also occurred following ODN treatment (Fig 2A). A 57% reduction in the level of Sp1 protein in cells containing Sp1 antisense but not sense oligonucleotides compared with nontransfected cells was detectable 36 h after treatment ($P<0.01$, Fig 2B).

Repression of telomerase activity to antisense Sp1 ODN

To evaluate the role of transcriptional factor Sp1 in the regulation of telomerase activity in Jurkat T cells, the effect of Sp1 antisense ODN treatment on the telomerase activity was measured by telomerase PCR-ELISA assay. To confirm the specificity of the telomerase signals and ladders, the cell extracts were pretreated with RNase as a control. The telomerase activity was significantly repressed after treatment with 0.25-2.0 µmol/L of Sp1 antisense ODN. A concentration-dependent repression of the telomerase activity was also observed in cells treated with antisense compound. Reduction of 27.1% in telomerase activity was detectable after treatment with ODN 0.25 µmol/L, and when the concentration of ODN rose to 2.0 µmol/L, 64.6% inhibition was achieved. Sense oligonucleotides exhibited little or no effect on telomerase activity (Fig 3). These results suggested that reduction of Sp1 protein levels blocked the induction of telomerase activity in Jurkat T cells.

Effect of inhibiting Sp1 gene expression on the induction of hTERT mRNA

To determine whether transcriptional factor Sp1 affected telomerase activity by regulating hTERT expression, hTERT mRNA was measured by RT-PCR after treatment of Jurkat T cells with Sp1 antisense ODN 1 µmol/L. Results showed that in the presence of antisense Sp1, hTERT mRNA decreased by 43.7% relative to control cells ($P<0.01$, Fig 4A, 4B). In contrast, the sense ODN that targets the same sequence in Sp1 mRNA had no effect on hTERT mRNA levels. These findings showed that inhibition of Sp1 antisense ODN on telomerase activity in Jurkat T cells was achieved at least in part through attenuating the hTERT mRNA levels.

DISCUSSION

The data presented here showed that Sp1 antisense ODN inhibited the telomerase activity and hTERT mRNA expression in Jurkat T cells. At 36 h after ODN 0.25 µmol/L treatment, telomerase activity was decreased by 27.1% when the concentration of the ODN rose to 2.0 µmol/L, 64.6% reduction was achieved. From 0.25 to 2.0 µmol/L, the repression of the telomerase
activity showed a dose-dependent manner. These indicate that transcriptional factor Sp1 precisely regulates telomerase activity. To identify whether transcriptional factor Sp1 affected telomerase activity by regulating hTERT expression, we measured the hTERT mRNA level in Jurkat T cells. Results showed that in the presence of antisense Sp1, hTERT mRNA level decreased dramatically. These suggest that Sp1 regulates telomerase activity in Jurkat T cells by modifying at least in part hTERT expression although another subunit comprising the human telomerase complex, human telomerase RNA component (hTR), is also regulated by Sp1. Our data also demonstrated that inhibiting hTERT transcription could decrease telomerase activity in Jurkat T cells.

Sp1 is initially thought to regulate ubiquitously expressed housekeeping genes, but there is considerable evidence that Sp1 participates in cell type-specific gene expression. Steady-state levels of the Sp1 message are 100-fold higher in some cells than in others, and the highest levels seen in developing hematopoietic cells, fetal cells, and spermatids in which many genes involved in cellular growth and differentiation are expressed. Most of these genes have no TATA boxes in their promoters. Many of these cells and other immortal cells and cancer cells express a high telomerase activities of which expression is required for cell proliferation, correlated with Sp1 expression. hTERT, the most important component responsible for the enzymatic activity of telomerase, is just a sort of the gene without TATA box in the promoter. These indicate that Sp1 may be a compartment of basal transcription factors for hTERT.

Of course, the hTERT promoter is probably regulated by multiple elements in addition to Sp1. c-Myc is another transcriptional regulator. Like other co-operative transcriptional factors, such as GATA-1, Ets, Ap2, and E2F1 which are required for Sp1 activation some cell-type specific genes, c-Myc-mediated activation of hTERT depends on Sp1 function. This was further demonstrated by recent publications of oncoprotein E6-mediated upregulation of hTERT expression and histone deacetylase inhibitors-activated the hTERT promoter through Sp1 but not c-Myc. A central role of Sp1 in hTERT transcriptional regulation can also be seen from which of p53 tumor suppressor protein powerful decreasing hTERT expression through inhibiting Sp1 binding to the hTERT proximal promoter by forming a p53-Sp1 complex.

Telomerase activity is highly induced in normal T lymphocytes and leukemia T cells by stimulators such as PHA. Jurkat T, a kind of leukemia T cell, expressed high telomerase activity and Sp1 protein. Sp1 antisense ODN dramatically inhibited telomerase activity and Sp1 protein. Sp1 antisense ODN may be an important way to inhibit telomerase expression.

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