Neurons and Stromal Stem Cells as Targets for Polycation-Mediated Transfection

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 1, pp. 22-25, January, 2011
Original article submitted May 26, 2010

Expression of transgenes in neurons and stromal/mesenchymal stem cells (MSC) can greatly enhance their therapeutic potential. In transfection experiments, we studied properties of linear and branched (dendrimers) polycations as transgene delivery vehicles. Linear polyethyleneimine transfected neurons, but was ineffective in MSC. Polyamidoamine dendrimers showed greater transfection efficiency and mean GFP fluorescence intensity compared to phosphorus dendrimers of the same (4th) generation. Expression of neurotrophic factor BDNF in MSC transfected with polyamidoamine dendrimers was also by more than 10 times higher.

Key Words: *neurons; stromal/mesenchymal stem cells; transfection; polyethylenimine; dendrimers; brain-derived neurotrophic factor*

In vitro culturing of cells and tissues is indispensable for studying the fundamental laws of their functioning in terms of genetics, biochemistry, and physiology, as well as for applied research, such as expansion to subsequent transplantation, forming of artificial organs, *etc*. [3,6]. The development of molecular genetic methods gave rise to a new trend: gene engineering of primary animal and human cells and tissues. In particular, expression of transgenes in stem cells can compensate for the lack of necessary body proteins, provide differentiation in a given direction, increase production of trophic factors, *etc*. [2,8,13]. In situations that do not require long-term functioning of the transgene and its integration into the cellular genome, transient transfection is optimal. In contrast to the transplanted immortalized cultures, transfection of primary cell populations is not always successful.

The purpose of research was to consider options for transfection with various cationic polymers capable of condensing vector DNA due to electrostatic interaction and delivering it through the cell membrane. The objects of research were neurons and stromal stem cells of the bone marrow also known as MSC.

MATERIALS AND METHODS

For isolation of human stromal stem cells, bone marrow aspirate from healthy donors was centrifuged in Ficoll density dragient, the mononuclear fraction was collected, washed twice with PBS, and seeded in α-MEM with 10% PBS (HyClone) [6]. MSC selected by adhesion to plastic were immunotyped using antibodies to CD90, CD105, CD45, and CD34. Primary neurons isolated from sympathetic ganglia of newborn rats were purified and cultured on collagen substrate in medium IMDM (Gibco) with 10% FCS as previously described [1]. All cell cultures were cultured in a CO_2

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0007-4888/11/1511-0126

incubator (5% CO_2) at 37°C, the growth medium was replaced after 2 days.

Basic vector used in transfection experiment was pAAV-IRES-hrGFP (Stratagene) carrying *GFP* reporter gene. pBDNF-IRES-hrGFP vector created on this basis was also used. Both plasmids were reproduced in *Escherichia coli* DH5a strain. To create pBDNF-IRES-hrGFP vector, the sequence of human *bdnf* gene (791 b.p.) was integrated into polylinker vector (MCS) pAAV-IRES-hrGFP, restriction sites *Cla*I and *Eco*RI (recognition sequences were introduced into the 5'-ends of primers) in accordance with standard guidelines [14]. The structure of pBDNF-IRES-hrGFP vector and the absence of mutations in the *bdnf* gene were confirmed by restriction analysis and sequencing. The presence of internal ribosome entry site (IRES) in pBDNF-IRES-hrGFP vector provided the expression of both the target BDNF gene and GFP reporter.

The plasmids for cell transfection were isolated isolation and purified using a Plasmid Maxi kit (Qiagen) according to manufacturer's instructions. The following cationic polymers were used for transfection: linear polyethylenimine ExGen500 (Fermentas), fourth-generation polyamidoamine dendrimers PAMAM-NH_2 G4 (Dendritic NanoTechnologies) and fourth-generations phosphorus dendrimers (Phos G4) [5,12,15]. Formation of DNA-polycation complexes was conducted in 150 mM NaCl at a charge ratio of 1:1 (for PEI ExGen500 according to manufacturer's recommendation), transfection was carried out for 2 h in culture medium containing 10% serum.

Detection of GFP fluorescence was performed on the next day after transfection under Carl Zeiss Axio Imager M1 microscope. GFP-positive cells were determined using a FACScan flow cytometer (BD) and BDNF secretion was analyzed by ELISA using BDNF E_{max} ImmunoAssay System® (Promega) according to manufacturer's instructions.

RESULTS

Recombinant GFP expression cassette under control of cytomegalovirus promoter in the presence of β-globin intron was used as a system for evaluation of transfection effectiveness and transgene expression. Polymer cations were able to deliver the transgene and ensure its expression up to 3 weeks; transfected neurons and MSC retained their morphology and viability. The results of monitoring of neuron transfection with linear polyethyleneimine by fluorescence microscopy showed that this reagent reproducibly provided delivery and expression of GFP-containing cassette. Transfected neurons retained their phenotype and participated in the formation of secondary nerve plexuses. Transfection efficiency of neurons with linear polyethyleneimine was 5.3±0.8%. However, this reagent was much less effective in transfection of human bone marrow MSC (<0.1%, Fig. 1).

Since linear polyethylenimine was little effective in transfecting human bone marrow MSC, the transgene in these clinically important type cells were delivered using dendrimers, polymeric cations of different structure consisting of nuclear spheres and polymer branches ended by charged groups [5]. In particular, we used polyamidoamine dendrimers with ethylenediamine core and branches of methyl acrylate and ethylenediamine, as well as dendrimers based on phosphorus [12,15].

Microscopy and flow cytometry data indicate that both polyamidoamine and phosphorus dendrimers provide a significantly larger ratio of GFP-positive MSC (Fig. 2). That allows us to consider these forms of polymer cation more efficient than linear polyethylenimine. However, the difference between dendrimer of the same generation (4th), but different chemical composition in transfecting bone marrow MSC is also significant (Table 1). Polyamidoamine dendrimers were significantly superior to phosphate dendrimers by the content of GFP-positive cells and mean fluorescence intensity.

For evaluation of the possibility of using polycations for expression of therapeutically relevant factors in MSC, these cells were transfected with plasmid pBDNF-IRES-hrGFP using both types of dendrimers created to express the neurotrophic factor BDNF. BDNF belongs to nerve growth factor family proteins.

TABLE 1. Parameters of MSC Transfection with Dendrimer Polycations

Variant	Transfection efficiency, %	Mean fluorescence intensity, arb. units	BDNF secretion, pg/ml
Control	–	–	34.4±11.2
PAMAM-NH_2 G4	4.7±0.8	626.0±59.7	607.0±34.9
Phos G4	1.8±0.2	127.0±20.5	43.6±9.81

Note. PAMAM: poliamidon dendrimer.

It is essential for maintaining brain neurons viability, participates in the regulation of the response to stress, triggers neuronal regeneration, *etc*. BDNF overexpression in MSC promoted, in particular, axonal growth after transplantation of MSC in damaged sites of the spinal cord [10]. Parameters of the BDNF expression in MSC after transfection with dendrimer polycations of different chemical composition corresponded to the results of expression of reporter *GFP* gene. Polyamidoamine dendrimers PAMAM-NH_2 G4 ensured sufficiently high level of BDNF secretion comparable or superior to the previously reported results of other authors [7,9,13], whereas transfection with phosphorus dendrimers provided very low expression (Table 1): significantly below the level expected on the basis of transfection efficiency ratio.

In our work we examined options of using polycationic vehicles for transgene delivery to neurons and stromal stem cells. There is evidence that efficient transgene expression in neurons can increase their therapeutic potential in transplantation [4,8,16]. For instance, expression of *Ngn2* gene encoding neurogenin ensures stabilization of the phenotype of trans-

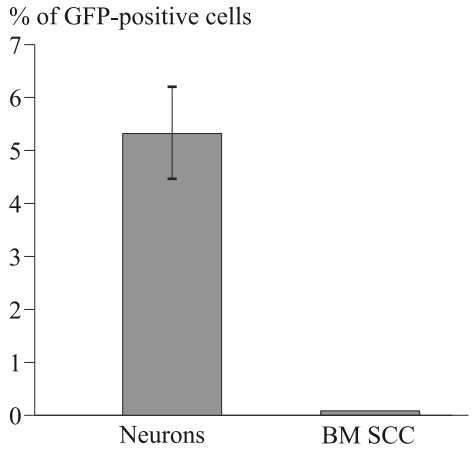
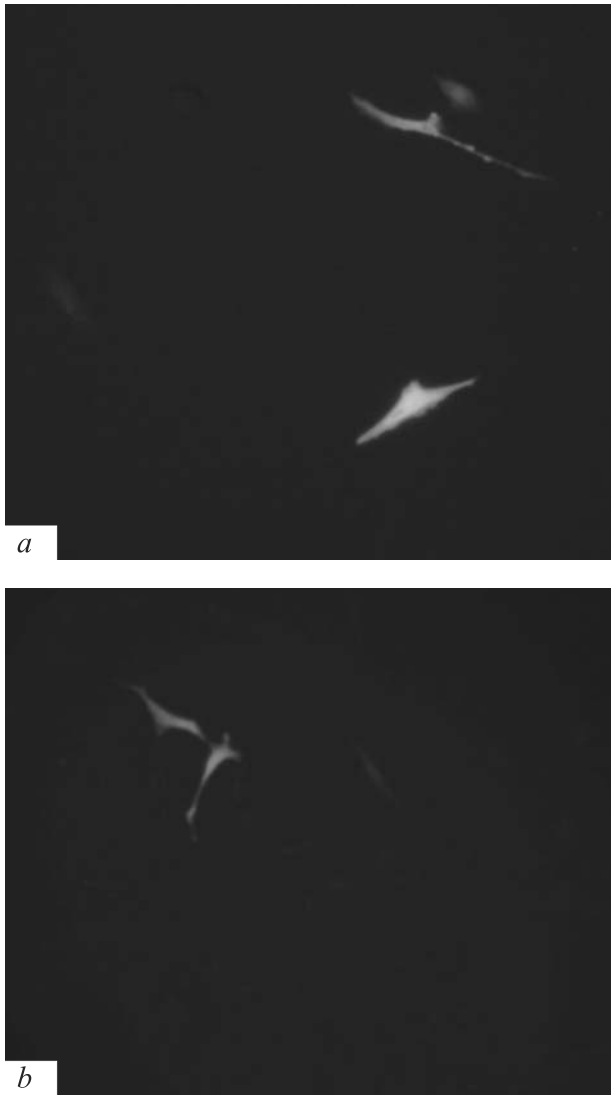


Fig. 1. Transfection efficiency in cells with the use of polyethyleneimine.



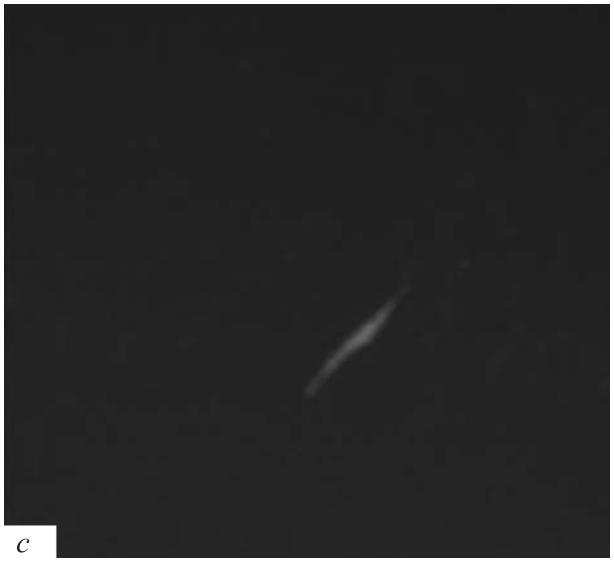


Fig. 2. Transfected MSC from human bone marrow (epifluorescence). *a*) MSC transfected with PAMAM-NH_2 G4 dendrimer; *b*) MSC transfected with Phos G4 dendrimer; *c*) MSC transfected with linear polyethylenimine.

planted neurons and promotes recovery of the recipient after spinal cord injury [8]. Introduction of *CNTF* gene in neuronal progenitors improved resistance to stress and reduces apoptosis [16]. Gene engineering expression of GAP-43 and CAP-23 protein promoted elongation of axons in sympathetic ganglia neurons [4]. The potential of MSC may also be significantly extended by introduction of one or more transgenes into their genome compensating for the functions disturbed in the recipient, improving cell resistance to stress, and promoting their differentiation in a given direction, *etc*. [2].

We studied a simple and very efficient method of delivering vector constructs in different populations of primary cells using a class of agents belonging to cationic polymers and compared transfection efficiency of some polycations. The advantages of these carriers over recombinant viruses are easy use and safety in terms of potential immune response and virus reversal to the wild type. Polycations do not integrate the vector into the chromosome, which reduces the risk of destabilizing the genome and activation of oncogenes.

Polycation-mediated transfection enables studies of activity of different genes, which are potentially therapeutically useful or interesting from the viewpoint of fundamental science, on the model of transient expression. Existing methods of cell sorting by flow cytometry make it possible, if necessary, to enrich the population of cells expressing fluorescent reporter protein bringing their content to 95% [11]. The development of gene-therapeutic approaches based on neurons and MSC will make it possible to realize the potential of gene and tissue engineering for the treatment of diseases, as well as to extent fundamental understanding of the functioning of potentially therapeutically useful genes.

This work was supported by grant ERA-NET NAN2007-31198-E.

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