Short Technical Report

Efficacy of an Amphipathic Oligopeptide to Shuttle and Release a *cis*-Acting DNA Decoy into Human Cells

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ABSTRACT

We investigated the ability of an amphipathic oligopeptide to carry a synthetic *ds*-DNA oligonucleotide inside human cells. The oligonucleotide was designed as a decoy binding site for the transcriptional activator of the methylguanine-DNA methyltransferase (MGMT) gene. The complex oligopeptide and decoy were administered to MCF10A exponentially growing cells, and the uptake was monitored by flow cytometry. After a 1-h exposure, almost all of the MCF10A cells were fluorescent, indicating that all of the cells had been transfected. By increasing the time, the fluorescence intensity per cell rapidly increased to a plateau at the 8-h time point. RT-PCR analysis of the MGMT gene was used as the molecular readout of the intracellular activity of the DNA decoy. MCF10A cells transfected with the oligopeptide/decoy complex showed a strong reduction in MGMT mRNA. Here, we discuss the advantages of using amphipathic oligopeptides as carriers of short DNA sequences.

INTRODUCTION

The mechanism by which cationic liposomes mediate the transfection of DNA is based on their ability to adsorb the negative charges of DNA. The DNA-liposome complex is then adsorbed into the cell membrane, leading to a transient destabilization that favors the endocytosis of the complex; after which, the disruption of endosomes allows the release of complexed DNA into the cytosol (17,18). The inhibition of a target gene function by transfecting DNA oligonucleotides requires prolonged exposure because the release of DNA from the transfection complex into the cytosol has been observed to be a rather slow process (2). Amphipathic oligopeptides derived from the third helix element of the Antennapedia homeodomain protein of *Drosophila* are able to shuttle peptides across the cell membrane into the cytosol (5,14). The internalization seems to be independent of endocytosis events; therefore, the release is not mediated by endosome formation and disruption. This feature prompted us to use oligopeptides instead of cationic liposomes as carriers of DNA oligonucleotides in living cells. The *cis*-acting DNA oligonucleotides are synthetic *ds*DNA sequences that mimic transcriptional elements and bind sequence-specific DNA-binding proteins (1,8,10,11,13). In this manner, binding of transcription factors to their native enhancer sequences is prevented, and gene expression is consequently down-regulated.

The O⁶-methylguanine-DNA methyltransferase (MGMT) gene, which encodes the enzyme that repairs DNA alkylation damage by transferring the alkyl group from the O⁶ position of guanine to a cysteine residue of its chain (12,15), confers resistance to antitumor alkylating drugs. We have already reported that the MGMT-positive cells (Mex⁺ phenotype) can be rendered MGMT-negative (Mex⁻ phenotype) by inhibiting the transcription of MGMT with a synthetic *ds*DNA oligonucleotide (9) acting as binding site for MGMT enhancer binding protein (MEBP), the transcriptional activator of MGMT (3). Here, we tested the ability of an amphipathic oligopeptide to shuttle that DNA oligonucleotide (MEBP decoy) and measured the down-regulation of the MGMT expression.

MATERIALS AND METHODS

RC355 Vehicle

The amphipathic oligopeptide (HCRRWRRWWRRWRR-NH₂) (16) was synthesized with an automatic 9050 model peptide synthesizer (PerSeptive Biosystems, Framingham, MA, USA) using conventional F-moc chemistry. Deprotection and purification were done as previously described (6). The purified oligopeptide was then covalently linked to a poly-L-lys molecule (M_r 40 000) (Sigma, St. Louis, MO, USA) using the SMCC according

to the manufacturer's instructions (Pierce Chemical, Rockford, IL, USA). The reaction product was characterized by Elmann assay of residual unreacted -SH groups. The resulting construct (named RC355) contained a mean of three oligopeptide copies for each poly-L-lys molecule.

MEBP Decoy

The MEBP decoy (Figure 1) was synthesized with an Expedite™ 8900 DNA Synthesizer (PerSeptive Biosystems) using phosphoramidite chemistry as previously described (9). The MEBP decoy, with a 5'-amino-modifier-C6 (Glen Research, Sterling, VA, USA) inserted at the 5' end, was reacted in the dark for up to 20 h at room temperature with fluorescein isothiocyanate (FITC) (Pierce Chemical) in 250 mM bicarbonate buffer, pH 9.0, according to the manufacturer's instructions. The resulting fluorescent oligonucleotide (FITC/MEBP decoy) was desalted and stored at -20°C.

Transfection

Recipient human breast epithelial cells (MCF10A) were grown in Dulbecco's F12 mixture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% horse serum, penicillin (50 U/mL), streptomycin (50 µg/mL), hydrocortisone (0.5 µg/mL), cholera toxin (0.1 µg/mL), epidermal growth factor (0.02 µg/mL), and insulin (10 µg/mL) (all from Sigma) at 37°C in a humidi-

fied atmosphere containing 6% CO₂.

The cells were seeded at a density of 2×10^5 cells/30-mm dish. After 24 h, 1.5 mL optiMEM™ (Invitrogen) containing 0.25 µM FITC/MEBP decoy (3.75 µg) complexed to RC355 or DOTAP (in a weight ratio of 1:1 or 1:2, respectively) were added to each dish. The uptake of the FITC/MEBP decoy was measured by flow cytometry.

Proliferation Assays

Cells were seeded in 96-well plates (10^4 cells/well), and 24 h later, the medium was changed and fresh medium containing the RC355, MEBP decoy, or both was added. The cell proliferation was determined at various time points by reduction assay based on tetrazolium salt. Twenty microliters of CellTiter 96® AQueous One Solution (Promega, Madison, WI, USA) were added per well, and an absorbance at 490 nm was recorded 2 h later with a DV990 BV4 96-well plate reader (Gio. De Vita, Rome, Italy).

Flow Cytometry

The cells were washed once with 0.2 M glycine, pH 2.8, to remove any extracellular complex (7), harvested, and resuspended in 100 µL PBS. The fluorescence from 5000 to 10 000 individual cells was analyzed with FACScan, Lysys II software (BD Biosciences, San Jose, CA, USA).

RT-PCR Analysis

The total mRNA from 2×10^6 cells was extracted with RNeasy™ (Tel-Test, Friendswood, TX, USA), quantified by UV absorbance at 260 nm, and treated with DNase I (Invitrogen). Reverse transcription of 3 µg DNA-free RNA was then performed in a 20-µL reaction mixture. Final concentrations were 1 mM dNTP mixture, 2.5 µM random nonamers, 40 U ribonuclease inhibitor (Sigma), and 1× reverse transcription buffer. Avian myeloblastosis virus reverse transcriptase (20 U) (Sigma) was added.

MGMT and hypoxanthine phosphoribosyl transferase (HPRT), which was used as reference gene, were amplified from the same PCR mixture. Briefly, we mixed 6 µL reverse transcription mixture, 10 µL 10× PCR buffer, 10 µL 25 mM MgCl₂, 2 µL 10 mM dNTP mixture, and water to a final volume of 100 µL. Then, half of the mixture was used to amplify the MGMT gene and the other half to amplify the HPRT gene, adding respective primers at a 0.2-µM final concentration. Forward (5'-CGAGCAGTGGGAGGAGCA-3') and reverse (5'-CATCCGATGCAGTGTTACACG-3') primers were used to amplify a 299-bp fragment of MGMT mRNA, while forward (5'-CAGATTCAACTTGAACCTCTC-3') and reverse (5'-AAAGGTGTTTATTCTCATGGA-3') primers were used to amplify a 522-bp fragment of HPRT mRNA. Five units of ThermalAce™

Figure 1. Schematic representation of the MEBP decoy. Briefly, the MEBP decoy is a DNA oligonucleotide (42 nucleotides) containing the minimal 9-nucleotide *cis*-element (5'-CTGGGTCGC-3') for specific binding of the transcription factor MEBP (3). Because the decoy contains complementary sequences, it structures itself as a double strand that reproduces the enhancer binding element and confers the resistance to exonucleolytic degradation.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Taq DNA polymerase (Invitrogen) were added during the initial denaturing step. The amplification conditions for the MGMT fragment were 23 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min. The HPRT fragment was obtained with 28 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min. Fifteen microliters of both PCR mixtures were then loaded on the same lane of a 1.5% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

We have already shown that the administration of free MEBP decoy for 24 h inhibited MGMT transcription with a consequent reduction of MGMT protein (9). Nevertheless, we observed that the fluorescence distribution profiles of control and transfected cells were, in part, overlapping. The explanations for that could either be the reduced stability that DNA oligonucleotides have in culture medium (4) or the slow rate of uptake. Because the MEBP decoy was designed and found to be resistant to extracellular degradation (9), the rate of uptake was probably the limiting step. Cationic liposomes are routinely used to enhance the internalization of oligonucleotides in living cells, even if there are concerns about the rapidity of the release of DNA oligonucleotides (2). In this study, we exploited RC355, an amphipathic oligopeptide derived from the third helix element of the Antennapedia homeodomain protein, as a carrier of the MEBP decoy. We compared the fluorescence distribution of MCF10A cells grown in the absence and presence of free 0.25 μ M FITC/MEBP decoy in culture medium (Figure 2, left panel). At various time points, the two distributions were largely overlapping, and the fluorescence intensity did not increase in relation to transfection time. The transfection of the FITC/MEBP decoy complexed to RC355 gave different results (Figure 2, right panel). After a 1-h exposure, almost all cells showed a higher fluorescence than the control cells. Furthermore, by increasing the exposure time, the fluorescence intensity was progres-

Figure 2. Time course of FITC/MEBP decoy uptake. The percentage of labeled cells (events) and the relative fluorescence intensity (FL1-Height) were determined at 1-, 2-, 4-, and 8-h time points. Left panel, the fluorescence distribution of cells grown in the presence (thick line) or absence (thin line) of the FITC/MEBP decoy. Right panel, the fluorescence distribution of cells after the administration of FITC/MEBP decoy either complexed (thick line) or not (thin line) with RC355.

Figure 3. DOTAP-mediated FITC/MEBP decoy uptake. The percentage of fluorescent cells (events) and the relative fluorescence intensity (FL1-Height) were determined at the 8-h time point. Left panel, the fluorescence distribution of cells grown in the presence (thick line) or absence (thin line) of the FITC/MEBP decoy. Right panel, the fluorescence distribution of cells after the administration of the FITC/MEBP decoy either complexed (thick line) or not (thin line) with DOTAP.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Figure 4. Proliferation curve of MCF10A cells growing in the presence of the RC355 (filled triangle), MEBP decoy (empty triangle), and RC355/MEBP decoy complex (circle).

Figure 5. Agarose gel electrophoresis of RT-PCR products. MGMT PCR fragment of untransfected (lane 1) and RC355/MEBP decoy-transfected cells at the 2-h (lane 2), 4-h (lane 3), and 8-h (lane 4) time points. M, 100-bp DNA ladder (upper panel) (New England Biolabs, Beverly, MA, USA). The MGMT and HPRT PCR fragments of untransfected (lane 1) and RC355/MEBP decoy-transfected cells at the 8-h time point (lane 2). DNA contamination was tested by performing PCR without reverse transcription (lane 3). M, 1-kb DNA ladder (lower panel). The pictures are inverted tone images.

sively higher. At the 8-h time point, the fluorescence intensity reached a plateau, indicating that the uptake mediated by RC355 was complete.

The efficacy of RC355 to deliver the MEBP decoy into living cells was also compared with that of the cationic lipid DOTAP (Figure 3). The uptake of the DOTAP/MEBP decoy at the 8-h time point was much higher than that of the free MEBP decoy, but not as rapid as that of the RC355/MEBP decoy (Figure 2). The cytotoxicity of the RC355/MEBP complex was tested by measuring the cell proliferation (Figure 4). It can be noted that at early and late time points cell proliferation curves were very similar. This indicates that RC355 neither alone nor complexed with MEBP decoy was cytotoxic.

The results reported here indicate that RC355 is an efficient carrier of the MEBP decoy inside MCF10A cells. To address whether the DNA is released from the complex, we measured the MGMT mRNA level at various time points (Figure 5, upper panel). The PCR band intensity decreased with the increase of exposure time. The densitometric analysis showed a 45% (lane 2), 74% (lane 3), and 84% (lane 4) reduction of bands at 2-, 4-, and 8-h time points, respectively, relative to that of untransfected cells (lane 1). Thus, only after 2 h did MGMT mRNA transcription decrease by 2-fold, indicating that the MEBP decoy release from RC355 was rapid. These findings also suggested that endocytosis and endosome disruption, which are critical steps of transfection mediated by cationic liposomes, are not involved. The specificity of MEBP decoy was tested using the HPRT gene as a reference gene (Figure 5, lower panel). At the 8-h time point, the intensity of the HPRT band of control cells was similar to that of the RC355/MEBP decoy-exposed cells, thus demonstrating that HPRT transcription was unaffected by the MEBP decoy. Conversely, the intensity of the MGMT band was strongly reduced. Furthermore, the inhibition of the MGMT transcription at the 8-h time point (Figure 5) is similar to that obtained after a 24-h exposure to 5 μ M free MEBP decoy (9), a concentration 25-fold higher than that used in complex with RC355. In conclusion, these

results indicate that the amphipathic oligopeptide RC355 is a rapid and efficient carrier of DNA oligonucleotides into cells so that a minimal MEBP decoy concentration is required to inhibit the transcription of the MGMT gene.

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