ONCOLOGY

Relationship between Suppression of E6 and E7 Virus Oncogenes and Expression of Apoptosis and Cell Cycle Genes in Cervical Cancer Culture

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 1, pp. 80-84, January, 2010
Original article submitted May 5, 2009

The effects of short interfering RNA suppressing the expression of E6 and E7 human papilloma virus (type 18) on the expression of apoptosis and cell cycle genes were studied in HeLa cells. Changes in the transcription profiles were evaluated using DNA microarray and real-time reverse-transcription PCR. Cell transfection with anti-E6 and anti-E7 short interfering RNA moderately reduced the expression of mRNA for CDC25C, GRB2, GTSE1, and PLK1 genes and induced expression of CDKN1A ($p21^{CIP}$) gene mRNA. In addition, culture proliferation was inhibited and morphological changes characteristic of differentiation and cell aging developed.

Key Words: *RNA interference; short interfering RNA; human papilloma viruses; viral oncogenes*

Human papilloma viruses (HPV) are the main etiological cause of cervical cancer, which ranks second among tumor diseases by its prevalence in women all over the world. Oncogenic HPV types 16 and 18 more often cause lesions progressing from grade III cervical intraepithelial neoplasia to *in situ* carcinoma and eventually to invasive cervical cancer. Study of the molecular mechanisms of the development of HPV-associated tumors showed that the main factors of malignant degeneration are virus oncoproteins E6 and E7 neutralizing activities of two most important tumor suppressors (p53 and pRb).

Therefore, the main trend in the search for new drugs for the treatment of cervical cancer and precancer is the development of target drugs specifically inhibiting activities of E6 and E7 oncoproteins or blocking the expression of the corresponding oncogenes in involved cells.

RNA interference (RNAi) is an evolutionally conservative mechanism suppressing the expression of cellular or viral genes under the effect of short (19-21 b. p.) molecules of double-stranded RNA (short interfering RNA; siRNA) [1,2].

High specificity and efficiency of this mechanism of gene expression control suggests the development of approaches to the use of synthetic siRNA molecules complementary to HPV genes E6 and E7 in the therapy of HPV infection-associated cervical cancer and precancer [3,8]. An important aspect in the development of these drugs is analysis of the effect of siRNA on the expression of various cell genes.

We studied the effect of siRNA suppressing the expression of HPV-18 oncogenes E6 and E7 on quan-

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0007-4888/10/1491-0073

titative levels of apoptosis and cell cycle gene mRNA in HeLa culture.

MATERIALS AND METHODS

HeLa cells were cultured in DMEM (Biolot) with 10% fetal calf serum (Biolot), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

siRNA transfection was carried out using HiPerfect reagent (Qiagen) according to manufacturer's instruction. Cell transfection with siRNA/MPGΔNLS peptide complexes was carried out as described previously [9]. The cells were inoculated in 24-well plates (1.5×10^5-7.5×10^5/well) and transfected with siRNA/HiPerfect or siRNA/peptide complexes (final concentration of siRNA was 0.1 μM).

DNA oligonucleotides used as primers in PCR were designed using PerlPrimer software. The unique design of PCR primers was verified by search for their sequences in the GenBank database using BlastN algorithm. The following primers were used: CDKN1A-F1 (TTGTACCCTTGTGCCTCGCTCAG), CDKN1A-R1 (ATGTAGAGCGGGCCTT-TGAGGC), GTSE1-FQ (ACAGGCGATGCCGTGGAAAG), GTSE1-R1(AGGAGCTGCAA-GACAGGAGGGA), GRB2-F1(CTTGCTCTTAGATTGGGCCATGG), GRB2-R1 (CCAATCTTG-CCTTCCCCAACTCT), CDC25c-F1 (TACTGTCGGGGAAGTTCCAGGG), CDC25c-R1 (GGC-CCCTCTCTGAGGAGAATTCA), PLK1-F1(GATCCTGCCTGCATCCCCATCT), PLK1-R1 (GGATGGGAACTCACGGTGAGGT), GAPDH-F2 (CTTTGACGCTGGGGCTGGCATT), GAPDH-R2 (TTGTGCTCTTGCTGGGGCTGGT).

The sequences of RNA oligonucleotides (E6-1, E6-2, E7-1, and E7-2) and negative control RNA oligonucleotides for obtaining siRNA were described previously [4]. Oligonucleotides were synthesized by the amidophosphite method and purified by preparative PAAG electrophoresis.

In order to obtain siRNA duplexes, single-strand RNA were dissolved in buffer (10 mM Tris-HCl (pH 7.5) and 20 mM NaCl) and mixed in equimolar proportions. The resultant solution was incubated for 2 min at 95°C and slowly cooled to ambient temperature.

The MPGΔNLS peptide (Ac-GALFLGFLGAAG STMGAWSQPKSKRKV-Cya) was synthesized by the solid phase method, purified by HPLC, and analyzed by MALDI-TOF mass spectrometry.

Total RNA was isolated from the cell strain using RNeasy mini kit (Qiagen). Reverse transcription (RT) of total RNA was carried out using Omniscript RT kit (Qiagen).

The transcription profiles of cell cultures after siRNA treatment was carried out on DNA microarrays (Helicon-Analytica) containing cDNA fragments of 500 genes involved in apoptosis and cell cycle. HeLa RNA specimens collected 72 h after treatment with anti-E6 or anti-E7 siRNA, were hybridized with the micromatrix by the dye swap method (the culture treated with control siRNA served as the reference sample). Each experiment was repeated 3 times.

Real-time RT PCR was carried out using 2.5-fold PCR mixtures (Sintol) containing EvaGreen intercaling stain. The cDNA (0.2 μl) and respective primers (to the final concentration of 0.2 μM) were added to the reaction mixtures (25 μl). PCR was carried out in an ANK-32 device (Sintol) by the following protocol: denaturation at 94°C, 20 sec; annealing at 60°C, 20 sec; elongation at 72°C, 20 sec; 45 cycles. GAPDH gene served as the reference gene for evaluation of mRNA levels. The concentrations of target gene mRNA were calculated by Pfaffl's formula [6]. Each specimen was tested 3 times with each pair of primers. The levels of mRNA were calculated using Microsoft Excel software.

RESULTS

The efficiencies of MPGΔNLS peptide and HiPerfect reagents as vectors for intracellular delivery of anti-E6 and anti-E7 siRNA into the cell were compared using an approach based on evaluation of changes in the level of expression of CDKN1 gene encoding $p21^{CIP}$ tumor suppressor. The expression of this gene increases many-fold after stimulation of p53 tumor suppressor and hence, can serve as a sensitive marker of E6 (and less so, of E7) oncogene suppression.

Transfection of HeLa cells with HiPerfect-anti-E6 and anti-E7 siRNA complexes and with their combina-

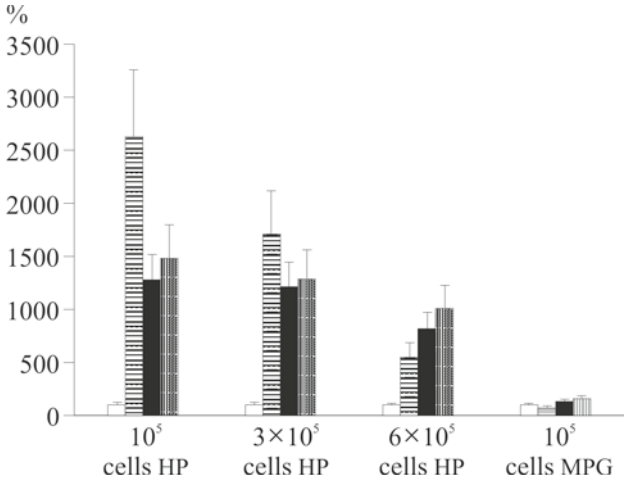

Fig. 1. Changed level of CDKN1A gene mRNA in HeLa cells treated with anti-E6 and anti-E7 siRNA complexes with HiPerfect reagent or MPGΔNLS peptide. Light bars: negative control; horizontally hatched bars: E6; dark bars: E7; vertically hatched bars: E6+E7. Abscissa: number of cells in the wells. HP: HiPerfect; MPG: MPGΔNLS.

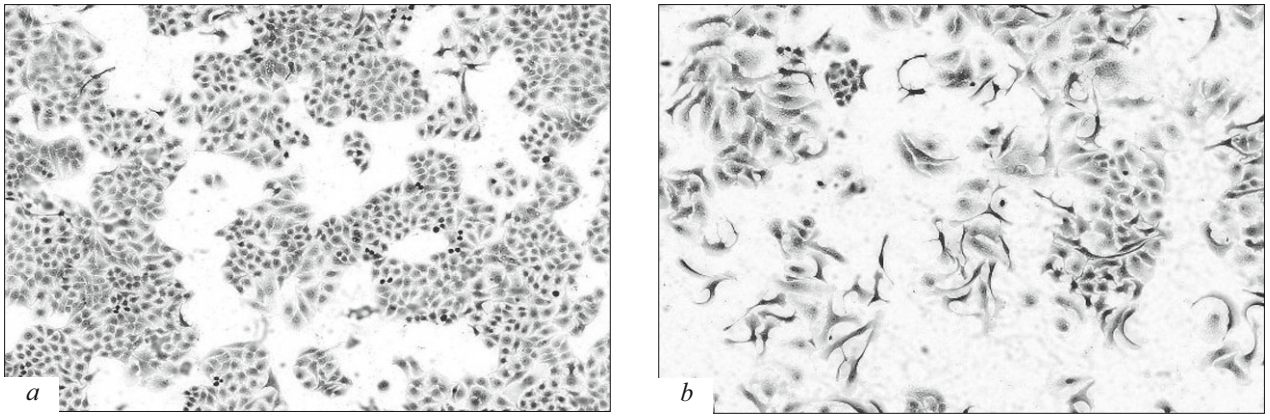

Fig. 2. Changed morphology of HeLa cells after treatment with anti-E6 siRNA. Hematoxylin and eosin staining, ×200. *a*) culture treated with control siRNA; *b*) culture treated with anti-E6 siRNA.

tion led to a many-fold increase in the CDKN1A gene expression. This fact indicated recovery of p53 activity in transfected cells and suppression of the expression of E6 and E7 virus oncogenes (Fig. 1). On the other hand, transfection with siRNA/MPGΔNLS complexes did not lead to statistically significant changes in the level of CDKN1A mRNA level in the culture (Fig. 1).

Microscopic examination of cultures transfected with siRNA/HiPerfect complexes detected pronounced inhibition of proliferation and showed the development of morphological changes (cell flattening, appearance of numerous filopodias), characteristic of differentiation and cell ageing (Fig. 2).

The profile of apoptosis and cell cycle genes expression in HeLa cells treated with siRNA was analyzed by DNA microarray method for detailed evaluation of the transcription effects of anti-E6 and anti-E7 siRNA. The genes expressed differentially were characterized by at least 2-fold differences in the levels of expression in experimental and control samples (Table 1).

TABLE 1. Apoptosis and Cell Cycle Genes Differentially Expressed in HeLa Cells after siRNA Treatment (Multiplicity of Change in Expression in Experimental *vs.* Control Samples)

Suppressed oncogenes	Apoptosis and cell cycle genes	Expression changed *n*-fold
E6	Ribosomal protein L5 pseudogene	2.3±0.3
	Cyclin-dependent kinase inhibitor p21CIP (CDKN1A)	3.41±0.21
	NGFB	2.67±0.51
	NAPOR-3 apoptotic protein	0.49±0.09
	SOD1	2.37±0.12
	A/C lamin (LMNA)	2.38±0.32
	Kunitz-like peptidase inhibitor (SPINT2)	2.47±0.08
	CLK4 protein	2.28±0.21
	Lymphotoxin-β (LTB, TNF superfamily)	2.97±0.34
	CDC25C proteinphosphatase	0.42±0.03
	Insulin-like factor receptor (IGF2R)	2.79±0.18
	Cell cycle phases G-2 and S protein (GTSE1)	2.67±0.24
	KIAA0720 protein	2.33±0.2
	SLC25A4 mitochondrial adenine-nucleotide translocator	0.45±0.11
	PLK1 polo-like proteinkinase	0.48±0.05
E7	CDKN1A cyclin-dependent kinase inhibitor	2.39±0.14
	GRB2 growth factor receptor-related protein	0.49±0.02

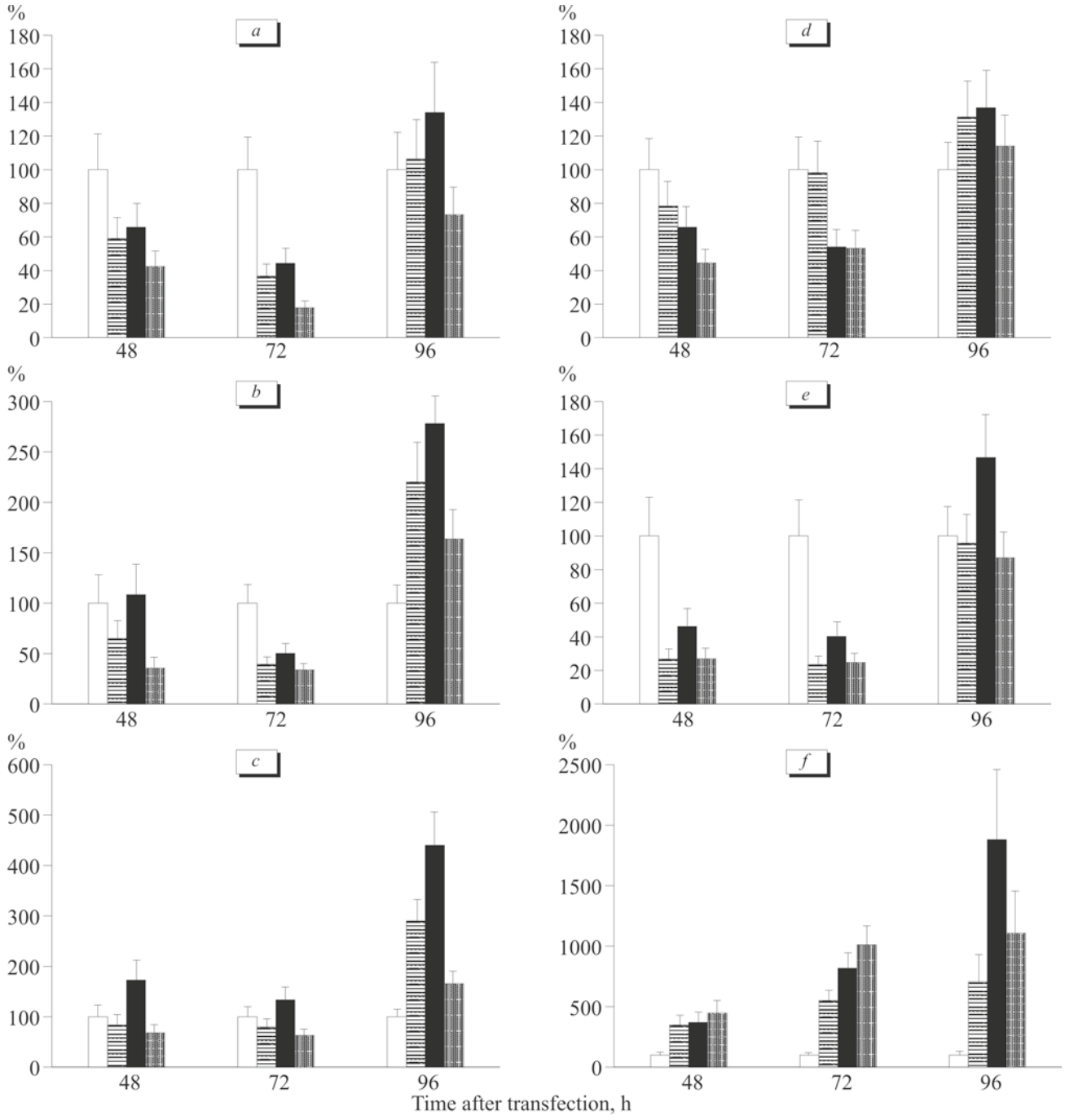

Fig. 3. Dynamics of gene expression in HeLa cells treated with anti-E6, anti-E7 siRNA, and their combination. *a*) CDC25C; *b*) GTSE1; *c*) NAPOR-3; *d*) GRB2; *e*) PLK1; *f*) CDKN1A. Light bars: negative control; vertically hatched bars: E6+E7; dark bars: E7; horizontally hatched bars: E6.

In order to verify the differences in the level of expression by real-time RT PCR, the following genes were selected (with consideration for functional significance): CDC25c encoding proteinphosphatase, involved in mitosis triggering; GTSE1, expressed in cell cycle phase G2 and involved in negative regulation of p53 tumor suppressor [5]; PLK1 encoding Polo-like kinase (proteinkinase) participating in metastasizing of malignant tumors [7]; GRB2 encoding adapter protein of the Ras signal pathway [10]; and NAPOR-3 gene, an RNA-binding protein, whose expression is associated with apoptosis development in neuroblastoma cells.

The expression of these genes was studied 48, 72, and 96 h after transfection in cells transfected with anti-E6 and anti-E7 siRNA in comparison with the cells transfected with control siRNA. The levels of CDKN1A gene transcription were also evaluated. The results of real-time RT-PCR are presented in Figure 3.

The most significant changes were observed in the expression of $p21^{CIP}$ tumor suppressor (CDKN1A). mRNA content for this protein increased more than 10-fold in cells treated with combination of anti-E6+anti-E7 siRNA after 72 h and was still increasing after 96 h. The levels of CDC25c, PLK1, and GTSE1 genes mRNA decreased to 20-40% of the initial level after 72 h. On the other hand, the level of GRB2 gene mRNA decreased significantly after anti-E7, but not after anti-E6 siRNA treatment. Later (96 h after transfection) the levels of almost all the studied genes virtually returned to the initial level, while the level of NAPOR-3 mRNA increased sharply in comparison with the negative control, particularly in the samples treated with anti-E7 mRNA, which can indicate the development of apoptosis during this period.

Hence, our findings indicate that the cytostatic effect of anti-E6 and anti-E7 siRNA towards HeLa cells is explained by changed expression of several genes involved in the regulation of cell cycle and apoptosis.

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