Photochemical Internalization of a Peptide Nucleic Acid Targeting the Catalytic Subunit of Human Telomerase¹

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Abstract

Because peptide nucleic acids (PNAs) are poorly taken up by mammalian cells, strategies need to be developed for their intracellular delivery. In the present study, we demonstrated the possibility to efficiently release a naked PNA targeting the catalytic component of human telomerase reverse transcriptase (hTERT-PNA) into the cytoplasm of DU145 prostate cancer cells through the photochemical internalization approach. After light exposure, cells treated with the hTERT-PNA and photosensitizer TPPS2a showed a marked inhibition of telomerase activity and a reduced cell survival, which was not observed after treatment with hTERT-PNA alone. In a direct comparison, photochemical internalization technology proved to be more efficient to internalize the hTERT-PNA than an HIV-Tat protein-based approach.

Introduction

Telomerase is a ribonucleoprotein that stabilizes telomere length in most tumor cells, thus preventing senescence and growth arrest (1). The enzyme consists of two main subunits: (a) the reverse transcriptase catalytic component (hTERT); Ref. 2) and (b) the RNA subunit (hTR; Ref. 2) bearing the template domain for the synthesis of telomeric repeats. Telomerase seems to play a crucial role in capping and protecting the telomere from signaling into cell cycle arrest and/or apoptosis (3). Moreover, it has recently been demonstrated that the enzyme has a pro-survival action independently of its catalytic activity (4). The notion that telomerase is involved in the attainment of immortality in cancer cells and therefore may contribute to tumorigenesis and neoplastic progression has led to its consideration as a promising target for developing new anticancer therapies (1). Successful approaches for the inhibition of telomerase have been developed by antisense oligonucleotides and PNAs (5) mainly targeting hT. PNAs are DNA mimics with a pseudopeptide backbone [composed of N-(2-aminoethyl) glycine units], which confers stability to the PNA/DNA (or PNA/RNA) duplex and affords greater resistance to protease and nuclease than unmodified oligonucleotides (5). Because naked PNAs are not efficiently taken up by mammalian cells (6), strategies have been developed for an efficient delivery to cells, some of which are based on the generation of conjugates between PNAs and import peptides (7). PNAs have also been delivered as complexes with DNA and cationic lipids or polycations (8) and by electroporation (9). It has been suggested that naked PNAs are taken up by cells through a liquid-phase endocytosis (10) and tend to accumulate in the endocytic compartment (6, 10). Therefore, the development of endosome-disruptive strategies is of great importance for allowing PNAs to reach the cytosol/nucleus and colocalize with their specific target. To this purpose, PCI represents a new approach to achieve photochemically inducible permeabilization of endolysosomal vesicles, leading to the release of endocytosed macromolecules in the cytoplasm, which has been already successfully used for intracellular delivery of protein toxins (11), plasmids (12), and adenoviral vectors (13).

In this study, we demonstrated the possibility to deliver a naked PNA targeting hTERT mRNA by the PCI technology in intact human prostate cancer cells with an efficiency superior to that obtained with an alternative strategy based on the use of the HIV-Tat internalization protein.

Materials and Methods

Cell Lines. The androgen-independent human prostate adenocarcinoma cell line DU145 and hTERT-negative human osteogenic sarcoma cell line U2OS (obtained from American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 µg/ml), at 37°C in 5% CO2 humidified atmosphere.

Chemicals. The photosensitizers AlPcS2a (aluminum phthalocyanine) and TPPS2a (meso-tetraphenylporphine) were purchased from Frontier Scientific (Logan, UT). A stock solution of AlPcS2a was made by dissolving the powder in 0.1 M NaOH and diluting in PBS to a final concentration of 5 mg/ml; a stock solution (2 mg/ml) of TPPS2a was obtained by dissolving the powder in DMSO and diluting in PBS to a concentration of 5 mg/ml; a stock solution (2 mg/ml) of TPPS2a was obtained by dissolving the powder in DMSO followed by a 5-min sonication. The photosensitizer solutions were protected from light and stored at −20°C until use.

PNA Synthesis. A 15-mer antisense PNA (5′-CCAGCCGCGACGCCT-3′) directed against the hTERT mRNA (hTERT-PNA) and a scramble sequence, used as a control, were manually synthesized using the standard method of solid-phase peptide synthesis, which follows the tert-butoxycarbonyl (Boc) strategy (14) with minor modifications (15). During the synthesis, both PNAs were labeled by direct coupling of biotin as described above for rhodamine B (Sigma Chemical) on the solid support to the NH2 terminal of the protected PNA sequences. Boc chemistry was also used for the hTERT-PNA conjugated with the HIV-Tat internalization protein (RKRRQRQR) synthesis. The PNA-peptide conjugate (Tat-hTERT-PNA) was labeled with biotin as described above for rhodamine B. All of the synthesized compounds were purified by reverse-phase high performance liquid chromatography, and their molecular weights were confirmed by electrospray mass spectrometry. PNAs were stored at −20°C and dissolved, just before use, in sterile water to the appropriate concentration. A conventional DNA oligonucleotide (PNA-like ODN) with a sequence identical to that of hTERT-PNA was obtained from MWG Biotech (Ebersberg, Germany), diluted in sterile water to the appropriate concentration, and stored at −20°C until use.
Fluorescence Microscopy. DU145 cells were seeded at a density of 1 × 10^4 cells/dish in Falcon 3001 dishes. After a 24-h incubation at 37°C in a humidified atmosphere, cells were treated for 18 h with 1, 10, and 20 μM hTERT-PNA (or scrambled PNA) in fresh medium. Cells were then washed three times with 1 ml of complete medium and incubated for 4 h in PNA-free medium. Cells were then washed three times with PBS and observed under a Zeiss Axiosplan fluorescence and phase-contrast microscope (Oberkochen, Germany). The filter combinations used to detect fluorescence from cells exposed to the single-agent treatment were the following: (a) a 365-nm band pass excitation filter, a 395-nm beam splitter, and a 610-nm long pass emission filter for samples treated with AlPcS_2a, and (b) a 546-nm band pass excitation filter, 580-nm beam splitter, and 590-nm long pass emission filter for samples treated with PNAs. For relocalization studies, DU145 cells were treated with 5 μg/ml AlPcS_2a and 10 μM PNAs and subsequently exposed to microscope light filtered through a 360–390-interference filter from 15 to 5120 s. One min later, fluorescence from PNAs was detected with the filter combinations described above. To cutoff the fluorescence signal caused by the excitation of rhodamine B and fluorescein, 16-nm beam split pass filter was also used on the emission side when fluorescence from rhodamine B was detected. Photographs from each sample were taken by a cooled charge-coupled device camera (TE2; Astromed 3200, Cambridge, United Kingdom). The images were then evaluated with an image analysis software program (analySIS PRO 3.0; Soft Imaging System GmbH, Münster, Germany).

Telomerase Activity Detection Assay. Cells were exposed to hTERT-PNA (or scrambled PNA) for 18 h, washed three times with fresh medium, and then after a 24-h incubation in PNA-free medium, harvested and assessed for telomerase activity. For PCI experiments, cells were treated with 0.7 μg/ml TPPS_2a and 2 μM hTERT or scrambled PNA for 18 h, washed three times with fresh medium, incubated for 4 h in drug-free medium, and washed once again with fresh medium. Cells were then collected and analyzed for telomerase activity. In an additional set of experiments, DU145 cells were exposed to PNA-like oligonucleotides or the use of DOTAP (Boehringer Mannheim, Mannheim, Germany). The oligomer and DOTAP were mixed in 20 μM HEPES, followed by incubation at room temperature for 15 min. Cells were then incubated in the presence of DOTAP-oligomer complexes for 18 h. The final concentration of the oligomer was 2 μM. After 48–96-h incubation in fresh medium, cells were collected and analyzed for telomerase activity.

For the detection of telomerase activity, cells were resuspended in ice-cold lysis buffer, and protein concentration of lysates was determined using the Bio-Rad assay. Telomerase activity was measured by the PCR-based TRAP assay using the TRAPEze kit (Intergen Co., Oxford, United Kingdom), as described previously (7). Products from the PCR reaction were analyzed by gel electrophoresis. Each reaction product was amplified in the presence of a 36-bp primer pair and TRAPeze kit (Intergen Co., Oxford, United Kingdom), as described previously (7).

Cell Survival Assay. Cell survival was assessed by the MTT-based assay. DU145 cells were seeded in 12-well plates (8 × 10^4/well) and treated with TPPS_2a and naked PNAs or with the Tat-hTERT-PNA, as described above. Moreover, in comparative experiments, DU145 and hTERT-negative U2OS cells were exposed for 18 h to 10 μM Tat-hTERT-PNA. Forty-eight h after each treatment, 50 μl of MTT solution (5 mg/ml) per ml culture medium were added to each well. After 4 h incubation at 37°C, the medium was removed, and 0.2 ml/well isopropanol was added to allow formazan crystals to solubilize. Absorbance at 570 nm was measured after dilution of each sample.

Results and Discussion

In the present study, we evaluated the possibility to efficiently deliver a 15-mer naked PNA targeting the catalytic component of hTR (hTERT-PNA) into DU145 human prostate carcinoma cells by the PCI approach. We first assessed the spontaneous uptake of PNA by the cells. Rhodamine B-labeling of the PNA was instrumental to follow the uptake of the molecule by fluorescence microscopy. After an 18-h exposure to 1, 10, and 20 μM rhodamine B-labeled hTERT-PNA or scrambled PNA, a strong fluorescent signal was observed in cells exposed to 10 and 20 μM hTERT-PNA, indicating that spontaneous uptake occurred at high PNA concentrations. Moreover, a typical granular distribution of PNA molecules was observed, suggesting that PNAs were taken up by cells through endocytosis and stored in endocytic vesicles. No fluorescence was detectable in samples exposed to 1 μM PNA, caused by a negligible level of signal intensity, or in untreated cells, demonstrating that the fluorescence detected was not attributable to autofluorescence of living cells (data not shown). Similar results were obtained when cells were exposed to the same concentrations of scrambled PNA, which suggests that the cellular uptake was not dependent on PNA sequence. Our finding is in agreement with the results of a previous study carried out on HIV-1 chronically infected H9 cells exposed to fluorescein-tagged PNAs targeting HIV genes, which indicated the fluorescent signal to be confined in the cytoplasm in most of the cells after an overnight incubation with 30–60 μM PNA (16). The cellular uptake of a naked PNA was also demonstrated in 504 and HL60 cells in which the kinetics of uptake of a 14-mer-[3H] PNA was compared with that of MP oligonucleoside with an identical sequence. The uptake of PNA was substantially higher than that of MP and characterized by a linear increase over 24 h (10). However, the uptake of PNAs and MP was lower than that of charged oligonucleotide analogues (phosphorothioates and phosphodiesters), probably reflecting differences in the mechanism of uptake between charged and uncharged oligomers and suggesting that PNAs, as already shown for MP, are also taken up by cells through liquid-phase endocytosis (10).

Because naked PNAs taken up by cells are relegated to the endocytic compartment, we investigated whether photochemical treatment could allow the release of PNAs from endocytic vesicles to the cytosol in DU145 cells. For fluorescence microscopy, AlPcS_2a was used because the compound has the same biological effects with respect to PCI as TPPS_2a but with spectral properties less overlapping those of rhodamine B than TPPS_2a (17, 18). However, TPPS_2a is preferable in most in vitro studies because of the lower concentration and light dose required to induce PCI. Nonetheless, the fluorescence signal from PNA was lower when the cells were cotreated with AlPcS_2a. Fluorescence spectroscopic analysis confirmed that this was caused by quenching of the rhodamine B signal and not fluorescence resonance energy transfer from rhodamine B to AlPcS_2a (data not shown). Despite the quenching of the signal, fluorescence microscopy results obtained in cells exposed to 5 μg/ml AlPcS_2a and 10 μM PNAs showed colocalization of the two molecules, thus confirming that PNA uptake occurred through endocytosis (Fig. 1). To evaluate the ability of light treatment to induce intracellular relocalization of PNAs, DU145 cells were exposed to microscope light filtered through a UV filter for 15–120 s, and, after 1 min in the dark, fluorescence generated from the photosensitizer and rhodamine B-labeled PNAs was followed. Results indicated that light exposure induced the relocalization of PNAs and photosensitizer from endocytic vesicles to cytosol, even at the shortest exposure time. Moreover, to some extent, PNAs were also able to enter the nucleus after light treatment, whereas AlPcS_2a showed a typical cytosolic distribution (Fig. 1). The cytosolic release of other cancer-specific molecules by PCI has been already demonstrated. Specifically, a fluorescein-labeled p21^waf1 peptide was found to be diffusely located in the cytosol of BL2.6-E6 mouse fibroblasts treated with AlPcS_2a and exposed to red light for 4 min (11). Similar results were obtained in THX melanoma cells by using a fluorescein-labeled, 60-base oligodeoxynucleotide (12). The
efficiency of PCI technology as a delivery system was also established in NHK 3025 cells treated with gelolin in combination with TPPS2a and light (11). In these cells, the effect of gelolin treatment combined with PCI resulted in a >300-fold reduction of protein synthesis as compared with each treatment alone. Moreover, the effect of gelolin internalized by photochemical treatment was demonstrated in vivo in BALB/c mice bearing WiDr tumors. In animals treated with gelolin followed by PCI, the tumors were almost completely eradicated 20 days after the treatment, and a complete response was obtained in 67% of the treated tumors (19).

In the present study, the ability of hTERT-PNA to inhibit telomerase activity as a function of the modality of delivery of the oligomer was also evaluated. At first, cells were exposed for 18 h to 2 μM naked hTERT-PNA or scrambled oligomer, and, after 48 h in PNA-free medium, telomerase activity was determined. TRAP results indicated a lack of enzyme inhibition by naked PNA treatment (Fig. 2A) according to localization of the PNAs, which, after spontaneous cellular uptake, were found to be entrapped in endocytic vesicles. We then evaluated the consequence of light-induced relocalization of PNAs on their antitelomerase activity. TRAP results obtained at different intervals after treatment of cells with TPPS2a and 2 μM hTERT-PNA, for 18 h and exposure to blue light for 60 s, showed a significant (P < 0.01, Student’s t test) reduction of enzyme catalytic activity at all time points (Fig. 2A). Such a reduction was maximum at 6 h after treatment (8.4 ± 0.79% of control) and slightly less pronounced (~30–35% of control) at later intervals (24 and 48 h; Fig. 2B). The greater extent of telomerase inhibition obtained in PCI experiments than in those carried out with naked PNAs without light exposure could reflect the higher bioavailability of PNAs at the level of their target (hTERT mRNA within cells). Conversely, TRAP results obtained in cell samples treated with hTERT-PNA and TPPS2a, but not exposed to light, failed to evidence any appreciable inhibition of telomerase activity (Fig. 2B). As expected, no telomerase inhibition was obtained after treatment of cells with the scrambled PNA, independently of light exposure (Fig. 2B).

In an additional step of the study, we evaluated the antitelomerase activity of a chimeric PNA molecule, made by conjugating the hTERT-PNA to the HIV-Tat internalization peptide (Tat-hTERT-PNA). The results of the TRAP assay carried out at different intervals after an 18-h exposure of cells to 2 μM Tat-hTERT-PNA indicated a very modest effect on telomerase activity, which was only appreciable at 48 h (73.3 ± 3% of control) and significantly lower (P < 0.02, Student’s t test) than that obtained with the hTERT-PNA and light exposure (Fig. 2B) at the same time point. Such results would demonstrate that the PCI approach represents a more efficient system for the delivery of PNAs than conjugation with internalization peptides.

In this context, we observed previously that to obtain a 50% inhibition of telomerase activity after an overnight exposure of OAW42 human ovarian carcinoma cells to the same Tat-hTERT-PNA, a concentration ~4-fold used for the PCI of PNAs was required (5). We also demonstrated that a 24-h exposure of JR8 human melanoma cells to a chimeric molecule made by coupling a 13-mer PNA, targeting the hTR component of telomerase, to the Antennapedia cell-penetrating peptide was able to induce a 50% inhibition of telomerase activity only at concentrations >30 μM (7).

To evaluate whether the greater resistance of PNA molecules to degradation compared with conventional oligonucleotides was reflected in a more prolonged inhibition of telomerase activity, we comparatively assessed telomerase catalytic activity in DU145 cells at different intervals (48–96 h) after an 18-h exposure to the photochemically internalized hTERT-PNA or PNA-like ODN delivered to cells by a DOTAP-mediated approach. Results from TRAP experiments (Fig. 3) indicated that a reduction of telomerase activity (from 39.4 ± 3.5% to 60.3 ± 8.1% of control) was appreciable ≈96 h after...
DU145 cells to photochemically internalized hTERT-PNA but not to Tat-hTERT-PNA would suggest that such an antiproliferative effect is, at least in part, the consequence of a prolonged telomerase activity inhibition. To further demonstrate that the decline of cell survival was a direct consequence of hTERT targeting by the PNA, we comparatively evaluated the effect induced by Tat-hTERT PNA in DU145 cells and the hTERT-negative U2OS cell line. Specifically, 48 h after an 18-h exposure of DU145 cells to 10 μM Tat-hTERT-PNA (which was able to decrease the level of telomerase activity by 60%), we found a 40 ± 5% reduction in cell survival compared with untreated cells. Conversely, no inhibition of cell survival was detected in U2OS cells exposed to the same Tat-hTERT-PNA concentration (data not shown).

Several reports have demonstrated a significant inhibition of telomerase activity by using PNAs that target the RNA component of hTR and delivered to intact cells with different modalities, including electroporation (9) and hybridization of PNAs, with the appropriate DNA oligonucleotide (8). However, in these studies, inhibition of cell proliferation was detectable only after several cell generations, as a consequence of telomere shortening. Such results are in accord with the classical mechanism by which telomerase inhibition induces a delayed cell growth impairment/arrest as a consequence of slow telomere shortening. The results we describe herein represent the first attempt to inhibit telomerase activity by using a PNA directed against hTERT mRNA. Moreover, we showed a marked antiproliferative effect in DU145 cells after only 2 days of treatment with the photochemically internalized hTERT-PNA. These findings are in line with a recent hypothesis about the role of telomerase in cell growth and apoptosis control. Specifically, an additional mechanism by which telomerase inhibition could lead to cell growth arrest has been proposed. Such a mechanism, which does not require telomere shortening, is probably attributable to the interference of telomerase inhibitors with the capping functions of telomerase (3).

There is substantial evidence which suggests that telomeres normally exist in a capped state but may switch to an uncapped state. The appropriate response to the uncapping of a telomere is action by telomerase to protect the telomere from signaling into cell cycle arrest/apoptosis pathways (3). On the basis of these suggestions, it could be hypothesized that, when there is marked inhibition of telomerase activity, the enzyme is no longer able to protect the telomere, and cells can die through a mechanism independent of telomere length. Such a possibility is consistent with the growth inhibition effect we observed in hTERT-PNA-treated DU145 cells as well as...
with results recently reported by Saretzki et al. (20), who demonstrated a massive cell death in four human ovarian cell lines 3 days after transduction with an adenoviral vector carrying a ribozyme sequence directed against hTERT mRNA.

Overall, our results demonstrate for the first time that: (a) the inhibition of telomerase activity can be efficiently achieved by a PNA directed against the catalytic subunit of the enzyme; and (b) the PCI approach represents a new and more efficient system for the internalization of naked PNAs that makes it possible to reduce the amount of PNA required to induce the biological effect compared with other delivery systems, such as those based on internalizing peptides. Such a new delivery approach could be exploited for in vivo applications of PNAs. In fact, the actual efficiency of PNAs as antisense molecules has been mainly determined in in vitro cell systems and needs to be validated also in animal experimental models before these molecules can be added to the clinical armamentarium.

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References

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