ISSN 0026-8933, Molecular Biology, 2008, Vol. 42, No. 2, pp. 253–260. © Pleiades Publishing, Inc., 2008.
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CELL MOLECULAR BIOLOGY

UDC 577.352.27,77.29,57.352.4,577113

Complexes of Telomeric Oligonucleotides with the PGEk Protein Vector: Internalization by Target Cells and Antiproliferative Activity

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Received September 20, 2007
Accepted for publication October 11, 2007

Abstract—Recombinant protein PGEk, consisting of the human epidermal growth factor (hEGF) and a DNA-binding domain, proved to be capable of interacting with the hEGF receptor and inducing cell proliferation, as characteristic of hEGF. PGEk complexes with the telomere-mimic oligonucleotide d(TTAGGG)$_4$ (TMO) and its thio analog (TMS) were efficiently and selectively internalized by cells with a high-level expression of the hEGF receptors. The extent of internalization was studied as a function of the PGEk : oligonucleotide ratio in the complex. The intracellular location of the oligonucleotides was determined. PGEk was found to ensure a more efficient delivery of the oligonucleotides and to protect them from nuclease degradation. The oligonucleotides contained in the complexes exerted a far greater cytotoxic effect as compared with the free oligonucleotides.

DOI: 10.1134/S0026893308020118

Key words: protein vectors, targeted DNA delivery, oligonucleotides, telomerase, protein–nucleic acid complexes, epidermal growth factor

INTRODUCTION

Medical application of DNA and RNA fragments is limited notwithstanding their great therapeutic potential. New means of delivering foreign genetic material are intensely developed taking account of the specifics of oligonucleotides as therapeutic agents, and various oligonucleotide derivatives and structural analogs are designed to reduce the effective therapeutic dose [1–6].

The application specifics and metabolism have been studied in most detail for phosphothioate oligonucleotide derivatives. Such derivatives are close in hybridization properties to phosphodiester analogs, but have appreciably higher affinity for membranes and are more resistant to nucleases. Thio analogs are effective as antisense oligonucleotides, although their systemic toxicity is rather high [7–12]. Yet modification of the sugar-phosphate backbone can induce functionally significant changes in the DNA secondary structure because of the nature of the thiophosphoryl bond. We thought it important in view of this to study the structure and to compare the properties for the natural phosphodiester oligomer d(TTAGGG)$_4$, which is a thelomeric DNA fragment (a telomere-mimic oligonucleotide, TMO), and its phosphothioate analog (TMS). Oligonucleotides consisting of human telomeric repeats presumably interact with several intracellular targets, including mRNA and telomerase [8, 9]. Phosphodiester TMO, forming an intramolecular G-quadruplex, inhibits telomerase [13, 14]. The TMO quadruplex probably acts as a telomerase trap. The inhibitory potential is enhanced when the quadruplex structure is stabilized with low-molecular-weight ligands, e.g., intercalating agents [13, 14].

To improve the biological activity of oligonucleotides, special transport systems are designed for their delivery into the cell. In addition to cationic lipofection, some amphiphilic peptides producing membranotropic complexes with oligonucleotides provide an efficient means for their delivery [3, 15–20]. Such peptides have DNA-binding and membranotropic domains. Some peptides protect the oligonucleotides from intracellular degradation when used in a high molar excess (peptide : oligonucleotide > 20 : 1) [16]. The main drawbacks are that peptide vectors are insufficiently selective for particular cells [16–20] and that the use of new peptides and viral protein fragments may have unpredictable biological consequences.

Unique properties are inherent in some protein DNA carriers, which attract increasing attention as new therapeutically important properties have been revealed for oligonucleotides (siRNA, aptamers, DNA–RNA hybrids, etc.). Their selective transport is based on the differences in the tissue composition of cell surface receptors or antigens and their changes in pathology. Targeted delivery of a complex or an oligonucleotide–peptide conjugate is due to the interaction of the protein component with the surface receptors of target cells. Growth factor fragments are often used as efficient targeting ligands and ensure a selective effect on actively proliferating (e.g., tumor) cells [1, 3, 4, 6, 21, 22]. However, the use of known constructs is hindered by several factors. For instance, conjugates of the epidermal growth factor (EGF) with polycations produce poorly soluble complexes of unacceptable geometrical dimensions with DNA and multidomain proteins are immunogenic [23–26].

We have earlier constructed a simple protein vector, PGEk (Protein Gene carrier based on EGF, 64 amino acid residues), which combines natural hEGF and an oligocationic nuclear localization signal (NLS). PGEk is capable of delivering plasmid DNA and oligonucleotides into target cells [27]. Physicochemical analysis has been used to estimate the thermodynamic parameters of PGEk complexation with oligonucleotides under physiological conditions. Adsorption of the first two PGEk molecules on TMO and TMS follows a noncooperative mechanism and is characterized by high association constants: $K_{a1(TMO)} = (7 \pm 1) \times 10^7$ M^{-1} and $K_{a1(TMS)} = (3 \pm 0.5) \times 10^7$ M^{-1}, respectively. Further adsorption (up to five or six PGEk molecules) on TMO is highly cooperative and has $K_{a2(TMO)} = (4.0 \pm 1.5) \times 10^6$ M^{-1}. Unlike TMO, TMS weakly binds the third and next PGEk molecules ($K_{a2(TMS)} \le (8 \pm 2) \times 10^5$ M^{-1}). Circular dichroism spectroscopy has shown that PGEk partly unfolds the G-quadruplex formed by TMO and has no effect on the single-stranded structure of TMS [28].

The objectives of this work were to study, with the example of TMO and TMS, the internalization of oligonucleotide–PGEk complexes varying in stoichiometric composition by target cells and to estimate the effect of the protein vector on the cytotoxic properties of the oligonucleotides.

EXPERIMENTAL

PGEk complexes with oligonucleotides. Recombinant PGEk was obtained as in [27] and was characterized by HPLC (an Agilent 1100 chromatograph, United States), MALDI TOFF mass spectrometry (M/z = 7623 ± 5 Da), and proteome analysis (an UltraFlex spectrometer, Bruker). Oligodeoxyribonucleotides were synthesized on an ASM 800 automated DNA synthesizer (Biosset, Russia) by the solid-phase method, using commercial nucleosides attached to a polymeric carrier and amidophosphite derivatives of nucleosides and fluorescein (5'-(6)-FAM amidite, Glen Research). To synthesize the phosphothioate TMS oligomers and the 5'-(6)-FAM TMS derivative (FTMS), 0.05 M 3H-1,2-benzodithiol-3-one-1,1-dioxide (Glen Research) in acetonitrile was used in place of the standard oxidizer. After ammonolysis (5 h, 50°C), the oligonucleotides were purified by HPLC (an Agilent 1100 chromatograph, Diasorb C16T column, 4 × 250 mm, Elsiko Lab, Russia, an acetonitrile concentration gradient in 0.1 M triethylammonium acetate aqueous solution, 50°C) and characterized by analytical HPLC, UV spectrophotometry (a Jasco V-550 spectrophotometer, Japan), and MALDI TOFF mass spectrometry (a MicroFlex mass spectrometer, Bruker). The oligonucleotide and PGEk concentrations were measured spectrophotometrically, using the molar extinction coefficient ε_{260} = 11000 M^{-1}1 cm^{-1} at 90°C for d(TTAGGG)$_4$ and computed ε_{280} = 19780 M^{-1}cm^{-1} for PGEk [29].

To obtain complexes, PGEk (40–100 μM in 20 mM Tris-HCl, pH 8, 0.17 M NaCl) was combined with oligonucleotides at necessary molar ratios and incubated at room temperature for 60 min.

Cell culture. We used the human cell lines SKOV3 (ovarian adenocarcinoma), HeLa (cervical carcinoma), and MCF7 (breast adenocarcinoma) and 3T3 mouse embryo fibroblasts. All cell lines were from the collection of the All-Russian Center of Molecular Diagnosis and Therapy. Cells were cultured in plastic flasks (Corning-Costar) in DMEM (Gibco) supplemented with 10% bovine fetal serum (FBS, Gibco) and 50 μg/ml gentamicin (Sigma) at 37°C in a moist atmosphere containing 5% CO_2. Cell passages were performed twice weekly, using trypsin–EDTA (0.05% trypsin, 0.02% EDTA) (Sigma).

Analysis of endocytosis of PGEk complexes with TMO and TMS by flow cytometry. SKOV3 cells of a logarithmic culture were washed twice with phosphate-buffered saline (PBS), incubated in DMEM without FBS for 2 h, harvested, and washed twice with PBS again. PGEk complexes with fluorescently labeled TMO or TMS (200 nM oligonucleotide, PGEk : oligonucleotide ratio from 1 : 1 to 4 : 1) were added to the cell suspension, and the mixture was incubated at 37°C for 1 h. Cells were washed thrice with PBS at 4°C and fixed with 2% paraformaldehyde. Fluorescence intensity was measured on an EPICS-XL flow cytometer (Beckman-Coulter) with excitation with an argon laser (Coherent) at 488 nm and emission at 515–520 nm. We examined 10^4 cells for each sample. Mean fluorescence intensity (in conventional units) was calculated using the XL SYSTEM II program (Bechman-Coulter).

Effect of PGEk–oligonucleotide complexes on the proliferative activity of synchronized fibro-

blasts. 3T3 mouse fibroblasts were plated in 96-well plates at 7000 cells/well. After 1 day, the medium was removed, cells were washed twice with FBS-free DMEM, 200 μl of DMEM containing 0.1% FBS were added to each well, and the cultures were incubated for 3 days to synchronize the cells in G1. Synchronized cells were supplemented with 10^{-12}–10^{-8} M hEGF, PGEk, PGEk–TMO, or PGEk–TMS (three wells for each concentration) and incubated under the standard conditions for 19 h. Two hours before the end of incubation, cells were supplemented with 1 μCi/ml [^{3}H]thymidine (Izotop, Russia, 50 Ci/mmol). Cells were harvested with trypsin–EDTA and transferred onto a filter with a Titertek semiautomated cell collector (United States). Radioactivity was counted using a RackBeta liquid scintillation counter (LKB). Cell stimulation was estimated relative to a nontreated control.

Cytotoxicity of PGEk complexes with TMO and TMS. MCF7 and HeLa cells were plated in a 96-well plate 1 day before the experiment. Cells were supplemented with 6–500 nM (oligonucleotide concentration) complexes or free oligonucleotides in triplicate and incubated under the standard conditions for 5 days. Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [30]. Four hours before the end of incubation, 50 μl of 1 mg/ml MTT (Sigma) in the culture medium was added to each well. After color development, the medium was removed, the resulting formazan crystals were dissolved in 100 μl of dimethyl sulfoxide, and A_{540} was measured using a plate spectrophotometer (Labsystems, Finland). Cell viability was estimated relative to a nontreated control.

Analysis of endocytosis of PGEk complexes with TMO and TMS by fluorescence microscopy. SKOV3 cells were seeded on coverslips in 24-well plates one day before the experiment. PGEk complexes with FTMS and FTMO (PGEk : oligonucleotide ratio from 1 : 1 to 5 : 1, 200 nM oligonucleotide) were added to the cells, and the culture was incubated for 24 h. Cells were washed thrice with PBS and fixed with 4% paraformaldehyde. Cell nuclei were stained with 5 μM Hoechst 33342 (Sigma) in BPS for 5 min. Cells were embedded in moviol. Fluorescence was examined using an Opton IM35 microscope (Carl Zeiss) at magnification × 400, using filters G450, FT510, and LP515–565 for FITC and G365, FT395, and LP420 for Hoechst. Images were obtained using a DXM 1200 digital camera (Nikon).

RESULTS AND DISCUSSION

Structure of the PGEk Recombinant Protein Vector

The PGEk vector [27] consists of 64 amino acid residues, of which 11 correspond to the NLS motif: NLS: KKKKRKVEDPYNSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR. Region 12–64 is identical in sequence to hEGF. The PGEk purity is no less than 97% by HPLC.

Mitogenic Activity of hEGF, PGEk, and PGEk–Oligonucleotide Complexes

Stimulating cell proliferation in a dose-dependent manner, EGF acts as a mitogen on many cells of the epithelial and epidermal origins [31]. The mitogenic activity of hEGF, PGEk, and PGEk complexes with TMO and TMS was inferred from the [^{3}H]thymidine incorporation in DNA of synchronized 3T3 fibroblasts. The observed dependences showed that hEGF and PGEk stimulated cell proliferation to the same extent (1300% relative to the nonstimulated control); i.e., N-terminal modification of hEGF exerted no effect on its mitogenic activity (Fig. 1).

The mitogenic activity of PGEk complexed with the oligonucleotides depended on the PGEk content in the complex. The highest activity was observed at a PGEk : oligonucleotide ratio of 1 : 1. As the PGEk : TMO ratio was increased to 5 : 1, the cell proliferative activity decreased in proportion (Fig. 1a). The mitogenic activity of PGEk–TMS complexes similarly decreased with increasing PGEk : oligonucleotide ratio, but also in the interval from 1 : 1 to 3 : 1, which agreed with the stoichiometry of complexes with TMO and TMS [29].

These dependences testify that the complexes specifically bind to the EGF receptors and suggest that only one EGF residue interacts with the receptor, while the complex acts as a single modified ligand X-EGF, where X = DNA + (n – 1) PGEk molecules.

Since free hEGF and PGEk contained in equimolar complexes with the oligonucleotides stimulated cell proliferation to the same extent, the receptor-binding site of the EGF domain of PGEk did not experience a functionally important structural alteration in the complexes.

Internalization of TMO and TMS Complexed with PGEk as Revealed by Flow Cytometry and Fluorescence Microscopy

To estimate the efficiency of oligonucleotide delivery, we compared the fluorescence intensity for cells incubated with the free oligonucleotides and the oligonucleotides complexed with PGEk. Experiments were performed with the 5'-endlabeled fluorescent derivatives FTMO and FTMS. Since the fluorescent label could affect the oligonucleotide properties, we ascertained in preliminary experiments that the introduction of 6-FAM did not appreciably change the oligonucleotide conformation and binding with PGEk [28, 29].

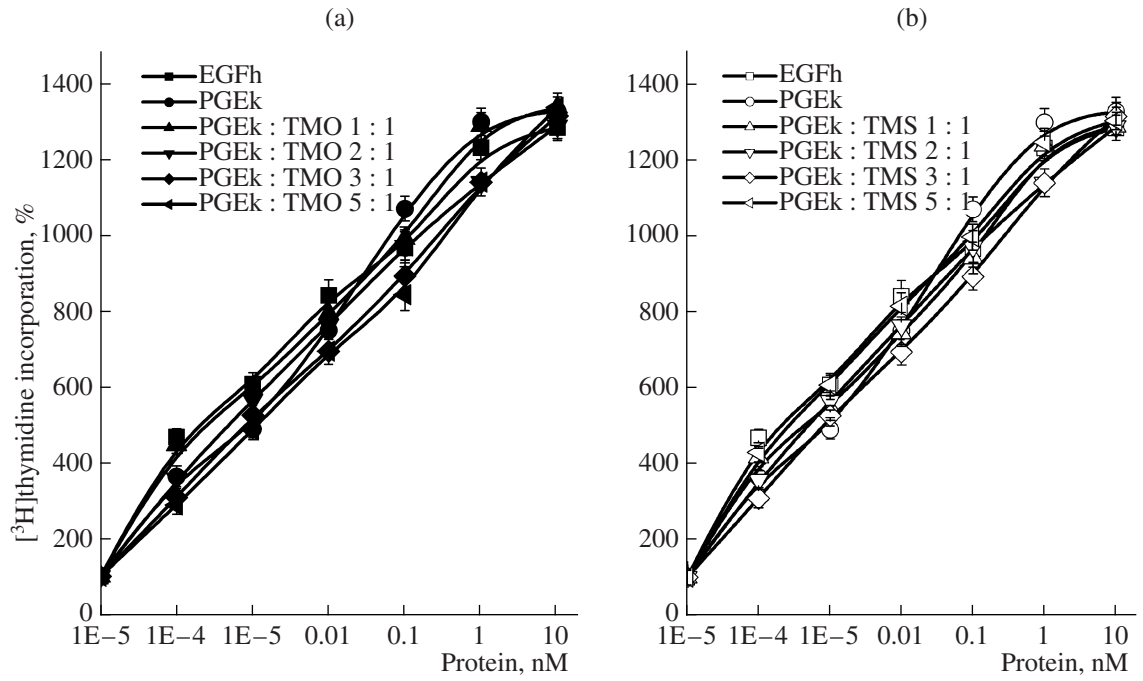

Fig. 1. Stimulation of proliferative activity of synchronized 3T3 mouse embryo fibroblasts by hEGF, PGEk, and PGEk complexes with (a) TMO and (b) TMS as estimated from the [^{3}H]thymidine incorporation in DNA. The activity of nonstimulated control cells was taken as 100%.

SKOV3 cells were incubated with free FTMO and FTMS and their complexes with PGEk at 37°C for 1 h, and the level of endocytosis was estimated by flow cytometry (Fig. 2). The fluorescence intensity of cells treated with the complexes having the equimolar PGEk : oligonucleotide ratio substantially (3- to 10-fold) exceeded that of cells treated with free FTMO or FTMS and remained almost unchanged as the ratio was increased to 4 : 1.

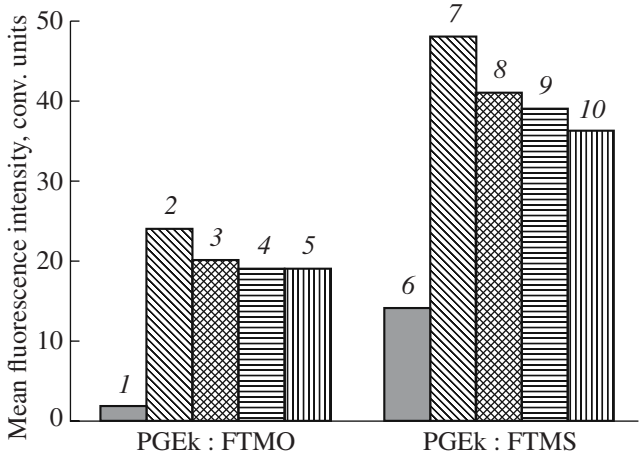

Fig. 2. Accumulation of FTMO, FTMS, and their complexes with PGEk by SKOV3 cells in 1-h incubation at 37°C as revealed by flow cytometry: *1*, FTMO; *2–5*, PGEk–FTMO with a ratio of (*2*) 1 : 1, (*3*) 2 : 1, (*4*) 3 : 1, or (*5*) 4 : 1; *6*, FTMS; *7–10*, PGEk–FTMS with a ratio of (*7*) 1 : 1, (*8*) 2 : 1, (*9*) 3 : 1, or (*10*) 4 : 1.

This result demonstrates that one vector molecule suffices to significantly increase the efficiency of oligonucleotide delivery and that the interaction with the EGF receptor is mediated, indeed, by only one PGEk molecule of a complex. The difference in internalization between FTMO and FTMS can be explained by the fact that the latter is more lipophilic and, consequently, capable of additional nonspecific transfer across the cell membrane and is more resistant to nucleases (Fig. 2).

When cell incubation was prolonged to 24 h, the accumulation of the labeled oligonucleotides in SKOV3 cells substantially depended on the PGEk content in the complexes and the oligonucleotide character (Fig. 3).

For instance, fluorescence was weak in cells treated with 200 nM FTMS for 24 h (Fig. 3b) and virtually undetectable in cells treated with FTMO (Fig. 3a). When cells were treated with PGEk–FTMO and PGEk–FTMS complexes, fluorescence was distinct and increased with increasing PGEk : FTMO molar ratio from 1 : 1 to 5 : 1 (Figs. 3c, 3e, 3g, 3i). In the case of FTMS, cell fluorescence was significantly higher than the control at the equimolar PGEk : FTMS ratio, increased with the ratio increasing to 3 : 1, and

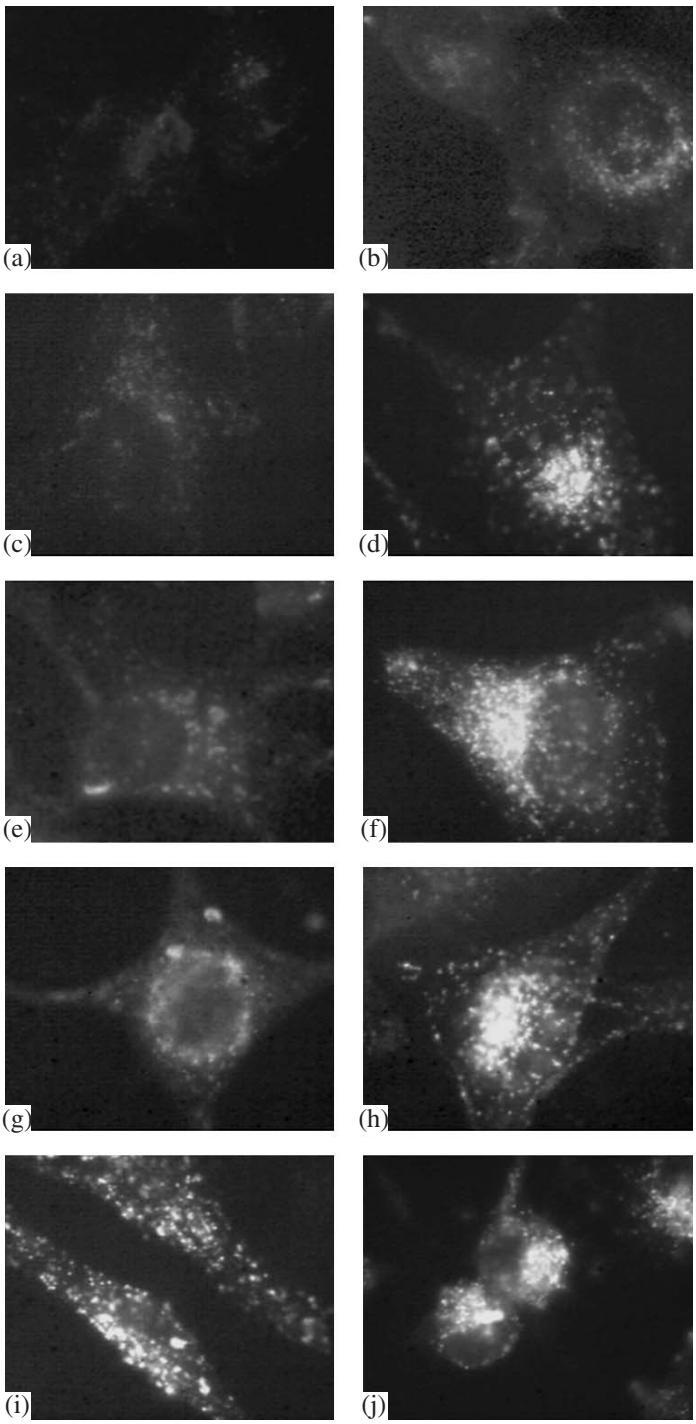

Fig. 3. FTMO and FTMS fluorescence in SKOV3 cells incubated for 24 h with (a) free FTMO, (b) free FTMS, (c, e, g, i) PGEk–FTMO, and (d, f, h, j) PGEk–FTMS. The PGEk : oligonucleotide ratio was (c, d) 1 : 1, (e, f) 2 : 1, (g, h) 4 : 1, and (i, j) 5 : 1. Fluorescence microscopy, ×40 objective.

was maximal at a greater excess of the protein (Figs. 3d, 3f, 3h, 3j).

A weaker fluorescence of cell treated with PGEk–FTMO compared with PGEk–FTMS can be explained by rapid degradation of phosphodiester oligonucleotides. Indeed, more stable FTMS efficiently accumulated in cells already in complexes with one to three PGEk molecules. FTMO was efficiently internalized in complex with one or two PGEk molecules, as found above (Fig. 2). However, it was only in complex with five PGEk molecules that FTMO was protected from degradation. This agrees with thermodynamic data:

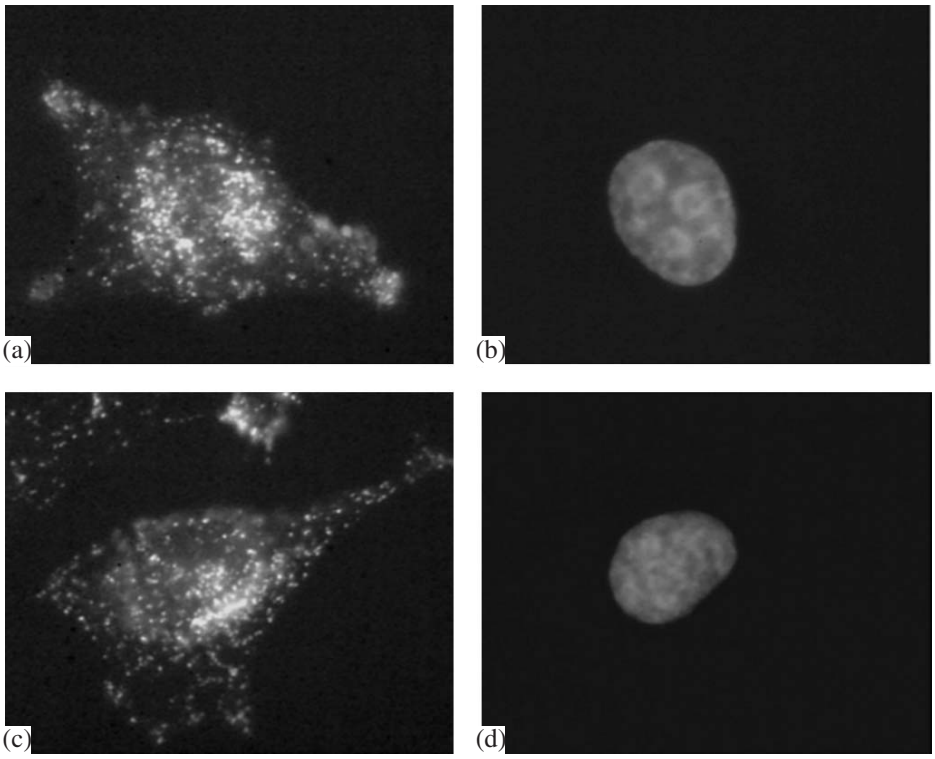

Fig. 4. Fluorescence distribution in SKOV3 cells incubated with the PGEk–FTMS complex for 24 h. (a, c) FTMS fluorescence; (b, d) Hoechst 33342 fluorescence (cell nuclei). FTMS is found in the (a, b) nucleus and (c, d) cytoplasm.

TMO is capable of binding with five or six PGEk molecules at maximum [29].

Intracellular Localization of d(TTAGGG)$_4$

Our data are insufficient for detailing the intracellular behavior of the PGEk–oligonucleotide complexes. Experiments with the fluorescently labeled oligonucleotides showed that the delivery in complex with PGEk did not prevent their transport into the nucleus. Fluorescence of labeled nucleotides is shown in Figs. 4a and 4c; fluorescence of cell nuclei stained with Hoechst 33342 in the same cells is shown in Figs. 4b and 4d. It is seen that the labeled oligonucleotides were localized in both the cytoplasm and nuclei of cells incubated with the complexes for 24 h (Figs. 4c, 4d).

Cytotoxicity of PGEk Complexes with TMO and TMS in Vitro

Both phosphodiester and modified oligonucleotides containing the d(TTAGGG) motif inhibit telomerase [8–11]. However, TMO did not exert a cytotoxic effect and TMS nonselectively suppressed cell viability only in millimolar concentrations.

The PGEk : oligonucleotide ratio in the complexes was based on the results of model experiments with the fluorescently labeled oligonucleotides (see above). Incubation with the PGEk–TMS complex for 5 days led to death of HeLa cells (2×10^4 receptors per cell [34]). The complex concentration suppressing the cell viability by 50% (IC_{50}) was 250 nM, tenfold lower than IC_{50} of free TMS (Fig. 5). In the case of MCF7 cells (3×10^3 receptors per cell [35]), IC_{50} of the PGEk–TMS complex was twofold higher (about 500 nM). Almost the same antiproliferative effect was observed for the PGEk–TMO complex (Fig. 6), whereas free TMO was inactive up to 5 μM (data not shown). This fact is the first to provide grounds for the efficient gene therapeutic application of nontoxic phosphodiester DNA fragments.

The regularities observed with the complexes differing in composition agree with the results of the model experiments with the delivery of the fluorescently labeled oligonucleotides. The antitumor activity of TMS substantially increased in the equimolar complex with PGEk and remained virtually unchanged after a three- to fivefold excess of PGEk was achieved (data not shown).

The mechanism sustaining telomerase inhibition by telomere-mimick oligonucleotides is poorly understood. Two binding sites for single-stranded telomere substrates (18 nt) have recently been identified in telomerase: one is in the catalytic subunit and the other is at the substrate recognition site of the telom-

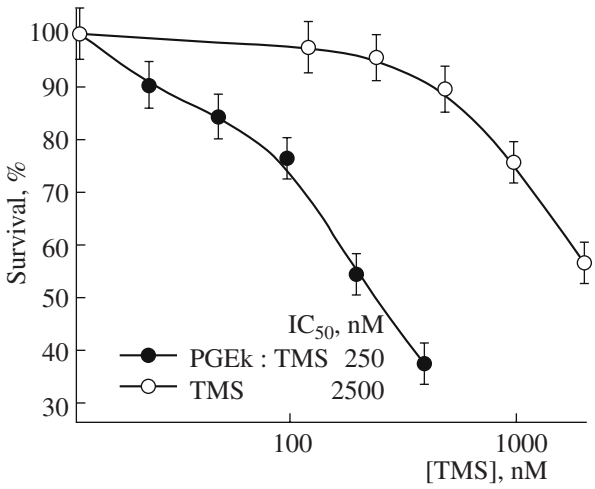

Fig. 5. Survival of HeLa cells after 5-day incubation with TMS and its complex with PGEk (PGEk : TMS = 4 : 1). Here and in Fig. 6, the survival of a control culture was taken as 100%.

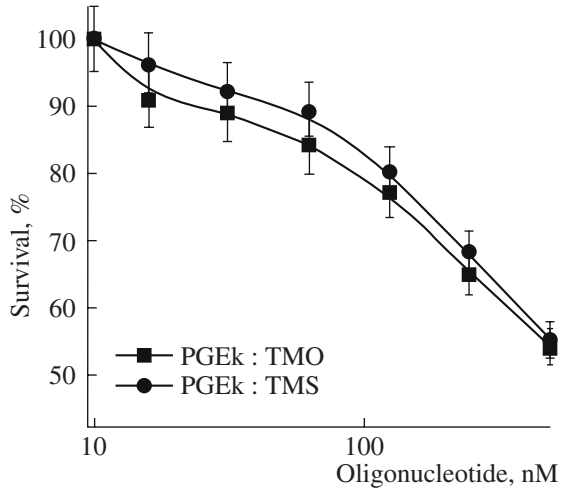

Fig. 6. Survival of MCF7 cells after 5-day incubation with PGEk–TMO and PGEk–TMS complexes (PGEk : oligonucleotide = 5 : 1).

erase RNA. The constant of the binding with the protein subunit is one order of magnitude higher than that of the complementary substrate–RNA interactions [36]. Our results indirectly support the assumption that the antitumor activity of TMO and TMS is similarly due to several different interactions.

To exert their biological effect, TMO and TMS have to pass through the cell membrane and reach their intracellular molecular target. An oligonucleotide released from the transport complex is capable of passive diffusion into the nucleus, bypassing the nuclear pore complex. If this were the case, degradation of phosphodiester TMO in the cytoplasm would reduce its cytotoxic effect at any PGEk excess in the complex, which disagrees with our experimental results. It seems that PGEk complexes with TMO and TMS are capable of NLS-mediated nuclear translocation, as other known nucleoprotein complexes [19, 32, 33].

Another assumption awaiting experimental verification is that the oligonucleotide contained in the complex is rendered available for interactions and can be trapped by an affinity molecular target.

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