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Specific inhibition of human telomerase activity by transfection reagent, FuGENE6-antisense phosphorothioate oligonucleotide complex in HeLa cells

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Abstract Human telomerase might be associated with malignant tumor development and could be a highly selective target for antitumor drug design. Antisense phosphodiester (ODNs) and phosphorothioate (S-ODNs) oligonucleotides were investigated for their abilities to inhibit telomerase activity in the HeLa cell line. The ODNs and S-ODNs were designed to be complementary to nucleotides within the RNA active site of telomerase. As a transfection reagent, FuGENE6 was used to enhance the cellular uptake of oligonucleotides in cell cultures. The results showed that S-ODN-3 (19-mer) encapsulated with FuGENE6 clearly inhibited the telomerase activity in HeLa cells, and the inhibitory efficiency increased with an increase in the S-ODN-3. However, free S-ODN-3 showed no inhibitory activity. On the other hand, ODN-3 encapsulated with FuGENE6 had no detectable inhibitory activity. The encapsulated S-ODNs exhibited higher inhibitory activities than the free S-ODNs, and showed sequence specific inhibition. Thus, the activities of the S-ODNs were effectively enhanced by using the transfection reagent. The transfection reagent, FuGENE6, may thus be a potentially useful delivery vehicle for oligonucleotide-based therapeutics and transgenes, and is appropriate for use in vitro and in vivo.

Key words: Antisense phosphorothioate oligonucleotide; Telomerase; Transfection reagent; HeLa cell

1. Introduction

The telomere is a specialized DNA-protein complex, located at the eukaryotic chromosome terminus. In humans and vertebrates, telomeres consist of hundreds to thousands of tandem repeats of the hexanucleotide sequence $(TTAGGG)_n$ [1,2]. The lengths of these repeat tracts vary from 15 to 20 kb in germ line cells, and from 5 to 12 kb in peripheral blood leukocytes [3]. They play an important role in cell replication by allowing the end of linear DNA to be replicated completely [4]. In addition, telomeres are proposed to be responsible for chromosome stabilization [5] by protecting the chromosome from enzymatic end-degradation and preventing its fusion with other chromosomes. In every cell division cycle, the telomeres continually shorten by about 50–200 nucleotides, and when they reach the critical length, then the cell stops dividing and becomes senescent. This means that maintaining the telomere at a constant length is necessary for cell immortalization and tumor cell division.

Telomerase is a ribonucleoprotein, in which the RNA component provides the template for the synthesis of telomeric repeats (TTAGGG) at the end of the chromosome to maintain the telomere at a constant length [6,7]. Telomerase activity can be detected in approximately 85–90% of primary human tumors [8] and is probably critical for sustained tumor proliferation, although this hypothesis remains in dispute. The introduction of a gene encoding an antisense RNA, targeted to the telomerase RNA component in the HeLa immortalized cell line leads to the proliferation crisis and cell death. Thus, the antisense approach seems to be a very attractive means of telomerase inhibition as a viable strategy for the suppression of tumor growth [9–11].

Antisense oligonucleotides have been used to regulate gene expression [12–15]. Antisense phosphodiester oligonucleotides have been reported to have an inhibitory effect against HIV-1 [16,17]. However, antisense phosphodiester oligonucleotides, in particular, have limited survival in vitro and in vivo, because they are rapidly degraded by nucleases present in serum and in cells [18,19]. Antisense oligonucleotides with phosphorothioate backbones exhibit several advantages over the other forms, including relatively high nuclease resistance and the capacity to induce the degradation of the target sequence by RNase H [20,21]. Another problem in the use of antisense oligonucleotides is their inefficient cellular uptake. They are mainly found in endosomes and lysosomes. The use of a delivery vehicle with specific uptake by cells would increase the circulation half-life of the oligonucleotides and improve their efficacy [22–24]. Liposome delivery addresses both of these concerns. Liposome delivery of oligonucleotides to cells in vitro has been shown to greatly reduce the effective concentration as compared to that of the free oligonucleotide [25–27].

Recently, it was reported that the telomerase inhibition by peptide nucleic acids (PNA) occurred in a sequence specific manner in cell-free systems [28,29]. However, PNAs do not appear to serve as substrates for RNase H.

In this paper, we present a detailed analysis of the inhibition of telomerase activity by ODNs and S-ODNs designed to be complementary to the template region of the telomerase RNA (Table 1). We also describe the potency of the transfection reagent (FuGENE6).

2. Materials and methods

2.1. Materials

Phosphodiester and phosphorothioate oligonucleotides were synthesized by Boston BioSystems Ltd (MA, USA). The following cationic liposomes, which are commercially available transfection reagents, were used: FuGENE6 (blended lipid with other compounds in 80% ethanol, Boehringer Mannheim, GmbH, Germany), DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate,

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Boehringer Mannheim), Tfx-10 (*N,N,N′,N′*-tetramethyl-*N,N′*-bis(2-hydroxyethyl)-2,3-dioleoyloxy-1,4-butanediammonium iodide) and L-dioleoylphosphatidyl-ethanolamine (DOPE) in a molar ratio of 1:9 (Promega, Madison, WI, USA).

2.2. Cell line

The HeLa cell line, which expresses a relative high telomerase activity was employed. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum (FCS).

2.3. Antisense oligonucleotides

The ODNs and S-ODNs were synthesized by J.BioS Co. and were purified by HPLC. They were designed to have 15- to 23-mer lengths and to be complementary to the template (5′-CUAACCCUAAC-3′) region in the telomerase RNA. S-ODN-1 and ODN-1 are 15-mer sequences, which overlap the target sequence from U42 to G56. On the basis of ODN-1 (15-mer) the lengths of the other oligomer sequences were elongated by two-base increments towards the 5′ end, according to the pairing bases of the target (Table 1).

2.4. Oligonucleotide transfection protocol

Before the transfection, the cells were plated on 6-well plates and were incubated until the cells were about 80% confluent. The cells were washed with fresh FCS-free DMEM, and 900 μl FCS-free DMEM were added into each well prior to transfection. For the preparation of the complex of antisense oligomers/transfector, the FuGENE6 transfection reagent (Boehringer Mannheim Co.) was diluted with serum-free medium and was incubated for 5 min at room temperature. Then, the desired amount of oligomer was added into the prepared transfection reagent, and the mixture was incubated for 15 min at room temperature to form the transfection complex. The final volume of the complex in each sample was 100 μl.

The complexes (100 μl each) were added dropwise to the prepared cell cultures containing 900 μl FCS-free medium, and the solutions were mixed gently. A FuGENE6 reagent only control and a normal cell control (untreated) were also included. After an incubation for 4 h at 37°C, 1 ml of 20% FCS in DMEM was added into each well, to make a final FCS concentration of 10%. The cultures were incubated for 24 h at 37°C in a humidified incubator with 95% air 15% CO_2.

2.5. Cell extract assay

The cell extract assay was performed according to the reported descriptions [28,30]. Treated cultures were washed once with phosphate-buffered saline (PBS), pelleted at 6000 rpm for 5 min, resuspended in PBS, and pelleted again. Cell extracts were obtained by resuspending 10^5 cells into 200 μl of lysis buffer (0.5% 3-(3-cholamidopropyl-dimethylammonio)-1-propane-sulfonate (CHAPS), 10 mM Tris-HCl, pH 7.5, and 5.1 mM ethylene bis-(oxyethylene-nitrilo) tetra-acetic acid (EGTA), 5 mM B-mercaptoethanol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 10% glycerol). The suspension was mixed and lysed for 30 min on ice. The lysates were centrifuged at 12000×*g* for 20 min at 4°C. A portion (160 μl) of the supernatant was collected and aliquoted into four tubes. The aliquots (equivalent to 500 cells/μl) were quickly frozen in liquid nitrogen and were stored at −84°C. The telomerase activity in the cell extract is stable for at least 6 months at this temperature.

2.6. Measurement of telomerase activity

The telomerase activity in the transfected cell extracts was measured by using the PCR-based telomere repeat amplification protocol (TRAP) [30]. The TS primer (AATCCGTCGAGCAGAGTT) in the TRAP Kit (Oncor Inc.) can serve as a telomerase substrate. The telomerase in the cell extract adds a number of telomeric repeats (GGTTAG) onto the 3′ end of the TS oligonucleotide. Then, the extended products are amplified by reverse transcription PCR. The amounts of the PCR products, which are visualized by polyacrylamide gel electrophoresis (PAGE), indirectly represent the telomerase activity.

The HeLa cell extracts were added directly to the TRAP reaction mixture (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 0.01% BSA, 1 μl dNTPs, 1 μl TS primer, 1 μl primer mix, and 0.5 μl (2 units) Taq-polymerase). After an incubation for 20 min at 30°C to allow the telomerase to elongate the TS primer, the reaction mixtures were immediately heated for 2 min at 94°C to inactivate the telomerase and to stop the TS primer elongation. Then, a three-step PCR (94°C for 30 s, 50°C for 30 s, and 72°C for 45 s) was performed for 30 cycles, in which the final step of the last cycle was an incubation at 72°C for 2 min. The PCR products were analyzed by 10% non-denaturing PAGE. The gels were stained by SYBR Green (FMC) and were visualized with a UV transilluminator (300 nM transillumination) and a FAS-II (Toyobo) image system.

The telomerase activities were quantified by means of the NIH Image 1.55 software. The mean density of each lane in the image was measured. The reported data of the treated samples are expressed as percent inhibition relative to the untreated control sample, in which the telomerase activity was defined as 100%. The density of the reagent control (no cell extract) was subtracted from the background.

3. Results and discussion

Telomerase consists of an essential RNA and a few proteins. The telomerase RNA contains a region complementary to the telomere repeats. This special region (3′-CAAUCCCAAUG-5′) serves as a template during the synthesis of telomeric DNA by telomerase [30]. Therefore, the template region is deduced to be one of the active sites in the enzyme, and was selected as the target of the antisense oligonucleotides for the regulation of the telomerase activity.

Norton et al. investigated the inhibition of telomerase ac-

Table 1
Sequences of antisense oligonucleotides

Name	Length (mer)	Sequence[a]
		60 55 50 45 40
Telomerase RNA		3′- GG AAG AGU **CAA UCC CAA UCU** GUU UU-5′
S-ODN-1 / ODN-1	15	5′- **GTT AGG GTT AGA** CAA -3′
S-ODN-2 / ODN-2	17	5′- CA **GTT AGG GTT AGA** CAA -3′
S-ODN-3 / ODN-3	19	5′- C TCA **GTT AGG GTT AGA** CAA -3′
S-ODN-4 / ODN-4	21	5′- TTC TCA **GTT AGG GTT AGA** CAA -3′
S-ODN-5 / ODN-5	23	5′- CC TTC TCA **GTT AGG GTT AGA** CAA -3′
S-ODN-3-ran[b]	19	5′ - CAT TTG CCT ATC CCG CCT GA -3′
S-ODN-3-sen	19	3′- G AGT CAA TCC CAA TCT GTT -5′

[a]The RNA active site and complementary nucleotides within the antisense oligonucleotides are in boldface.
[b]The random sequences (S-ODN-3-ran) with the same base composition as antisense oligomers (S-ODN-3).

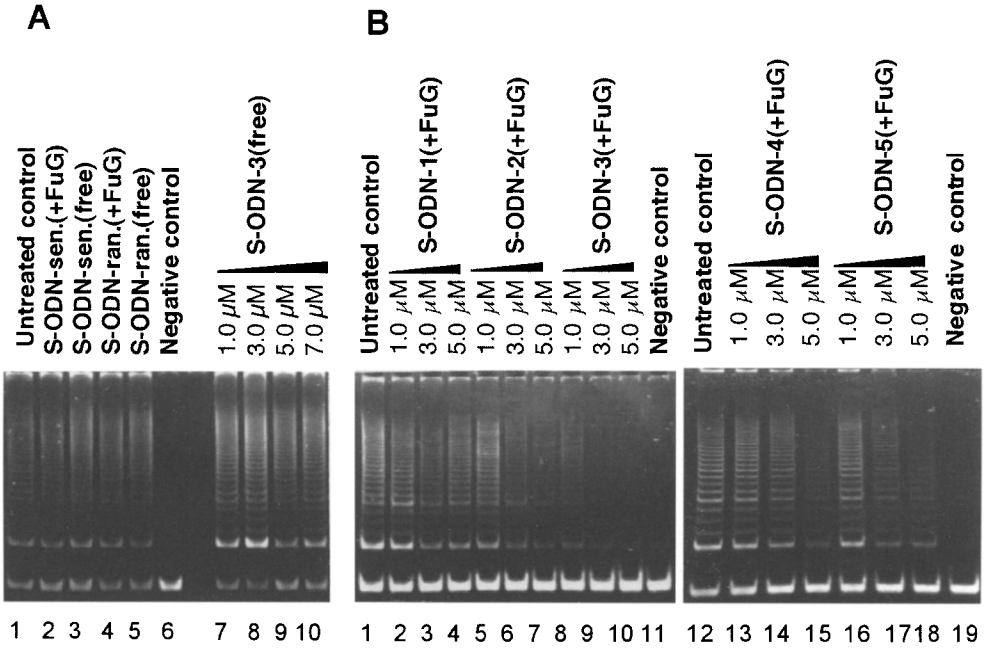

Fig. 1. Inhibition of telomerase activity by S-ODNs. The TRAP protocol was employed to measure the telomerase activity. In each measurement, 160 HeLa cell equivalents were used. The PCR products of the telomerase in the gel were visualized by SYBR Green staining. A: Inhibition effects were detected using free and encapsulated S-ODN-3, S-ODN-3-sen, and S-ODN-3-ran. Lane 1, untreated control cells; lane 5, negative control; lanes 2–4, sense and random control oligonucleotides with or without the transfection reagent, FuGENE6; lanes 7–10, treated with free S-OND-3. B: Inhibition of telomerase activity by the S-ODN-1-3 encapsulated with transfection reagent, FuGENE6. Lanes 1 and 12, untreated control cells; lanes 11 and 19, negative controls (without cell extract added).

tivity in cell-free systems by S-ODNs [28]. The inhibition by S-ODNs in cell-free extracts was due to non-specific binding to non-template RNA or protein components, rather than Watson–Crick pairing, because the random S-ODN control could also suppress the telomerase activity. The same phenomenon was also observed in our laboratory (data not shown). When cell-free extracts of HeLa cells were treated with the sense or random S-ODNs, telomerase activity was also significantly inhibited (even in a dose-dependent manner), due to non-specific binding. In common with other DNA and RNA polymerases, it is likely that telomerase also binds DNA substrates via association between its protein component and the negatively charged phosphodiester bonds of DNA, and similar interactions might lead to enhanced binding and inhibition. S-ODNs are negatively charged and may associate with proteins in this manner. This suggests that tests of the inhibition of telomerase activity by S-ODNs in a cell-free system are not suitable.

The in vitro telomerase activity of the antisense oligonucleotides was assessed on the basis of their inhibitory effects in HeLa cell lines. To clarify the sequence specificity, we tested the phosphorothioate oligonucleotides with antisense sequences as targets to the nucleotides (11 nucleotides) within the RNA active site of telomerase, as well as sense sequences and random sequences with the same base composition as the antisense oligomers (Table 1). We also evaluated the inhibitory effects of free ODNs and S-ODNs and those encapsulated with FuGENE6 on the telomerase activity.

First, we tested the inhibitory effects on the telomerase activity, using free ODNs and S-ODNs that progressively overlapped the telomerase RNA template region in HeLa cells. The HeLa cell line possesses high telomerase activity, which presents a linear response with the cell number. The inhibition of telomerase activity using oligonucleotides was assayed using the telomerase repeat amplification protocol (TRAP) [31]. In this protocol, telomerase extends an oligonucleotide primer to form elongation products. These products are then amplified by PCR to facilitate their detection. TART affords a sensitive and linear response over the range of telomerase activity used in these studies, and inclusion of an internal amplification strand in each sample permits reproducible quantification.

We could not detect any inhibitory effects on the telomerase activity with the free S-ODN-3 at 1–7 μM concentrations in HeLa cell cultures (Fig. 1A). On the other hand, the free ODN-3 was also tested, and showed no inhibitory effects at 1–7 μM concentrations in the HeLa cell line (data not shown). These results suggest that the negative results on the inhibitory effect of the free antisense oligonucleotides might be associated with poor delivery to HeLa cells. Applications of antisense oligonucleotides in vitro and in vivo are hampered by several limiting factors, such as oligonucleotide stability, cellular uptake, subcellular availability, and other pharmacokinetic parameters [32–35]. These factors lead to relatively poor delivery of the oligomers to the targeted molecular sites, and make treatments cost prohibitive. Thus, efficient transport and intracellular delivery are important and fundamental considerations when developing an effective oligonucleotide-based therapy. Liposomally encapsulated antisense oligonucleotides have significantly enhanced intracellular delivery, they have simultaneously introduced new disadvantages of their own [36,37]. Cationic lipids are being used in vitro and in vivo for the delivery of oligonucleotides for therapeutic or researches purpose, and are the vehicles of choice for some forms of gene therapy [38–42]. Therefore, we evaluated the inhibition of telomerase activity by S-ODNs encapsulated

with FuGENE6 (non-liposomal formulation) as a transfection reagent to enhance the delivery of the oligonucleotides into HeLa cells. Every transfection experiment included control cells treated with FuGENE6 alone and oligonucleotide alone. These additions did not inhibit telomerase activity.

The encapsulated S-ODN-3 showed the highest inhibitory effect on the telomerase activity (87% inhibition at 3 μM), and the inhibition occurred in a dose-dependent manner (Fig. 1B and Table 2). For the control sequences of the sense and random oligonucleotides (S-ODN-3-sen and S-ODN-3-ran), we could not detect any inhibitory effects on the target telomerase RNA at a 5 μM concentration. In this assay system, the phosphorothioate oligonucleotides cannot inhibit the telomerase activity because the sense and random phosphorothioate oligonucleotides, which lacked complementary to hTR, did not detectably inhibit telomerase activity. These results suggest that the encapsulated S-ODN-3 has a significant advantage over the use of S-ODN alone for delivery into HeLa cells and conferred sequence specific inhibition of the telomerase activity. However, if a more direct measurement of the antisense oligonucleotide binding to targeted telomerase RNA can be identified, it should help to clarify the mechanism of antisense inhibition of telomerase activity. On the other hand, the encapsulated ODN-3 (5 μM concentration) did not show any inhibitory effects on the telomerase activity (data not shown). Furthermore, we examined the inhibition of human telomerase activity by S-ODN-3 encapsulated with several types of cationic liposomes in HeLa cells. The S-ODN-3 encapsulated with FuGENE6 showed higher inhibitory effects than the S-ODN-3 encapsulated with other cationic liposomes, DOTAP and Tfx-10 (data not shown). The transfection reagent, FuGENE6, has a significant advantage over the use of S-ODN alone for delivery of S-ODN into HeLa cells.

The lengths of the S-ODN sequences were studied, and the overlapping S-ODNs were used to identify the nucleotides (11 nucleotides) within the RNA active site of telomerase (Table 1). S-ODN-1 (15-mer), which is complementary to the telomerase RNA template (C46-C56) as well as its adjacent four bases upstream (Table 1) showed weaker inhibitory efficiency with only 64% of the telomerase activity inhibited at a 5 μM concentration (Table 2). Therefore, when the S-ODN-1 sequence was extended by two bases at its 5′ end (the paired bases include U57 and G58 in the target) to form a 17-base sequence (S-ODN-2), the inhibition efficiency increased from 64% to 87% (Table 2). Furthermore, while the S-ODN-1 sequence was further elongated for another two bases (A59 and G60) in the same direction to form S-ODN-3 (19-mer), an obvious increase of telomerase inhibition was observed as compared to S-ODN-1 and S-ODN-2, and the telomerase activity was inhibited up to 93% at a 5 μM concentration. The increase of the inhibitory efficacy of S-ODN-3 is not entirely dependent on its longer sequence length, because S-ODN-4 (21-mer) and S-ODN-5 (23-mer), which are two and four bases longer than S-ODN-3 (19-mer), possessed less telomerase inhibitory activity. Presumably, addition bases of the longer S-ODNs offer more potential for non-specific contacts in the yield stronger intramolecular secondary structure, thereby reducing the likelihood of binding the telomerase target. It is known that the phosphorothioate oligonucleotides blocked the proliferation HIV-1 in infected cells in a non-sequence specific manner [43], probably the inhibition of reverse transcriptase [44,45] and/or the viral entry process [46,47]. Our previous study of the anti-HIV activities of antisense oligonucleotides indicated that a phosphorothioate oligonucleotide targeted to the HIV-1 *gag* gene was inhibitory, and the sense and the random sequence oligomers were also able to protect against HIV-1 induced CPE, but their anti-HIV-1 activities were weaker than that of the antisense phosphorothioate oligonucleotide (S-ODN-*gag*-AUG) [48]. Furthermore, the inhibition of HIV-1 in de novo infected cells by the phosphorothioate homooligonucleotides is related more to their length than to their sequence [49,50]. These results suggest that S-ODN-3, which progressively overlaps the targeted sequence downstream from C46 to G60, might induce more significant inhibition in a sequence specific fashion.

Table 2
Inhibition of telomerase activity by S-ODNs encapsulated with FuGENE6

Name	Length	Inhibition (%)[a,c]			IC_{50} (μM)[b,c]
		1 μM	3 μM	5 μM	
S-ODN-1	15	42	63	64	1.62
S-ODN-2	17	36	75	87	1.50
S-ODN-3	19	59	87	93	0.61
S-ODN-4	21	25	71	81	1.94
S-ODN-5	23	13	42	84	2.69
S-ODN-3-ran	19			7	
S-ODN-3-sen	19			15	

[a]Inhibition (%) represents the percentage of telomerase activity inhibited by S-ODNs, as compared with the untreated HeLa cell culture control (expressed as 100% telomerase activity).
[b]IC_{50} represents the concentration of S-ODNs at which 50% of the telomerase activity was inhibited.
[c]Data represent average values for at least three different experiments.

In conclusion, the antisense phosphorothioate oligonucleotides (S-ODN-3, 19-mer) complementary to the template region of the telomerase RNA showed the greatest inhibition of telomerase activity in HeLa cells. The use of the transfection reagent, FuGENE6, provides a simple and successful method for the intracellular delivery of oligonucleotides. The antisense phosphorothioate oligonucleotides encapsulated with FuGENE6 exhibited higher inhibitory activities than the free oligonucleotides, and showed sequence specific inhibition, whereas the free antisense phosphorothioate oligonucleotides suffered from poor delivery to the HeLa cells. Furthermore, the S-ODNs encapsulated with FuGENE6 did not appear to be cytotoxic in cell growth rate. The transfection reagent may have an advantage over conventional encapsulating liposomes for delivery of high levels of oligonucleotides in vitro and in vivo. Consequently, these specific antisense phosphorothioate oligonucleotides appear to be favorable therapeutic agents for cancer.

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