Immunoliposomal Containers as Systems of Directed Transport of Minor Interfering RNA into Schwann Cells

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Immunoliposomal container system for targeted delivery of minor interfering RNA into Schwann cells was developed. Monoclonal antibodies to myelin basic protein served as the vector. Analysis by sandwich ELISA of myelin basic protein showed specific suppression of the target protein gene expression in Schwann cells after incubation with PEG-conjugated immunoliposomes loaded with minor interfering RNA blocking the synthesis of myelin basic protein. Prospects of effective use of this system for targeting of minor interfering RNA for the treatment of diseases of genetic etiology are discussed.

Key Words: *PEG-conjugated immunoliposomes; minor interfering RNA; Schwann cells; myelin basic protein; targeting*

Specific posttranscription degradation of matrix RNA (mRNA) associated with RNA interference (RNAi) is a new promising approach to target inhibition of gene expression in cell cultures and *in vivo*. The effectors of this process are minor interfering RNA (miRNA) duplexes of about 21-26 b. p. directly responsible for specific degradation of target mRNA [6]. RNAi method is a prospective new technology of functional genomics, while miRNA are potential therapeutic agents for the treatment of diseases of genetic etiology [6,12,13]. However, because of high molecular weight (~13 kDa) and polyanionic structure (~40 negatively charged phosphate groups) free miRNA do not penetrate through the cell membrane, which impedes wide use of RNAi *in vivo* [5]. Therefore, the development of systems for directed transport and targeting of miRNA acquires special significance in modern medicine [5,10,11].

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The choice of object of the study (target cell cultures) is based on the possibility to solve one of the priority problems of neuropharmacology: creation of systems for realization of specifically directed transport of drugs from systemic bloodflow to the CNS and peripheral nervous system cells [9,14].

Myelin basic protein (MBP) is the main structural component of the membrane of myelin-forming cells. This protein is the main marker of states associated with impairment of myelin membrane integrity [1], such as acute demyelination process, multiple sclerosis, brain injuries, and CNS tumors involving its glial component [2].

Transport systems specifically directed towards myelin-forming cells, *e.g.* Schwann cells, can be used for the diagnosis and regulation of demyelination or detection of the pathological process.

The aim of our study was to create PEG-conjugated immunoliposomal systems specifically binding to Schwann cells and delivering miRNA blocking MBP synthesis into these cells. Monoclonal antibodies to MBP specifically binding to Schwann

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cells (previously obtained in our laboratory [4]) were selected as the optimal vector for these systems.

MATERIALS AND METHODS

Liposomes were prepared on the basis of the method proposed by J. A. Kamps *et al.* [7] and modified for our study.

The following lipid components were used for obtaining cationic liposomes: chicken egg yolk lecithin, cholesterol, DSPE (distearoylphosphatidylethanolamine) conjugated with PEG-200 (polyethylene glycol with a mol. weight 2000 Da), DDAB (dimethyldioctadecylammonium bromide) cationic lipid, MBP-PE (maleimido-4-(n-phenylbutyryl)-phosphatidylethanolamine), realizing covalent binding to protein (all lipid components from Avanti Polar Lipids). Other chemicals, solvents, and accessory substances were of maximum possible purity. All manipulations on preparation of liposomes (except short ones, such as weighing, centrifugation, *etc.*) were carried out in an atmosphere with highly pure argon. Selected lecithin, cholesterol, MBP-PE, DSPE-PEG, and DDAB were dissolved in chloroform-methanol (9:1) mixture in a concentration of 10 mg summary lipids/ml solvent in 23:16:1:1.6:4.4 molar proportion. The mixture was dried in a rotor evaporator at low pressure. Dry lipid film was dissolved in absolute cyclohexane, frozen in liquid nitrogen, lyophilized, after which 100 μl miRNA (21 b. p.) blocking MBP synthesis (SYNTOL) was added to the lipid mixture and the preparation was emulsified in 0.1 M phosphate buffer. Hydrated emulsion was filtered through a series of polycarbonate membrane filters with 400, 200, 100, and 50-μ pores (15 times through each filter) using an Avanti Polar Lipids miniextruder (Alabaster).

Monoclonal antibodies to MBP obtained at our laboratory [4] served as the specific vector. Liposomes were conjugated with thiolated antibodies by the maleimide method [8]. Antibody activity was tested immunohistochemically at all stages of thiolation and conjugation [3].

The method for measurement of total protein and summary lipids in water emulsions (immunoliposome preparations) was described previously [2].

The specificity of binding of PEG-conjugated immunoliposomes to Schwann cells was verified in fixed and living cell cultures derived from rat embryonic spinal ganglia. The method and results of the study were described in detail [3].

Solution of PEG-conjugated immunoliposomes charged with miRNA blocking MBP synthesis were added (100 μl) to living cultures of rat embryonic Schwann cells (2,000,000 cells) under sterile conditions. The system was incubated for 5 days. Growth medium (DMEM-F12 with 10% fetal calf serum) was replaced after 2 days. After incubation the cells were lyzed with PBS (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.4), 3 times frozen and defrosted, and the supernatant was collected.

The concentration of MBP in Schwann cell lysate was measured by a variant of sandwich ELISA developed in our laboratory. Human polyclonal antibodies to MBP were immobilized on a sublayer of the plate wells (Sarsdedt). Native MBP (obtained previously at our laboratory [1]) in concentrations of 0.25 to 32 ng/ml served as the reference antigen for plotting the calibration line, the optical density was measured 6 times for each concentration. Mouse monoclonal anti-MBP reactive with antispecies (to mouse IgG) goat antibodies (Sigma) conjugated with horseradish peroxidase IV (Sigma) served as the second antibodies. Human polyclonal anti-MBP antibodies were obtained from the serum of patients with multiple sclerosis during therapeutic plasmapheresis. Monoclonal antibodies to MBP were obtained at our laboratory [4]. Peroxidase activity was developed using 0.04% ortho-phenylenediamine with 0.3% H_2O_2 in substrate buffer (0.04 M $C_6H_8O_7$, 0.27 M $NaHPO_4 \times 2H_2O$, pH 4.5). Optical density (OD) of the reaction product was measured at λ=450 nm on an Elx 800 multichannel spectrophotometer (Bio Tek Instruments Inc.).

The calibration plot represents a linear relationship between optical density and antigen concentration in the studied sample (Fig. 1). Working range was determined for MBP concentrations from 0.5 to 16 ng/ml.

RESULTS

A preparation of PEG-conjugated immunoliposomes charged with miRNA (diameter 100 nm) was obtained in the form of emulsion (1 ml). Evaluation of the lipid and protein composition showed the following proportion: 150 μg conjugated antibodies/μmol phospholipids, this corresponding to reference data on the procedure for immunoliposome preparation [8]. Each liposome contained about 60 conjugated antibody molecules and 10 miRNA molecules.

The adequacy of vector function of prepared PEG-conjugated immunoliposomes was confirmed experimentally *in vitro* [3]. The specificity of immunoliposomal system binding to Schwann cells was proven; it was due to affinity interactions between liposomal immunochemical vector (monoclonal antibodies to MBP) and MBP presented on the surface of Schwann cells (Fig. 2).

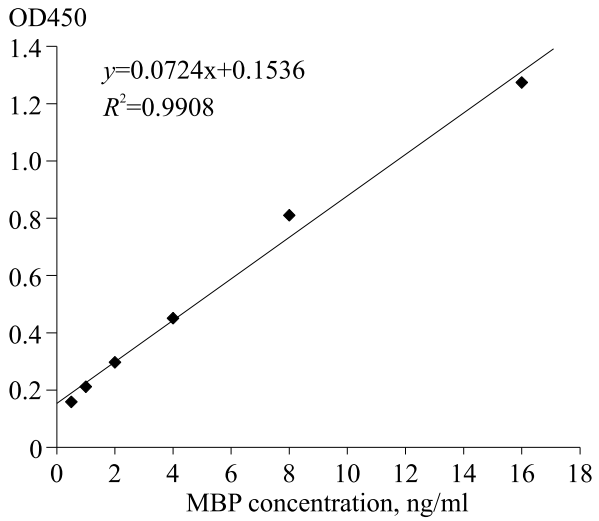

Fig. 1. Calibration plot of MBP enzyme immunoassay.

The results were presented in detail previously [3].

The efficiency of delivery of the preparation by PEG-conjugated immunoliposomes into Schwann cells was evaluated by the physiological response of the target cells to penetration of miRNA into them (by reduction of MBP synthesis level). The concentration of MBP in a sample of 2×10^6 Schwann cells lysate after incubation with PEG-conjugated immunoliposomes with anti-MBP charged with miRNA, blocking MBP synthesis, was measured by sandwich ELISA. The mean concentration was 0.93± 0.02 ng/ml (n=4), which was 10-fold lower than in the control. In system 1, concentration of MBP in lysate of 2×10^6 Schwann cells after incubation with immunoliposomes with nonspecific mouse IgG (used instead of specific immunochemical vector) charged with miRNA was 9.55±0.27 ng/ml, the corresponding parameter in system 2 (no liposomes added) and in system 3 (incubation with free miRNA without delivery system) was 9.05±0.18 and 9.43± 0.28 ng/ml, respectively.

A dose-dependent effect of adding PEG-conjugated immunoliposomes charged with miRNA was demonstrated: the same volume of immunoliposomes was added to cell preparations containing 2 and 4×10^6 Schwann cells. The concentrations of

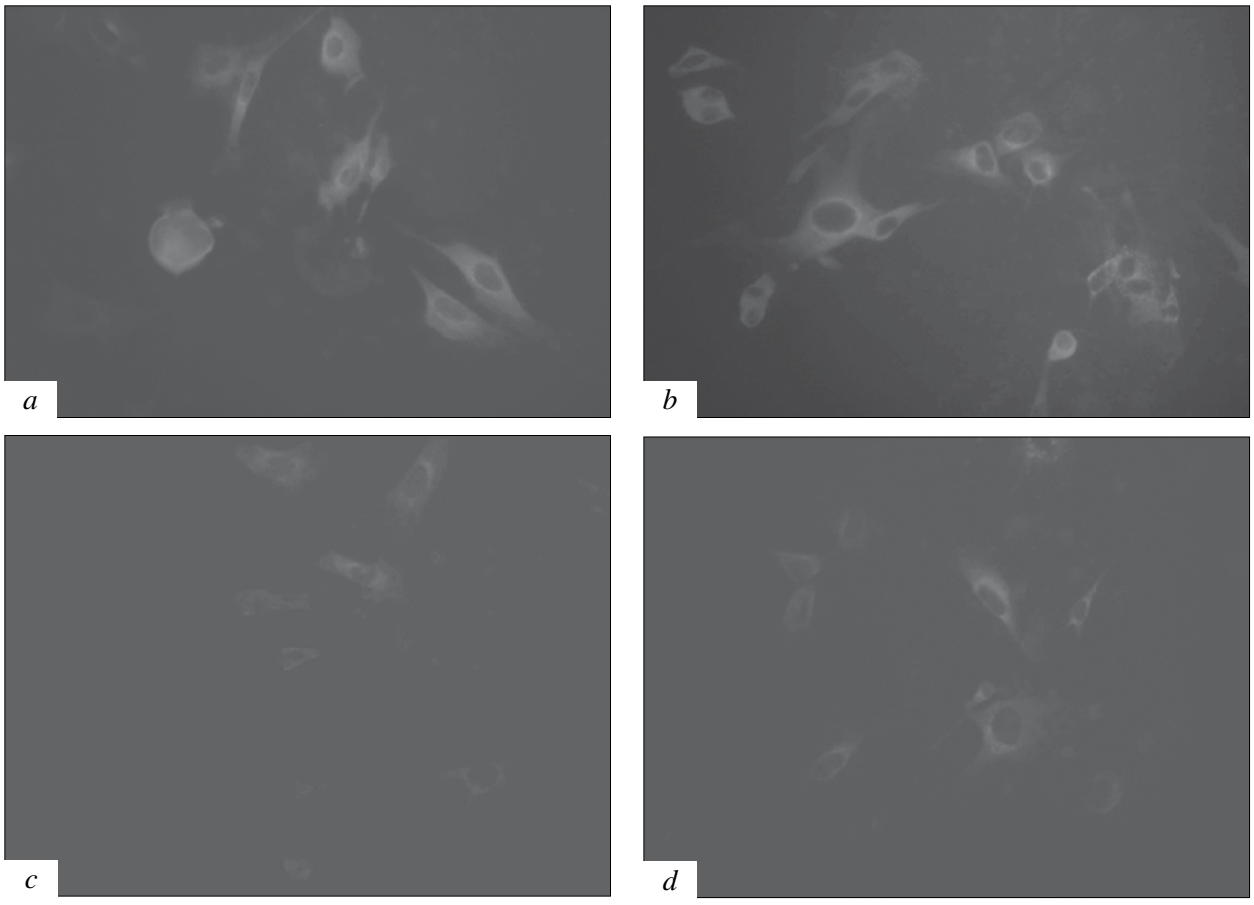

Fig. 2. Culture of rat embryonic Schwann cells. Staining with Dil fluorescent indicator (×400). *a*, *b*) incubation with DiL-labeled PEG-conjugated immunoliposomes (with anti-MBP vector) for 3 (*a*) and 6 h (*b*); *c*) incubation with PEG-conjugated immunoliposomes, containing the vector (Dil labeled nonspecific mouse IgG), for 6 h; *d*) 6-h incubation with preparation of Dil labeled non-vector cationic liposomes.

MBP in these lysates were 0.93±0.02 and 3.50± 0.14 ng/ml, respectively.

These results prove that due to vector orientation determined by anti-MBP, the preparation of PEG-conjugated immunoliposomes charged with miRNA specifically delivers nucleic acids to living target cells and promotes penetration of miRNA into the cells. The delivered miRNA retain their capacity to inhibit specifically MBP translation, that is, after release from the liposomal compartment they trigger the mechanism of RNA interference in the cells, which leads to 90% suppression of the synthesis of target protein.

Hence, due to technology of obtaining vector immunoliposomal systems, a highly specific container is created, capable of targeting of nucleic acids into Schwann cells. Intracellular delivery of minor interfering RNA is confirmed by detection of 10-fold reduction of the intensity of the target protein biosynthesis. We can expect that these systems will be effective in the diagnosis and treatment of diseases associated with myelin abnormalities.

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