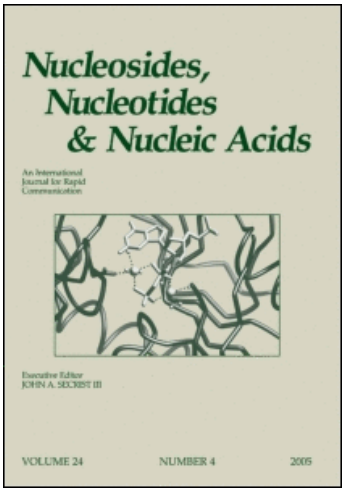

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Silencing of c-myc Expression in Tumor Cells by siRNA

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ABSTRACT

Suppression of c-myc protooncogene expression in KB-3-1 cells by siRNA was investigated. The siRNA duplex targeted to the exon 3 of c-myc mRNA was prepared by in vitro transcription with T7 RNA polymerase on short dsDNA-templates. It was found that incubation of KB-3-1 cells in the presence of 75 nM siRNA results in decrease of the c-myc mRNA level down to 5% of the level in the control cells and significant decline of KB-3-1 cell proliferation rate. Using 200 nM siRNA four-fold decrease of KB-3-1 cells proliferation rate was observed and this effect was stable at least 96 h after transfection.

Key Words: c-myc; siRNA; KB-3-1 cells; Gene silencing.

Discovery of gene silencing by double-stranded RNA (RNA interference, RNAi)[1–4] opened new possibilities for development of therapeutics for treatment of tumor, infectious and other diseases. Short double-stranded RNAs (siRNAs) were shown to efficiently arrest functions of the target mRNAs in mammalian cells.[4]

We investigated effect of siRNA on expression of the proto-oncogene c-myc. The c-myc gene product belongs to a family of transcriptional regulators and is involved in control of cell growth.[5] C-myc misregulation is associated with tumor formation;[5,6]

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oncogenic activation and tumor development occurs mainly through constitutive and elevated expression of c-myc gene product.[7]

MATERIALS AND METHODS

Preparation of siRNA Duplex

The siRNA duplex was prepared by in vitro transcription with T7 RNA polymerase on dsDNA-templates (antisense templates: 5′-AAGAGGTGCCACGTCTC-CACACCTGTCTC-3′) as proposed by Ambion (http://wws.ambion.com/techlib/tn/103/2.html) (Fig. 1) with some modifications. Briefly, in separate reactions, the two DNA oligonucleotides, consisting of 21 nt encoding the siRNA and 8 nt leader sequence, optimized for maximal RNA yield, were hybridized to the universal T7 Promoter Primer that contains a T7 promoter sequence and 8 nt complementary to the leader sequence. The 3′ ends of hybridized oligonucleotides were extended by the Klenow fragment of DNA polymerase to create double-stranded siRNA transcription templates that were transcribed by T7 RNA polymerase. In transcription assay we used natural

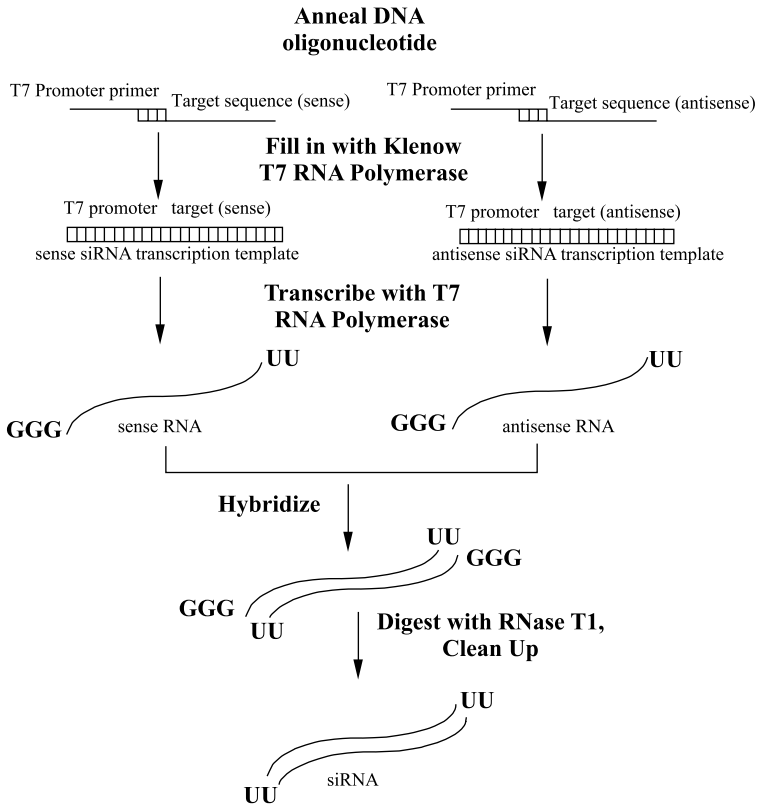

Figure 1. Scheme of siRNA preparation by in vitro transcription.

UTP instead of modified as proposed by Ambion. Obtained transcripts contained additional 5′-terminal universal leader sequences that were removed by T1 RNase after hybridization of sense and antisense transcripts. The resulting siRNA was purified by reverse HPLC, which removes excess nucleotides, short oligomers, proteins, and salts from the reaction mixture. The yield was 5 μg of siRNA from 1 μg dsDNA-template.

siRNA Treatment

KB-3-1 cells were grown to 20–50% confluence in complete medium without antibiotics. siRNA (at various concentrations) and oligofectamine™ (Invitrogene) were added to IMDM medium, the solution was incubated at room temperature for 15–20 min (as recommended by the manufacturer) and added to KB-3-1 cells. Cells were incubated 24–96 h and then harvested for the analysis.

Reverse Transcription-PCR (RT-PCR)

Quantification of specific mRNAs by RT-PCR was done essentially as described.[8] Briefly, total RNA was isolated from KB-3-1 cells monolayers using SDS/phenol-based standard method.[9] Equal amounts of total RNA were subjected to reverse transcription using Oligo(dT)15 primers and murine leukemia virus reverse transcriptase (MuLV RT) at 42°C for 1 h, followed by PCR at 25 cycles in standard conditions using c-myc and β_2-microglobuline specific primers. The primers were as follows (sense and antisense, respectively): c-myc: 5′-ctcctcggtgtccgaggaac-3′ and 5′-gacagcagctcgcccaa-3′; β_2-m: 5′-atcttcaaacctccatgatg-3′ and 5′-acccccactgaaaaagatga-3′. The RT-PCR products 455 and 120 bp long, respectively, were analyzed by standard agarose gel electrophoresis followed by ethidium bromide staining.

MTT Assay

Cell number was assessed using a colorimetric assay based on reduction of the dye 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) by living cells.[10] Exponentially growing KB-3-1 cells were plated at density 1000 cells/well in 96-well microtiter plates (100 μl/well) and incubated at 37°C for 24 h. Then siRNA (25 to 400 nM) was added in mixture with oligofectamine™. KB-3-1 cells were exposed to siRNA twice at 24 h intervals, total incubation time was 24 to 96 h. 0.5 mg/ml MTT was added three hours before cell harvesting. MTT Formosan crystals in each well were resolubilized by addition of 100 μl DMSO, and absorbances were measured spectrophotometrically at wavelength of 570 nm.

RESULTS AND DISCUSSION

In this study we evaluated the ability of 21 nt siRNA with 3′ overhanging uridine dinucleotides against sequence (1452–1470) of c-myc mRNA (antisense strand of the duplex: 5′-UGUGGAGACGUGGCACCUCUU-3′) to induce silencing of the gene. The

siRNA was designed as described by Tuschl and colleagues[4] (target sites beginning with AA, 3′UU overhangs for both the sense and antisense siRNA strands, ~50% G/C content). The target sequence was aligned to the human genome database in a BLAST search to eliminate the significant homology to other genes.

The siRNA duplex was prepared by in vitro transcription with T7 RNA polymerase on dsDNA-templates according to Ambion's manufacture (http://www.ambion.com/techlib/tn/103/2.html) (Fig. 1) as described in materials and methods. This easy method provides an economical alternative to chemical synthesis of siRNAs and allows to avoid chemical modifications. KB-3-1 cells were exposed to siRNA complexed to oligofectamine™. 24 h after the treatment, total RNA was isolated from the cells and the c-myc mRNA level was evaluated by RT-PCR. It was found that incubation of KB-3-1 cells in the presence of 75 nM siRNA results in effective silencing of the c-myc expression and mRNA level fells down to 5 % of the level in the control cells (Fig. 2). The decrease of the c-myc mRNA level occurs in a dose-dependent manner with the bell-shaped concentration-response curve that can be explained by the limited amounts of proteins involved in processing of RNA duplexes providing the dsRNA silencing effect in the cells. Apparently the excess amount of siRNA can bind different proteins of RISC complex thus interfering with their processing of the oligonucleotide-mRNA complexes. Since decrease of c-myc expression level can lead to a reduction in cell proliferation rate, we monitored cell proliferation by MTT assay at 24 h intervals following the siRNA treatment. Figure 3 shows results of this experiment, four-fold decrease of KB-3-1 cells proliferation rate was observed 48 h after incubation with 200 nM siRNA and effect was still visible at least 96 h after transfection. The transfection and cell proliferation assays were reproduced in independent experiments and the differences in relative c-myc mRNA levels and cell proliferation rates were shown to be statistically significant, deviation was less 15%.

In conclusion, we designed siRNA, which able effectively decrease the c-myc mRNA level in KB-3-1 cells, arrest proliferation of the cells and does not show any toxicity at used concentrations. The observed significant inhibition of cell proliferation

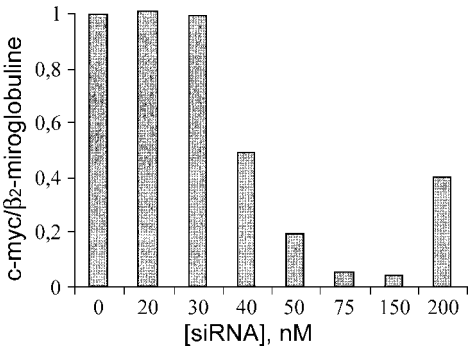

Figure 2. Inhibition of c-myc gene expression in KB-3-1 cells by siRNA. KB-3-1 cells were incubated in the presence of siRNA at 37°C for 24 h. Oligofectamine™ was used to improve the siRNA delivery into the cells. C-myc mRNA levels were assayed by RT-PCR using beta-microglobulin mRNA as internal standard.

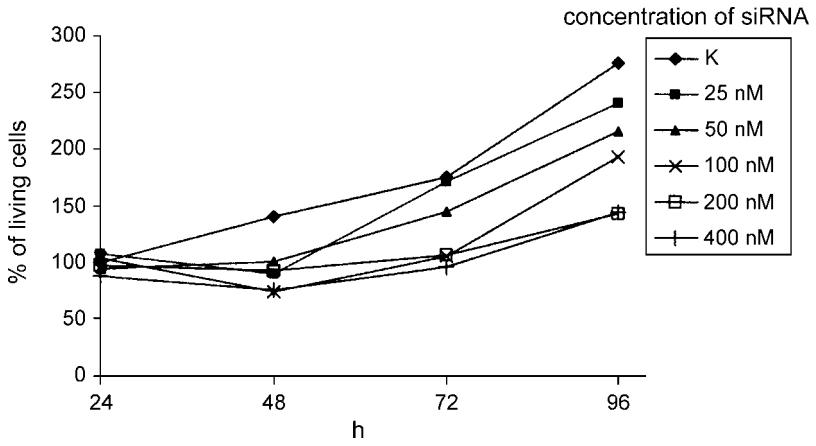

Figure 3. Kinetics of KB-3-1 cells proliferation inhibition by siRNA. KB-3-1 cells were exposed to siRNA (0–400 nM) in the presence of oligofectamine™ twice, at 24 h intervals, at 37°C, and analyzed using MTT-assay.

suggests using of the designed siRNA as potential therapeutics for regulation of c-myc expression in tumor cells.

ACKNOWLEDGMENTS

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