Antitumor Activity of Small Double-Stranded Oligodeoxynucleotides Targeting Telomerase RNA in Malignant Melanoma Cells

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Human telomerase RNA (hTR) is an intrinsic component of telomerase enzyme. Small interfering RNAs (siRNAs) and single-stranded antisense oligonucleotides have been used previously for silencing of the hTR. The objective of this study was to investigate the effect of partially double-stranded oligodeoxynucleotides (ODNs), in vitro and in vivo in comparison to single-stranded antisense ODNs and siRNAs. ODNs were designed on the basis of structural properties of an ODN from previous studies on HIV, to target the hTR in the human cervical carcinoma HeLa cell line and mouse telomerase RNA (mTR) in the murine metastatic melanoma B16-F10 cell line, respectively. Our results indicate that ODNs were able to inhibit the hTR by 68% and the mTR by 81% in the respective cell lines. This correlated with ODN-mediated rapid inhibition of cell proliferation and induction of apoptosis excluding slow effects on telomerase function. The inhibition of the hTR was decreased by knock-down of the cellular RNases H suggesting their contribution. Furthermore, we showed a reduction in numbers of metastases by 70% after intravenous administration of ODN-transfected B16-F10 cells in C57BL/6 mice. Our study demonstrates the potential utility of these hairpin-loop-structured ODNs as a different group of nucleic acids for telomerase-based antiproliferative strategies.

Introduction

Telomeres are specialized DNA–protein structures located at the ends of chromosomes (Blackburn, 1991). Human telomeric DNA, usually 5–20 kb in length, consists of repetitive 5'-TTAGGG-3' sequences (Greider, 1991; McElligott and Wellinger, 1997). Telomeres serve to protect chromosomes against rearrangement, end-to-end fusion, degradation, and chromosomal loss, thus contributing to chromosomal stabilization (McEachern et al., 2000). In human somatic cells telomeres are shortened between 50 and 200 base pairs with each cell division (Allsopp et al., 1995; Blackburn et al., 2006) leading to telomere dysfunction and senescence.

A telomere maintenance mechanism is provided by the telomerase, a cellular reverse transcriptase (RT)-like enzyme complex (Greider and Blackburn, 1985). It is a ribonucleoprotein whose catalytic function depends on telomerase reverse transcriptase (TERT) protein (hTERT in humans) and telomerase RNA (TR, hTR in humans) (Blackburn et al., 2006). The hTR contains a short segment, the template sequence, which encodes the cognate telomere repeat TTAGGG (Fig. 1A), and this segment serves as the template for reverse transcription by hTERT (Nakamura et al., 1997). Repeated cycles of extension and translocation reactions (Lue, 2004) lead to the elongation of the telomeres. This ability of telomerase is predicated on telomerase-specific structures located both within and outside of the RT domain (Autexier and Lue, 2006).

Telomerase is strongly repressed in normal somatic tissues but expressed in highly proliferative ones, including ovaries, testis, and hematopoietic tissues (Wright et al., 1996). Furthermore, telomerase is also strongly up-regulated in most cancer cells (Blackburn, 2005). In fact, the ectopic expression of hTERT in combination with two oncogenes, the simian virus 40 large T oncoprotein and an oncogenic allele of H-ras, results in tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn et al., 1999a, 1999b). Therefore, the telomerase has attracted attention...
FIG. 1. Oligonucleotides targeting the human telomerase RNA (hTR). (A) Model on telomeric DNA synthesis catalyzed by human telomerase. The human telomerase catalytic protein (hTERT) is shown with the target sequence of the hTR for oligonucleotides. The template sequence is indicated by a dotted arrow. The 3′ end of the telomeric DNA can form base pairs with the 3′ end of the template sequence of the hTR and is extended until the 5′ end of the template sequence (thick arrow). Then the newly synthesized 3′ end translocates to the 5′ end of the template sequence to allow a further round of extension. (B) The sequences of the hTR target site and hTR targeting ODN (ODN-hTR), antisense oligonucleotide (as-hTR), and siRNA (si-hTR) are shown. The complementary sequences of the oligonucleotides to the template sequence (dotted arrow) are indicated by solid arrows. The nonspecific ODN-sc served as control. Phosphorothioation of the phosphodiester backbone is indicated by asterisks. Vertical bars indicate Watson–Crick bonds. (C) HeLa cells were transfected at 0 h (filled arrowhead) with the 50 nM of the indicated oligonucleotides. RNA was extracted at 16, 24, and 48 h (vertical arrows) and analyzed by qRT-PCR using a set of primers recognizing the hTR Human GAPDH (hGAPDH) was used for standardization. Bars represent mean values of relative hTR RNA levels (+SD) from three different independent experiments. (D) Same as C except that HeLa cells were transfected with 25, 50, or 80 nM of the indicated oligonucleotides for 48 h.

as a target for cancer therapy (Shay and Wright, 2002). The first report of successful inhibition of telomerase activity involved the use of a single-stranded antisense oligonucleotide against the hTR (Feng et al., 1995). Later, others have used different expression vectors and modified oligonucleotides against hTR and hTERT (Bisoffi et al., 1998; Zhang et al., 2000; Braasch and Corey, 2002; Zhang et al., 2003). We investigated here the effect of a partially double-stranded oligodeoxynucleotide (ODN) against hTR.

We have recently applied hairpin-loop-structured ODNs against HIV and targeted them to the highly conserved polypurine tract (PPT) required for the second strand DNA synthesis of HIV-1 (Jendis et al., 1996; Jendis et al., 1998; Moelling et al., 2006; Matskevich et al., 2007; Matzen et al., 2007). The ODN forms local RNA–DNA hybrids in cell-free HIV virus particles, which are recognized as substrate by the virion-associated ribonuclease (RNase) H, which then hydrolyzes the viral RNA, so that the virus is no longer infectious (Matskevich et al., 2007). The ODN can also be applied to HIV-infected cells and leads there to an RNase H-mediated inhibition of DNA provirus formation and viral replication (Jendis et al., 1998; Moelling et al., 2006). This effect of the ODN was highly sequence-specific and more effective than a single-stranded antisense strand (Jendis et al., 1998; Matzen et al., 2007). This may be attributed to the partially double-stranded structure of the ODN, which protects against nuclease.

Since the telomerase is related to an RT, we investigated the use of telomerase-specific ODNs against hTR in vitro and in vivo. In the present study, ODNs targeting the human and murine TRs were designed in analogy to the HIV-ODN. An antisense strand of 25 nucleotides targeting hTR comprising the template sequence is linked by four thymidines to a second strand, which is partially complementary to the antisense strand thereby allowing a partially double-stranded structure similar to that expected for the HIV-ODN. The
ODNs were tested for their inhibitory effect on gene expression in HeLa and B16-F10 cells, respectively. The inhibition was confirmed in a surrogate model for pulmonary metastases by the reduction of the number of colonies formed in the lung in C57BL/6 mice after intravenous (i.v.) administration of ODN-treated B16-F10 cells. These data provide a good basis for the use of this group of nucleic acids for further studies in telomerase-based antiproliferative strategies.

Materials and Methods

Cell lines

The human cervical carcinoma cell line (HeLa cells) and the murine metastatic melanoma cell line (B16-F10 cells) were purchased from the American type culture collection (ATCC; Manassas, VA, USA). Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Brunschwig AG, Basel, Switzerland) in the presence of 5% CO₂ at 37°C.

Oligonucleotides

ODN-hTR and ODN-mTR were designed for targeting the human and murine telomerase RNA template sequences, respectively. Briefly, ODNs were designed as hairpin-looped structures where the antisense strand was fully complementary to the target site and linked by four additional thymidines (T4 linker) to the second strand. All ODNs used were phosphorothioated at each end (three bases) and in the T4 linker. Antisense ODNs (as-hTR and as-mTR) were designed complementary to their respective target site and were phosphorothioated at each end (three bases). All ODNs and antisense oligonucleotides were synthesized by Integrated DNA Technologies, Coralville, IA, USA. The small interfering RNAs (siRNAs; si-hTR and si-mTR) were supplemented by dinucleotides dTdT overhangs at the 3’ end of each strand (Dharmacon, Lafayette, CO, USA). A nonspecific scrambled ODN (ODN-sc) and an irrelevant siRNA (si-neg, Allstar siRNA; QIAGEN, Hilden, Germany) were used as negative controls.

Transfection

HeLa cells were seeded in the 12-well plate at the density of 16 × 10⁴ cells/mL overnight. The following day cells were transfected with ODN-hTR, as-hTR, si-hTR, ODN-sc, or si-neg. Each oligonucleotide was diluted with 50 μL of OPTIM-MEM (Invitrogen, Carlsbad, CA). In a separate tube, 8 μL of Lullaby (OZ Biosciences, Marseille Cedex, France) was diluted with OPTIM-MEM and the contents of the first tube were then added drop by drop into this tube. The mixture was incubated at room temperature for 20 min. This complex was then gently overlaid on culture cells in each well and mixed by gentle rocking for 30 s. Mock-treated cells were transfected with Lullaby alone. The first time of treatment was defined as 0 h (0 h). Both cell lines were transfected with 50 nM of each oligonucleotide for 16, 24, and 48 h.

B16-F10 cells were transfected as described above for HeLa cells, except that they were transfected with mTR targeting oligonucleotides: ODN-mTR, as-mTR, si-mTR.

RNA preparation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cellular RNA was isolated using the QIAamp RNA Blood Mini kit (QIAGEN). Subsequent cDNA synthesis and qRT-PCR were performed in one step using the QuantiTect probe RT-PCR kit (QIAGEN) as recommended by the manufacturer. The primers and Taqman probes were as follows: the hTR was amplified using forward primer hTR-F (5'-GGTGGCAAGGTTGGC-3'), reverse primer hTR-R (5'-AACGGTGAGGCAGCAG-3'), and probe hTR-P (FAM-5'-TGGCCGGTCTTTTTGC TC-3'-TAMRA). The human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) was used for normalization and was amplified by using forward primer hGAPDH-F (5'-GAAGGTGAAGTCCAGTTC-3'), reverse primer hGAPDH-R (5'-GAAGATGGTGATGGAGTTTC-3'), and probe hGAPDH-P (FAM-5'-AACGTTCCCTGGTCAGCT-3'-TAMRA). The mTR was amplified using forward primer mTR-F (5'-GTCTTTTTGTCCTCGCCCG-3'), reverse primer mTR-R (5'-CCCGCAACTGGAGGCTC-3'), and probe mTR-P (FAM-5'-CCTCCCCAGCCTCAAACAAAGC 3'-TAMRA). The mouse GAPDH (mGAPDH) was amplified by using forward primer mGAPDH-F (5'-CTTCA CCACCATGGAGAAGGC-3'), reverse primer mGAPDH-R (5'-GGCATGGACTGTGGTCATGAG-3'), and probe mGAPDH-P (FAM-5'-CCTGCGCAAAGGCTCATGCAGA ACTTT-3'-TAMRA). Cycling conditions were used as: 48°C for 30 min (one cycle for cDNA synthesis), 95°C for 10 min (one cycle), and 95°C for 15 s followed by 60°C (69°C for hTR) for 1 min (50 cycles). GAPDH was used for standardization. Percentage RNA values were compared to mock-treated cells. Bars represent means of relative hTR RNA level ± standard deviation (+/−SD) from three independent experiments.

Cell proliferation and viability

The effect of ODNs on cell proliferation was determined by the cell proliferation kit I (Roche Diagnostics GmbH, Mannheim, Germany) using [4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) reagent. Cells were seeded in triplicates in 12-well plate with the density of 8 × 10⁴ cells/mL. On the following day, cells were transfected with oligonucleotides as described above (t = 0 h). After 48 h of transfection, cells were trypsinized and 2 × 10⁵ cells from each well were transferred in 100 μL into a 96-well plate and incubated overnight. The MTT assay was performed at 72 h according to the manufacturer. Absorbance was measured at 550/650 nm to determine the cell viability and was compared to mock-treated cells to obtain the relative cell proliferation. Bar represents relative cell proliferation + SD from two independent experiments with two replicates in each.

Apoptosis assay

Apoptotic HeLa cells were detected using the M30-Apoptosense ELISA kit (PEVIV A AB, Bromma, Sweden). The M30-Apoptosense ELISA is a solid-phase sandwich enzyme immunoassay. M30 antibody recognizes a neoepitope exposed after caspase cleavage of cytokeratin 18 (CK 18) after aspartic acid residue 396 (Leers et al., 1999). Cells were seeded in triplicates in a 12-well plate with a
density of \(8 \times 10^6\) cells/mL and incubated for 24 h at 37°C. At 0 h, cells were transfected using a procedure as described above for HeLa cells. After 72 h of transfection, the apoptotic assay was performed as recommended by the manufacturer. Unbound conjugate was removed by washing, then 3,3',5,5'-tetrathymethylbenzidine (TMB) substrate was added and absorbance was measured at 450 nm. The resulting color was directly proportional to the concentration of the analyte. By plotting a standard curve from known concentrations versus measured absorbance, the amount of antigen in the sample was calculated. Bars represent the concentration [U/L] of CK18Asp396 from two independent experiments with two replicates each. Positive control (pos con) represents the M30-high control as provided in the kit.

Effect of ODNs on telomerase RNA template

The RNA component of telomerase is essential for telomerase reverse transcription and is therefore a natural target for anti-telomerase agents (Fig. 1A). We designed a partially double-stranded DNA targeting hTR, ODN-hTR, against a sequence comprising the template sequence based on previously described structural properties of an ODN against the HIV PPT (Jendis et al., 1996; Jendis et al., 1998; Moelling et al., 2006; Matskevich et al., 2007; Matzen et al., 2007). The ODN-hTR is composed of a fully homologous 25-mer antisense strand, and a second arm, which is partially complementary to the antisense strand thereby allowing a partially double-stranded structure similar to that expected for the HIV-ODN. The two arms are connected by a T4 linker and the linker and the three terminal nucleotides were protected against nuclease by phosphothioate modifications (Jendis et al., 1996; Jendis et al., 1998; Moelling et al., 2006). We tested the ODN-mediated hTR inhibition in comparison with its 25-mer antisense (si-hTR) and 21-mer siRNA (si-hTR) analogs (Fig. 1B). The target sequence selected was identical to a previously described one for hTR (Kondo et al., 1998; Kosciele et al., 2003), which allows a direct comparison.

To evaluate the onset and duration of the action of each oligonucleotide, HeLa cells were transfected with 50 nM of each oligonucleotide and the hTR RNA level was measured at 16, 24, and 48 h of transfection using qRT-PCR. A minimal decrease in the hTR RNA was observed after 16 and 24 h of transfection. However, at 48 h the level of hTR RNA was strongly reduced to 32 ± 8% by ODN-hTR, 48 ± 4% by as-hTR, and 36 ± 8% (SD) by si-hTR relative to the mock-treated cells (Fig. 1C). Further decrease or increase in concentration did not improve the hTR knockdown level (Fig. 1D).

In order to facilitate the functional utility of ODN in a mouse model in vivo, we used ODN to target the mouse telomerase as well. Here, we designed a set of oligonucleotides; ODN-mTR, as-mTR, si-mTR targeting mTR (Fig. 2A) and first tested their efficiency and specificity in vitro using B16-F10 cells. The cells were transfected with 50 nM of each oligonucleotide and the expression level of mTR was measured at 16, 24, and 48 h of transfection using qRT-PCR. At 48 h, the level of mTR RNA was strongly reduced to 29 ± 4% by ODN-mTR, 34 ± 2% by as-mTR, and 18 ± 1% (SD) by si-mTR relative to mock-treated cells (Fig. 2B).

A control ODN-sc and si-neg were used, which showed no effect on RNA levels in both cell lines, suggesting sequence specificity of the hTR and mTR targeting oligonucleotides.

Cell proliferation assay

Using phase-contrast microscopy, we noticed that oligonucleotides had variable effects on cell proliferation during telomerase RNA knockdown studies (data not shown). In order to quantify the potential effect of these oligonucleotides...
on cell proliferation, an MTT assay was performed after 72 h of oligonucleotides transfection. This has been used by others as a mean of assaying the quantification of viable cells (Fu et al., 2005; Chen et al., 2006), because only metabolically active cells cleave the MTT salt to form formazan dye (Vistica et al., 2005; Chen et al., 2006), because only metabolically active cells cleave the MTT salt to form formazan dye (Vistica et al., 2005; Chen et al., 2006). We designed the ODN-hTR to allow the formation of RNA–DNA hybrids, so that cellular RNase H might have a role in ODN-mediated hTR down-regulation in HeLa cells. In order to test this hypothesis, cellular RNases H were reduced by knockdown in HeLa cells. First, a control (without knockdown) study was performed. HeLa cells were pretransfected (−48 h) with control irrelevant siRNA (si-neg) and later, at 0 h, transfected with the hTR-specific oligonucleotides. The hTR RNA level was reduced to 36 ± 8 by ODN-hTR, 51 ± 0.29 by as-hTR, and 34 ± 5% (SD) by si-hTR relative to mock-treated cells (Fig. 4B). In the next step, cells were pretransfected (−48 h) with siRNAs specific to RNase H1 (RNase H1 knockdown) or RNase H2A (RNase H2A knockdown). The down-regulation was tested by qRT-PCR using primers specific for RNase H1 and RNase H2A, respectively. The level of RNase H1 was reduced to 59 ± 0 and that of RNase H2A to 36 ± 7% (SD). At 0 h, these cells were transfected with the hTR-specific oligonucleotides. In RNase H1 knockdown cells, the hTR RNA level was reduced to 58 ± 13 by ODN-hTR, 67 ± 0.16 by as-hTR, and 38 ± 0.15% (SD) by si-hTR compared to mock-treated cells. In RNase H2A knocked down cells, the hTR RNA level was reduced to 57 ± 3 by ODN-hTR, 73 ± 10 by as-hTR, and 36 ± 5% (SD) by si-hTR compared to mock-treated cells (Fig. 4B). These results showed a decrease in inhibition of hTR by ODN- and as-hTR in knockdown cells compared to the control without knockdown. Thus, both cellular RNases H appear to contribute to the antiproliferative effect of the ODNs.

**Role of RNase H**

The ability of antisense ODNs to elicit RNase H-mediated degradation of target mRNA is one of the most important criteria for antisense effectiveness (Braasch and Corey, 2002). We designed the ODN-hTR to allow the formation of RNA–DNA hybrids, so that cellular RNase H might have a role in ODN-mediated hTR down-regulation in HeLa cells. In order to test this hypothesis, cellular RNases H were reduced by knockdown in HeLa cells. First, a control (without knockdown) study was performed. HeLa cells were pretransfected (−48 h) with control irrelevant siRNA (si-neg) and later, at 0 h, transfected with the hTR-specific oligonucleotides. The hTR RNA level was reduced to 36 ± 8 by ODN-hTR, 51 ± 0.29 by as-hTR, and 34 ± 5% (SD) by si-hTR relative to mock-treated cells (Fig. 4B). In the next step, cells were pretransfected (−48 h) with siRNAs specific to RNase H1 (RNase H1 knockdown) or RNase H2A (RNase H2A knockdown). The down-regulation was tested by qRT-PCR using primers specific for RNase H1 and RNase H2A, respectively. The level of RNase H1 was reduced to 59 ± 0 and that of RNase H2A to 36 ± 7% (SD). At 0 h, these cells were transfected with the hTR-specific oligonucleotides. In RNase H1 knockdown cells, the hTR RNA level was reduced to 58 ± 13 by ODN-hTR, 67 ± 0.16 by as-hTR, and 38 ± 0.15% (SD) by si-hTR compared to mock-treated cells. In RNase H2A knocked down cells, the hTR RNA level was reduced to 57 ± 3 by ODN-hTR, 73 ± 10 by as-hTR, and 36 ± 5% (SD) by si-hTR compared to mock-treated cells (Fig. 4B). These results showed a decrease in inhibition of hTR by ODN- and as-hTR in knockdown cells compared to the control without knockdown. Thus, both cellular RNases H appear to contribute to the antiproliferative effect of the ODNs.

**Suppression of lung metastasis in mice by intravenous injection of mTR-transfected B16-F10 melanoma cells**

As oligonucleotides targeting the mTR in B16-F10 cells inhibited both the mTR RNA level and cell proliferation in vitro, we explored their activity on in vivo tumor growth. The animal model used here was the well-described B16-F10/C57BL/6 surrogate model for metastatic malignant melanoma (Schultz et al., 1999). B16-F10 cells were transfected with ODN and 16 h later the cells were injected into the tail vein of C57BL/6 mice. The metastases observed in the lungs were pigmented by melanin and were easily detectable upon opening the chest of mice after 21 days (Fig. 5A). The numbers of metastases were counted and found to be decreased to 26 ± 3 by ODN-mTR, 25 ± 4 by as-mTR, and 8.6 ± 3 (SD) by si-mTR (Fig. 5B) compared to 65 ± 5 (SD) by untransfected control cells. Injection of ODN-sc- or si-neg-transfected B16-F10 cells had no significant effect on whether ODN-mediated hTR down-regulation was correlated with apoptosis. HeLa cells were transfected with oligonucleotides at 0 h and apoptosis ELISA assay was performed after 72 h of transfection. The concentration of apoptosis-associated antigen CK18Asp396 neoepitope was increased to 572 by ODN-hTR, 486 by as-hTR, and 503 U/L by si-hTR compared to 183 U/L in mock-treated cells (Fig. 4A). These data indicate that the apoptotic cell death was responsible for rapid inhibition of HeLa cell proliferation.

**Apoptosis**

As inhibition of hTR in vitro is known to limit cell life span by triggering cell apoptosis (Feng et al., 1995), we tested whether ODN-mediated hTR down-regulation was correlated with apoptosis. HeLa cells were transfected with oligonucleotides at 0 h and apoptosis ELISA assay was performed after 72 h of transfection. The concentration of apoptosis-associated antigen CK18Asp396 neoepitope was increased to 572 by ODN-hTR, 486 by as-hTR, and 503 U/L by si-hTR compared to 183 U/L in mock-treated cells (Fig. 4A). These data indicate that the apoptotic cell death was responsible for rapid inhibition of HeLa cell proliferation.

**Role of RNase H**

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the formation of metastases. Results from two independent experiments showed a reduction in the numbers of metastases to 30% by ODN-mTR, 29.5% by as-mTR, and 8.5% by si-mTR when compared to the control (Fig. 5C).

Discussion

This study shows that ODNs, partially double-stranded ODNs, targeting the RNA component hTR of telomerase, lead to an inhibition of gene expression in human cervical carcinoma HeLa cells and in murine metastatic melanoma B16-F10 cells.

FIG. 3. In vitro analysis of tumor cell proliferation. (A) HeLa cells were transfected at 0 h with 50 nM of the indicated oligonucleotides. MTT assay was performed at 72 h (vertical arrow) as described in Materials and Methods. Absorbance was measured at 550/650 nm to determine the cell viability and was compared to mock-treated cells to obtain relative cell proliferation. Bars represent relative cell proliferation rates (+SD) from two independent experiments. (B) Same as A except that murine B16-F10 cells were transfected with 50 nM of the indicated mTR-specific oligonucleotides.

FIG. 4. Mechanism of ODN-mediated hTR down-regulation. (A) Quantification of cell apoptosis. HeLa cells were transfected at 0 h with the indicated oligonucleotides. An apoptosis ELISA assay was performed at 72 h (vertical arrows) as described in Material and Methods. Absorbance was measured at 450 nm. Bars represent the mean of the concentration of the apoptosis indicator CK18Asp396 units per liter [U/L] (+SD) from two independent experiments using two replicates in each experiment. The positive control (pos con) represents the M30-high control provided in the kit. (B) HeLa cells were pretransfected (−48 h, open arrowhead) with irrelevant control siRNA (without knockdown) or with siRNAs specific for RNase H1 (RNase H1 knockdown) and RNase H2A (RNase H2A knockdown). At 0 h cells were transfected with the indicated oligonucleotides (filled arrowhead). RNA was extracted at 48 h (vertical arrow) and analyzed by hTR-specific qRT-PCR. Relative RNA values were obtained by comparison to mock-treated cells. Bars represent relative hTR RNA levels normalized to GAPDH RNA levels (+SD) from two independent experiments.
B16-F10 cells in vitro. ODNs reduce cell proliferation in both cell lines. Furthermore, they induce apoptosis in HeLa cells (it was not tested in B16-F10 cells). Most importantly, they lead to strongly reduced numbers of metastases of malignant melanoma B16-F10 cells, which had been pretransfected with ODNs before injection. Our study confirms that telomere or telomerase maintenance is a key regulatory mechanism determining the fate of cancer cells, which can be disturbed by oligonucleotides targeting the telomerase RNA component.

In the present study, we compared the effects of a set of single-stranded antisense ODNs, partially double-stranded ODNs, and double-stranded siRNAs targeted to the same region. Single-stranded antisense DNA and siRNA have been applied recently against the same region and showed some effect in all cases (Norton et al., 1996; Glukhov et al., 1998; Kondo et al., 1998; Kosciolek et al., 2003). In order to evaluate the quality of the ODNs used here, we directly compared the various methods.

In cell culture antisense DNA was less efficient than the ODNs and the efficiency of siRNA was similar to the ODN approach described here. We have observed before that the partially double-stranded nature of the DNA is superior to a single-stranded DNA in the case of HIV (Matzen et al.,...
2007), presumably due to higher resistance against nucleases. The partially double-stranded ODN proved previously to be more effective than a fully self-complementary double-stranded DNA, which was less effective in RNA–DNA hybrid formation (Moelling et al., 2006). Thus we based the design of the ODN used here on the structure, which proved most efficient in the previously described HIV case.

Previously we selected a highly conserved PPT as target for an HIV-specific ODN. We designed the second strand in order to allow triplex formation with the target sequence. We never were able to prove the formation of a triple helix, but cannot exclude such a structure, which may even form only transiently (Moelling et al., 2006). Here we target a sequence of TR, which has an average purine content. Therefore we decided to adopt the pattern of Watson–Crick bonds instead of the capacity to form triplex structures.

The ODN-mediated inhibition of cell proliferation is most likely not or not only due to shortening of the telomeres, which requires more time than the 48 h tested here (Yatabe et al., 2002). Blackburn and coworkers (Li et al., 2005) proposed that reduction of telomerase riboprotein complex expression levels rather than inhibition of telomerase function is responsible for rapid inhibition of proliferation. Most oligonucleotides targeting TR or TERT, which reduce expression levels of their target genes, cause rapid inhibition of proliferation (Folini et al., 2003; Li et al., 2004; Hao et al., 2005), whereas short PNAs, which mask the template sequence, but do not interfere with expression, lead to slow inhibition of proliferation (Asai et al., 2003). Furthermore, the observed inhibition of proliferation can be attributed to alterations in the gene expression profile upon reduction of telomerase levels as described previously (Li et al., 2005).

We demonstrated a role of cellular RNases H for the ODN-mediated reduction of TR levels in HeLa cells (Fig. 4B). Knockdown of RNase H1 or RNase H2A leads to an increase of TR levels in the ODN-hTR-transfected cells (Fig. 4C) suggesting a role of these enzymes in reduction of TR. This effect was sequence-specific, since it was not observed with irrelevant controls ODN-sc or si-neg. Incomplete knockdown of RNases H may explain the residual ODN-mediated reduction of TR levels. Overexpression of the HIV RT/RNase H only slightly enhanced the inhibition of hTR by ODN- and as-hTR (data not shown).

Antisense studies have previously demonstrated that sequence-specific RNases H-mediated effects lead not only to gene inactivation but also to translational arrest (Chan et al., 2006). Since TR is not a mRNA, a translational arrest is not involved. However, binding of the ODN to TR might interfere with the proper formation of the telomerase holoenzyme. Furthermore, TR not properly bound to TERT might be more accessible to degradation. Finally, ODN binding to TR in the holoenzyme would mask the template sequence thereby decreasing the concentration of active telomerase. The latter effect could also occur in the nucleus, where telomerase function is localized due to its nuclear localization signal in the hTR (Mitchell et al., 1999; Lukowiak et al., 2001), and where siRNA effects on hTR levels were observed as well (Koscielek et al., 2003).

Other mechanisms may be involved. Recently G-quadruplex oligonucleotides have been described to inhibit proliferation and induce apoptosis in tumor cells (Qi et al., 2006). The ODN-TRs have several G-clusters (Fig. 1 and 2), which could contribute to G-quadruplex formation. Furthermore, ODN-mTR exhibits a decreased electrophoretic mobility in native gels and an increase in the adsorption at 295 nm in comparison to its unfolded form in vitro (data not shown) typical for G-quadruplexes (Mergny et al., 2005). Antisense also have some G-clusters. In both cases we cannot exclude the potential effect of G-quadruplexes on proliferation, apoptosis, and antitumor effects.

It is conceivable that the ODNs interrupt the ends of the telomeres, which may lead to the induction of apoptosis shortly after treatment prior to any decrease in telomerase activity and telomere length. The end structure of telomeres, such as loops or single-stranded overhangs, are important triggers of cells entering senescence or apoptosis (Karslender et al., 2002). Thus, telomere or telomerase impairment in cancer cells may be mediated by several mechanisms including several ways of induction of apoptosis (Counter et al., 1998; Holt and Shay, 1999; Zhang et al., 1999; Ren et al., 2001).

In summary down-regulation of the TR by ODNs might occur in two compartments, in the cytoplasm as shown in Figure 4C, where cellular RNases H contribute to its down-regulation. In the nucleus ODNs might interfere with the telomere structure and binding of telomeric DNA to the telomerase RNA template or distort the telomerase itself. These various effects could result in reduced telomerase expression levels and activity and lead to apoptosis.

The effect of ODN-mTR was also tested in an in vivo mouse tumor model by injecting B16-F10 cells, already transfected with ODN-mTR, into the tail vein of mice. A significant reduction in the numbers of lung metastases was found by this approach (Fig. 5). Similarly as- or si-mTR-transfected B16-F10 cells also showed reduced numbers of metastases, whereby si-mTR showed a stronger effect. In summary, we demonstrate that ODNs specific for telomerase RNA may be worth combining with conventional antisense ODNs for further studies with tumor cells in general.

Note in Proof

Recently we demonstrated an antiviral effect of a similar ODN against influenza A virus (KWOK et al. 2008).

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