Intracellular Localization of Natural and Modified Oligonucleotides in Primary Human Endothelial Cells

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Intracellular localization of natural and fluorescent-labeled oligonucleotides in primary human endothelial cells was studied by means of fluorescence microscopy and radioisotope analysis. Transport and distribution of oligonucleotides in endotheliocytes depended on their structure and resistance to hydrolysis under the effect of cell nucleases. Modification of 5'-terminal phosphate and 3'-terminal oligonucleotide increased the stability and ensures nuclear localization of oligonucleotides in cells.

Key Words: *endothelial cells; intracellular localization; deoxyribooligonucleotides*

Deoxy- and ribooligonucleotides can inhibit gene expression due to complementary interactions *in vitro* and in mammalian cells [3,7,8]. For the development of chemotherapeutic drugs based on oligonucleotides (ON) new information about their transport in cells is required. It is known that ON administered via different routes are transported with the blood and enter various organs within the first 5-10 min [1]. Hence, vascular endothelial cells are in constant contact with ON. Intracellular localization of ON can be determined by means of fluorescence microscopy and radioisotope analysis.

This work was designed to compare transport of natural and fluorescein- modified deoxyribo-ON in human endotheliocytes by means of fluorescence microscopy and radioisotope analysis and to evaluate the effects of cell nucleases on the transport and intracellular distribution of ON and their fragments.

MATERIALS AND METHODS

Experiments were performed with ON synthesized on an ASM-700 automatic synthesizer by the phosphoroamide method using deoxyuridine phosphoramidite modified with aminohexofluorescein (Glen Research) by the 5th position of nitrogen base and (dimethoxytrityl)-diethyleneglycol-β-(β-cyanethyl) (*N*, *N*-diisopropyl) phosphoramidite ribouridine [4]. A radioactive label was introduced into ON by means of 5-terminal phosphate exchange using T4-polynucleotide kinase. The following ON were used: pTACAGTAAATATCTAGGAATGdegrU (pN_{21}degrU), pTACAGT^{Flu}AAATATCTAGGAATGdegrU ($pN_5T^{Flu}N_{15}$degrU), and Flu-Lys-pTACAGTAAATATCTAGGAATGdegrU (Flu-Lys-pN_{21}degrU). Primary human endothelial cells were isolated from the umbilical vein, cultured, and characterized by expression of E-selectin and von Willebrand factor [5]. To study stability and transport of ON, the cells were transferred to wells of 8-well plates 2 days before the study. Cell viability was estimated by trypan blue staining.

Before addition of radioactive ON, the cells were washed with DMEM and incubated with 0.5 or 2.5 μM ON in DMEM at 37°C and 5% CO_2. After incubation, the cells were washed 2 times with phosphate buffered saline, harvested with 0.1% collagenase in a buffer with 2.5 mM EDTA, and pelleted by centrifugation at 1200 rpm for 10 min. The cell pellet was separated into the membrane-cytosolic (MCF) and nuclear fraction (NF). Radioactivity of fractions was measured [3].

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For the analysis of distribution of ON by fluorescence microscopy, endothelial cells (<70% confluence) were incubated with fluorescent-labeled ON and preparations were made as described previously [6].

RESULTS

Fluorescence microscopy showed that Flu-Lys-pN_{21}degrU is accumulated in the nuclei of endothelial cell. Increasing the time of incubation was accompanied by an increase in fluorescence of cell nuclei (Fig. 1). Incubation of cells with $pN_5T^{Flu}N_{15}$degrU was mainly followed by cytoplasmic and perinuclear localization of the fluorescence signal. The distribution of fluorescence in cells remained unchanged in experiments with $pN_5T^{Flu}N_{15}$degrU, but the signal intensity increased with lengthening of cell incubation in the presence of ON (Fig. 1).

During cell incubation with 0.5 μM [^{32}P]-labeled ON, ^{32}P accumulation in cell fractions depended on the structure of ON and time of incubation with ON (Table 1). Increasing the time of cell incubation with pN_{21}degrU was accompanied by ^{32}P accumulation in MCF and NF. Experiments with Flu-Lys-pN_{21}degrU showed that ^{32}P content in cell fraction decreased with increasing the incubation time. Accumulation of $pN_5T^{Flu}N_{15}$degrU in MCF and NF of endotheliocytes reached maximum after 1-h incubation with ON (Table 1). Increasing the time of cell incubation with 2.5 μM [^{32}P]-labeled pN_{21} degrU was followed by primary accumulation of the radioactive label in MCF. The time dependence for radioactivity accumulation in NF had a maximum at 1 h incubation. Increasing the time of cell incubation with 2.5 μM radioactive $pN_5T^{Flu}N_{15}$ degrU was accompanied by primary accumulation of the label in NF of cells. The time dependence for accumulation of the radioactive label in MCF had the maximum at 1 h (Table 1). Accumulation of Flu-Lys-pN_{21}degrU in NF and MCF was maximum after 30-min incubation of cells with ON. ON accumulation decreased in the follow-up period. Radioisotope analysis showed that accumulation of pN_{21}degrU and $pN_5T^{Flu}N_{15}$degrU is most significant in MCF and NF, respectively.

ON with modified 3'-terminal end were stable in a culture medium with 10% fetal bovine serum containing supernatants and A431 cells over 6 h [6]. Endothelial cells and their supernatants are

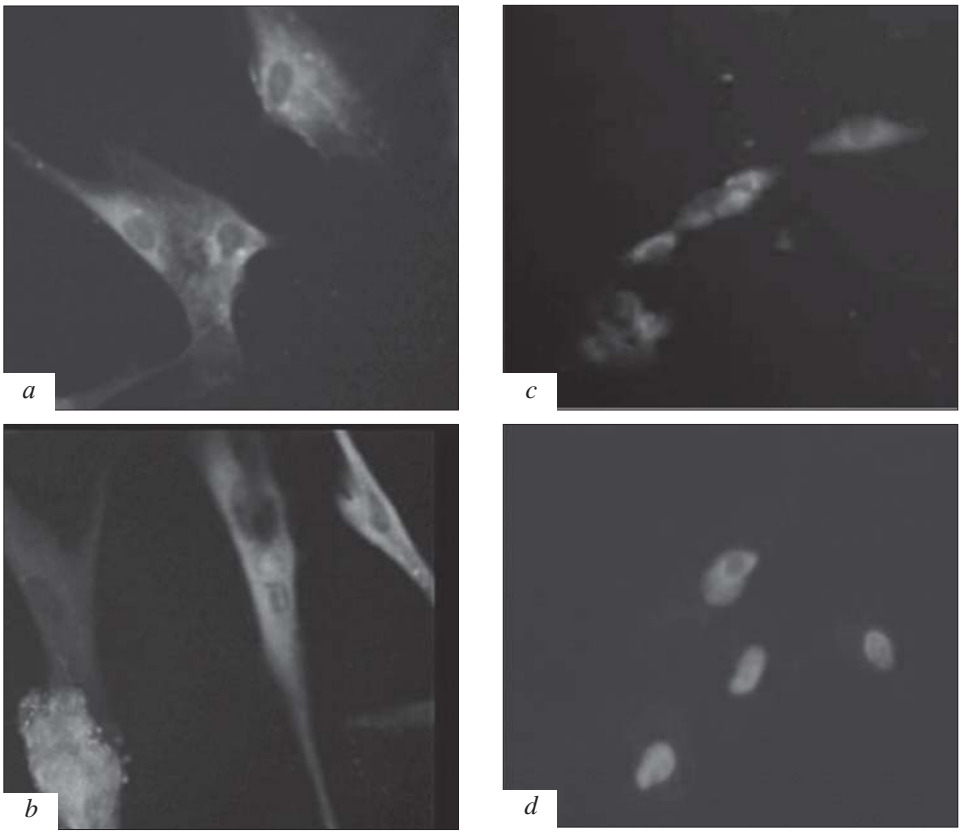

Fig. 1. Dependence of distribution of the fluorescence signal in human endothelial cells on the time of cell incubation with fluorescent-labeled ON (×400). $pN_5T^{Flu}N_{15}$degrU (*a*, *b*) and Flu-Lys-pN_{21}degrU (*c*, *d*). Incubation for 10 (*a*, *c*) and 30 min (*b*, *d*).

TABLE 1. Dependence of ON Distribution (pmol/10^6 cells) in Endotheliocytes on the Time of Incubation with Cells ($M\pm m$, n=3)

ON	Incubation time, h	ON concentration, μM			
		0.5		2.5	
		MCF	NF	MCF	NF
$pN_5T^{Flu}N_{15}$degrU	0.5	0.61±0.06	0.94±0.10	4.15±0.55	1.68±0.18
	1	1.24±0.09	2.48±0.16	6.25±0.63	3.16±0.23
	2	1.99±0.16	3.95±0.31	4.80±0.39	6.54±0.59
	4	1.15±0.7	2.72±0.18	3.30±0.27	8.56±0.78
Flu-Lys-pN_{21}degrU	0.5	4.76±0.61	3.38±0.27	6.69±0.64	5.89±0.54
	1	4.21±0.42	2.90±0.30	3.33±0.41	3.91±0.26
	2	2.86±0.24	2.44±0.16	3.13±0.27	3.78±0.28
	4	2.68±0.24	2.86±0.21	4.15±0.33	4.48±0.35
pN_{21}degrU	0.5	2.51±0.18	1.42±0.17	5.24±0.52	1.65±0.15
	1	3.65±0.26	1.93±0.11	5.93±0.50	2.80±0.18
	2	4.16±0.21	3.29±0.23	13.23±0.91	2.04±0.14
	4	4.98±0.32	4.32±0.21	12.73±0.88	1.00±0.07

characterized by higher content of nucleases. Flu-Lys-pN_{21}degrU was most stable in endotheliocyte-conditioned medium, while $pN_5T^{Flu}N_{15}$degrU and pN_{21}degrU were hydrolyzed after 30 min by 30 and 70%, respectively. Only the triplet of 7-8-9-unit nucleotides was found after 1-h incubation of endothelial cells with $pN_5T^{Flu}N_{15}$degrU. pN_{21}degrU was hydrolyzed to fragments of 7-9 and 2 nucleotides. Flu-Lys-pN_{21}degrU underwent hydrolysis to fragments of 2, 7-9, 16, and 19 nucleotides.

Contradictory data on cellular localization of ON obtained by using two different methods are probably related to nuclease activity of endotheliocytes. All ON except for Flu-Lys-pN_{21}degrU are hydrolyzed to short fragments (<9 nucleotides). According to fluorescence microscopy, Flu-Lys-pN_{21}degrU is primarily accumulated in endotheliocyte nuclei. The data on stability of ON and results of fluorescence microscopy illustrate primary transport of long ON fragments into the nucleus. The data on distribution of the radioactive label in cell fractions are less informative due to high activity of specific endonucleases in endothelial cells, which results in rapid hydrolysis of ON. Modification of 5'-terminal phosphate and 3'-terminal end of ON improves stability and provides nuclear localization of ON in endothelial cells.

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