Symptomatic Improvement, Increased Life-Span and Sustained Cell Homing in Amyotrophic Lateral Sclerosis After Transplantation of Human Umbilical Cord Blood Cells Genetically Modified with Adeno-Viral Vectors Expressing a Neuro-Protective Factor and a Neural Cell Adhesion Molecule

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Abstract: Amyotrophic lateral sclerosis (ALS) is an incurable, chronic, fatal neuro-degenerative disease characterized by progressive loss of moto-neurons and paralysis of skeletal muscles. Reactivating dysfunctional areas is under earnest investigation utilizing various approaches. Here we present an innovative gene-cell construct aimed at reviving inert structure and function. Human umbilical cord blood cells (hUCBCs) transduced with adeno-viral vectors encoding human VEGF, GDNF and/or NCAM genes were transplanted into transgenic ALS mice models. Significant improvement in behavioral performance (open-field and grip-strength tests), as well as increased life-span was observed in rodents treated with NCAM-VEGF or NCAM-GDNF co-transfected cells. Active trans-gene expression was found in the spinal cord of ALS mice 10 weeks after delivering genetically modified hUCBCs, and cells were detectable even 5 months following transplantation. Our gene-cell therapy model yielded prominent symptomatic control and prolonged life-time in ALS. Incredibly survivability of xeno-transplanted cells was also observed without any immune-suppression. These results suggest that engineered hUCBCs may offer effective gene-cell therapy in ALS.

Keywords: Adeno-virus, amyotrophic lateral sclerosis (ALS), gene-cell therapy, glial cell-derived neuro-trophic factor (GDNF), human umbilical cord blood cell (hUCBC), human umbilical cord blood mono-nuclear cell (hUCB-MC), neural cell adhesion molecule (NCAM), vascular endothelial growth factor (VEGF), viral vector.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an incurable, chronic, fatal motoneuron disease whereby progressive degeneration of neurons in the central nervous system (CNS) leads to paralysis of skeletal muscles. An important approach in treating this slowly progressive disorder is to support or even replace lost motoneurons.

Strategy for ALS gene therapy unfortunately mostly is still at pre-clinical stage. There are a variety of pre-clinically proofed therapeutic genes for ALS treatment which belong to different functional groups, such as: (1) neurotrophic factors, eg. brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotumrin; (2) growth factors, eg. nerve growth factor (NGF), insulin-like growth factor-1 (IGF1), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2), angiogenin (ANG); (3) cell adhesion molecules, eg. neural cell adhesion molecule (NCAM) [1-5]. Plasmid vectors which were commonly used for gene delivery at the earlier attempts of gene therapy recently were changed to more effective viral systems with or without possibility to integrate into host genome. Viral delivery of VEGF, GDNF or IGF genes is actively tested on ALS animal models [5-7]. There are few ongoing clinical trials in treatment of Alzheimer’s and Parkinson’s diseases based on adeno-associated virus serotype 2 (AAV2) vector encoding NGF and neurotumrin genes which are directly delivered into the patients’ brains [8].
Meanwhile, new gene, stem cell, or gene-stem cell technologies are employed to produce an effective drug for ALS treatment. Stem cells are gaining ground in restoring dysfunctional tissues associated with cell death in general, and current investigations into ALS therapy includes transplantation of neural stem cells, mesenchymal stem cells, hematopoietic stem cells, CD34+ and CD133+ stem cells [9, 10]. These cells are available from fetus, bone marrow, peripheral blood, or umbilical cord blood.

Variability of therapeutic genes, gene vectors, types of stem cells as well as possible different combinations between recombinant genes and cells selected for gene delivery in our studies keep us as pioneers in development of gene-cell approaches for ALS treatment [11-14]. Here we present new gene-cell constructs for ALS treatment, based on human umbilical cord blood mono-nuclear cells (hUCBMCs) and adenoviral vectors encoding human VEGF, GDNF and NCAM genes in different combinations. hUCBMCs after transduction with one or two viral vectors encoding different therapeutic genes (VEGF and NCAM, GDNF and NCAM, etc.) were transplanted into ALS mice. The efficacy of gene-cell therapy of ALS mice was evaluated employing behavioral tests, life span measurement and immuno-fluorescent study of spinal cord sections at the terminal stage of disease.

MATERIAL AND METHODS

Adenoviral Vectors Generation

Recombinant adenoviruses Ad5-EGFP, Ad5-GDNF, Ad5-NCAM1 and Ad5-VEGF165 were generated using Gateway cloning technology according to manufacturer’s instructions (Invitrogen, USA). VEGF165 is a predominantly expressed VEGF isoform, which plays a central role in vascular development [15]. In 2002 plasmid construct containing human VEGF165 gene was recommended for clinical use [16].

Cloning of vegf165 and gdnf cDNA into The plasmid Vector pENTR-D/TOPO

Amplification of cDNA fragments of vegf and gdnf was performed in a thermocycler C1000 Thermo Cycler (BioRad, USA) using Phusion High fidelity DNA Polymerase (FINNZYMES). Primers were synthesized by "Synthol" (Russia), nucleotide sequences are presented in Table 1. Purified products were cloned using Gateway recombination into a plasmid vector pENTR-D/TOPO (Invitrogen, USA) followed by transformation into E.coli Top 10 competent cells. Colony PCR was carried out using vector-specific primers (Table 1). Generation of the recombinant plasmids was confirmed by sequencing and restriction analysis (data not shown).

Cloning of cDNA of Ncam1 gene into Plasmid Vector pDONR221 Using Gateway Recombination Technology

PCR amplification of gene fragment of ncam1 was carried out in two rounds: the first round is used to amplify cDNA with short flanking attB sites using primers hNCAM - attB1 and hNCAM - attB2, nucleotide sequences are presented in Table 1. Second round of PCR amplification was conducted using adapter primers GW-attB1 and GW-attB2 intended to generate full length flanking attB sites. BP-recombination was performed under standard protocol according to manufacturer’s instructions (Invitrogen, USA), followed by transformation into E.coli Top 10 competent cells. PCR screening of the colonies and confirmation of target insert in recombinant plasmids were carried out as described above. Donor Gateway-compatible plasmid encoding enhanced green fluorescent protein (EGFP) cDNA (pDONR-EGFP, plasmid 25899) was obtained from Addgene (USA).

Table 1. Nucleotide sequences of primers and probes for PCR and RealTime PCR amplification (uppercase - coding sequence).

<table>
<thead>
<tr>
<th>Name of the Primer</th>
<th>Nucleotide Sequence</th>
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<td>hVEGF - TOPO-F*</td>
<td>caccATGAATTTTCTTCGTCCTTGG</td>
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<td>hVEGF - Stop - SalI*</td>
<td>gttcagTCACCGCCTGGCTGTC</td>
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<td>caccATGAAGTTAGGATGTCTGG</td>
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<td>hNCAM - attB1**</td>
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<tr>
<td>hNCAM - attB2**</td>
<td>AGAAAGCTGTTGTCGTTGGTTCTC</td>
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<tr>
<td>GW - attB1**</td>
<td>ggggacagtctgtaaaacagcgct</td>
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<tr>
<td>GW - attB2**</td>
<td>ggggacagtctgtaaaacagcgct</td>
</tr>
<tr>
<td>M13-Forward***</td>
<td>gTAAACAgCCgCAGGACgTg</td>
</tr>
<tr>
<td>M13-Reverse***</td>
<td>ggAAACAGCTATgACCATg</td>
</tr>
<tr>
<td>VEGF-TM-Forward</td>
<td>ATACACCTCGAGATTTCCG</td>
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<tr>
<td>VEGF-TM-Reverse</td>
<td>TGACATCCATTTGTTGTC</td>
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<tr>
<td>VEGF-TM-Probe</td>
<td>[FAM]TCAAACCTCAACGACCAGGAC[BJH]</td>
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</tr>
<tr>
<td>EGFP-TM-Probe</td>
<td>[FAM]CCGCGATCATGGTCTGTCG[BJH]</td>
</tr>
</tbody>
</table>

* PCR amplification for first round amplification
** PCR amplification for introduction of attB Gateway sites
*** Vector-specific primers for colony PCR screening
Creation of Expression Adenoviral Vectors Using Gateway Cloning Technology

To generate expression constructs based on adenoviruses LR-recombination was carried out from donor plasmids pENTR-VEGF165, pENTR-GDNF, pDONR-EGFP and pDONR-NCAM1 into destination vector pAd/CMV/VS-Dest (Invitrogen, USA). After transformation into competent E.coli cells, positive colonies were screened using genus-specific PCR. Correct insert confirmation was confirmed by restriction analysis and sequencing. Preparative plasmid DNA isolation was performed using QIAFilter Plasmid Midiprep Kit (QIAGEN, Netherlands) according to producer’s recommendations.

Transfection of HEK293A Cells with Recombinant Plasmids

HEK293A cell line (Invitrogen, USA) was cultured at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM, PanEco, Russia), containing 10% fetal bovine serum (FBS, Gibco, USA), 1% antibiotic mixture of penicillin and streptomycin (PanEco, Russia), and 2 mM of L-glutamine (PanEco, Russia). Transfection of HEK293A cell lines with genetic constructs (pAd-EGFP, pAd-GDNF, pAd-NCAM1, pAd-VEGF165) was carried out using the transfection reagent TurboFect (Fermentas Inc., Canada) according to the procedure recommended by the manufacturer. Conformation of recombinant proteins expression was performed 48 hours after transfection by immunofluorescent analysis.

Immunofluorescent Analysis of Recombinant Protein Expression

For fixing transfected HEK293A cells in the wells of the culture plate after removing the culture medium, pre-chilled methanol was added with further incubation at -20°C for 10 minutes. After incubation, cells were washed with Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.64; reagents obtained from Sigma, USA). Cell membranes were permeabilized by 0.1% solution of Triton X-100 (Helicon, Russia). Incubation with primary antibodies (Ab) was performed in TBS for 1 hour; the cells were then washed with TBS and incubated with secondary Ab for 1 hour (Table 2). Preparations were observed using fluorescent microscope Axio Observer Z1 (Carl Zeiss, Germany).

Production of Recombinant Adenoviruses

To produce recombinant adenovirus Ad5-EGFP, Ad5-NCAM1, Ad5-GDNF, and Ad5-VEGF165 an adenoviral vector plasmid was transferred from the ring into a linear form with a restriction enzyme PacI (Invitrogen, USA). Purified linear plasmid was used for genetic modification of HEK293A cell line using transfection reagent TurboFect. After transfection we replaced media every 2-3 days with fresh one until the formation of visible cytopathic regions characterizing with changing of cell morphology. On day 10 after transfection, cell suspensions were collected in sterile 2 ml tubes. After collection, cell suspensions were conducted several freeze/thaw cycles followed by centrifugation to prepare a crude viral lysate. Viral stock was stored at -80°C.

<table>
<thead>
<tr>
<th>Antigene</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>*GDNF</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>*VEGF</td>
<td>Goat</td>
<td>1:125</td>
<td>Sigma</td>
</tr>
<tr>
<td>*GFP</td>
<td>Mouse</td>
<td>1:100</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>*NCAM1, PE conjugated</td>
<td>Mouse</td>
<td>1:100</td>
<td>Sorbent (Moscow, Russia)</td>
</tr>
<tr>
<td>*Human nuclei antigen (HNA)</td>
<td>Mouse</td>
<td>1:150</td>
<td>Millipore</td>
</tr>
<tr>
<td>*CD34</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>*Neuronal β III-tubulin</td>
<td>Rabbit</td>
<td>1:2,000</td>
<td>Covance</td>
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<tr>
<td>*β-actin</td>
<td>Mouse</td>
<td>1:2,000</td>
<td>GenScript</td>
</tr>
<tr>
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<td>Rabbit</td>
<td>1:2,000</td>
<td>Abcam</td>
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<tr>
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<td>DakoCytomation</td>
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<td>Invitrogen</td>
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<td>**Anti-mouse Alexa 647</td>
<td>Donkey</td>
<td>1:150</td>
<td>Invitrogen</td>
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<tr>
<td>**Anti-goat Alexa 488</td>
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<td>Invitrogen</td>
</tr>
<tr>
<td>**Anti-rabbit IgG, HRP conjugated</td>
<td>Goat</td>
<td>1:5,000</td>
<td>Sigma</td>
</tr>
<tr>
<td>**Anti-mouse IgG, HRP conjugated</td>
<td>Goat</td>
<td>1:5,000</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

* primary antibodies
** secondary antibody
§§ for western blotting
§§ for immunofluorescent staining

Amplification and Titration of Viruses

To obtain preparative amounts of adenovirus encoding egfp, vega165, gdnf and ncam1 genes HEK293A cell line was infected with derived viruses. After 72 hours, cell lysates were collected in 15 ml tubes and subjected to several freeze/thaw cycles according to procedure described above. The supernatant was filtered and stored at -80°C. Determination of viral titers was performed concerning to Invitrogen proposed plaque assay.

Cell Preparation and In vitro Analysis

Umbilical cord blood was taken after obtaining informed consent of the pregnant and prenatal screening for contraindications to blood donation. All manipulations were performed on the basis of stem cell bank of Kazan State
Medical University. Study was approved by Local ethical committee of Kazan State Medical University.

Isolation of nuclei containing red blood cells was performed as described previously [17]. To each tube 25 ml of Ficoll with 1.077 g/ml density (PanEco, Russia) were added by gentle using an automatic dispenser overlaid with an equal volume of umbilical cord blood mixed with anticoagulant (blood and anticoagulant ratio range 1:1-3:1). After centrifugation at 720×g for 20 minutes, clear separation of blood into 4 fractions were seen: erythrocytes, Ficoll, leucocytes and plasma. Leukocyte fraction was collected into a separate tube, resuspended in Dulbecco’s phosphate-buffered saline (DPBS, PanEco, Russia) at a ratio of 1:2 and centrifuged at 305×g for 15 minutes. The resulted cell pellet was resuspended in 10 ml of DPBS and again centrifuged at 305×g for 15 minutes. To remove the erythrocytes, cell pellet was resuspended in a hypotonic cell lysis buffer (0.168 M NH₄Cl, 0.1 M KHCO₃, 1.27 mM EDTA (Acros organics, Belgium), pH 7.3), at the final stage the cells were washed with DPBS solution.

After purification, fraction of mononuclear cells from human umbilical cord blood was cultivated in RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS and a mixture of antibiotics penicillin and streptomycin (100 U/ml, 100 µg/ml).

Immediately after isolation, mononuclear cells were seeded on 10 cm culture dish and transduced with recombinant adenoviruses with MOI 10. Cells were incubated for 12-16 hours in a humid environment at +37°C with 5% CO₂ content.

**RNA Isolation and cDNA Synthesis**

hUCBMCs modified with one type of adenoviruses or viral cocktails (MOI 10) were collected 5 days after transduction. RNA isolation was performed with RNA isolation kit from cell cultures (BioSilica, Russia) according to the manufacturer’s recommendations.

Synthesis of cDNA first strand was achieved with use of 100U Maxima Reverse Transcriptase (Thermo Scientific, USA). For cDNA synthesis, an RNA/primer/dNTPs mix of 100 ng RNA, 1µl of Random hexamer primers (LifeTech, Russia), 1 µl of dNTP mix (10mM) and 8.5 µl of H₂O was denatured at 65°C for 5 minutes and chilled quickly to 4°C. cDNA was synthesized by adding 5× RT buffer (Thermo Scientific, USA), 200U of Maxima Reverse transcriptase (Thermo Scientific, USA), and 20U of Ribolock RNase inhibitor (Thermo Scientific, USA) in a 20 ml volume of reaction mix. After incubation for 10 minutes at 25°C, the reaction was proceeded for another 30 minutes at 42°C and finally terminated by heating at 95 °C for 5 minutes.

**TaqMan Real Time PCR**

Samples of cDNA obtained were analyzed by CFX 96 Real-Time PCR System (BioRad, USA). Each PCR reaction (15 µl) consisted of 0.5 µl of cDNA, 2.5× Reaction mixture B (Syntol, Russia), 200 nM of each primer and 100 nM of probe (Table 1). Amplification parameters were the following: preheating at 95°C for 3 minutes, 39 cycles: 95°C for 10 seconds, 55°C - 15 seconds including plate-read. Amount of RNA was normalized using 18S ribosomal RNA as housekeeping gene. Standard curves for relative quantitation of the NCAM1, VEGF and GDNF were generated using serial dilutions of plasmid DNA with corresponding cDNA inserts. Expression level of genes in no treatment control (NTC) was considered as 100%. All RT-PCR reactions were performed in triplicates. All data are presented as mean ± S.D. with statistical significance assessed by Student’s t-test, with P<0.01 being regarded as a statistically significant difference.

**Western Blotting**

Adenovirus infected cells were lysed in 1.5× sample buffer (10% glycerol (Panreac, Spain), 50 mM Tris-HCl (pH 6.8, Helicon, Russia), 2 mM EDTA, 2% sodium deoxycholate (SDS, Helicon, Russia) 144 mM 2-mercaptoethanol (Sigma-Aldrich, USA), 0.0084% bromphenolic blue (Helicon, Russia)) and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) [18]. Proteins were transferred (45 minutes, 118 mA) onto PVDF cellulose membranes (Millipore, USA) using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA) and blocked for 4 hours at room temperature with 5% non-fat dry milk (Helicon, Russia) in phosphate-buffered saline (PBS, Biolot, Russia) and 0.5% Tween 20 (AppliChem GmbH, Germany). After three washes, membranes were incubated (18 hours, 4°C) with Ab against GFP, β-actin and GDNF. Antigen-antibody complexes were identified with horseradish peroxydase conjugated anti-IgG Ab and visualized using ECL Western Blotting substrate kit according to the manufacturer’s instructions.

**Animals and Treatments**

ALS mice, transgenic for mutant human SOD1 gene (B6SJL-TG(SOD1-G93A)1Gur/J, Stock No. 002300) were obtained from Jackson Laboratory (Bar Harbor, USA) and bred at animal facility of Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Russia). This strain is characterized by reduced transgene copy number compared to the original high copy number strain (B6SJLTG(SOD1-G93A)1Gur, Stock No. 002726) which results in delayed onset of the ALS phenotype and mean survival of 266 days. Mature 22-week old male and female mice were delivered to Kazan State Medical University and housed one per cage under standard laboratory conditions. Monitoring of the disease stages development, holding of suffering animals and evaluation of survival rate (definition of the endpoint) were performed based on the Jackson Laboratory guidelines “Working with ALS Mice” (http://jaxmice.jax.org). Animal treatment protocol was approved by the Kazan State Medical University Animal Care and Use Committee.

Female and male ALS mice were randomly assigned to few groups, including (1) control group (n=9) and 6 groups underwent transplantation of hUCBMCs transduced by adenoviral vectors expressing: (2) EGFP (n=9), (3) VEGF (n=9), (4) GDNF (n=9), (5) VEGF and NCAM (n=9); (6) GDNF and NCAM (n=9), (7) VEGF and GDNF (n=9). 2×10⁶ cells were transplanted retroorbitally in presymptomatic 27-29-week old ALS mice without immunosuppression; control ALS mice were injected with saline.
We assume that blood-brain barrier makes hUCBMCs invisible for healthy immune system of ALS mice. Previously we demonstrated hUCBMCs survival in lumbar spinal cord of ALS mice for several weeks without any immunosuppression [12, 13]. Immune privilege status of CNS helps the grafts to survive for a longer period comparing with other recipient tissues [19]. The retro-orbital injection of cells is equal to intravenous administration and was chosen based on our previous work where the effective migration of hUCBMCs into the CNS was shown after injection of 1-2 x10^6 cells [12, 13, 20].

For histological analysis mice under ketamine-xylazine anesthesia were transcardially perfused first with cold PBS and then with cold 4% paraformaldehyde (Panreac, Spain) in PBS (pH 7.4). The whole spinal cord was removed, post-fixed with paraformaldehyde overnight at 4°C and then immersed in 30% sucrose solution in PBS (pH 7.4) at 4°C. Afterwards lumbar spinal cords were embedded in TBS tissue freezing medium (Triangle Biomedical Science, USA).

**Behavioral Tests**

The clinical signs of the disease have been developed typically both in males and females. Animals become paralyzed in rear legs, then front legs. Endpoints have been determined when the animals could no longer forage for food or water. Behavioral examination has been started prior to development of clinical signs as abnormalities in gait, impaired grooming, problems with extension of hind legs.

Two weeks before cell transplantation, mice were trained to perform two types of behavioral tests: open field and grip strength. In the open field test, mouse was placed to the commercially obtained arena (Otkrytaya Nauka, Russia) for 3 minutes and the number of crossed lines (horizontal activity), vertical stands (vertical activity), and the number of explored holes in arena floor (exploration activity) were analyzed as described previously [11]. Grip strength test was performed on the next day after open field testing. The mouse was allowed to grip a metal grid with all four paws. Then, the grid was slowly rotated over so that the mouse was turned upside down. The time of mouse hanging to metal grid was evaluated. The test included 3 trials and the maximal result was recorded. Behavioral tests were started on days 2-4 after cell transplantation. Each test in each group was repeated twice per week before paralysis stage ends.

All data are presented as mean ± S.E.M. with statistical significance assessed by Student’s t-test. P value of less than 0.05 was considered as statistically significant difference (shown in Fig. 4). Life span of every animal was also calculated. The significance of differences in survival rate of various groups was assessed with Fisher’s exact test, while the difference was considered statistically significant when q Emp > 1.64 (data given in Results section).

**Immunofluorescence**

Frozen free-floating (20 µm) coronal serial sections of lumbar spinal cord were prepared. To detect and phenotype transplanted cells, to verify expression of therapeutic genes in ALS mice spinal cord sections were subjected to immunofluorescent staining. After blocking of non-specific binding sites with normal donkey serum the primary Ab (Table 2) were applied overnight at 4°C. The subsequent incubation of the sections with proper secondary Ab (Table 2) for 2 h at room temperature was followed with cell nuclei staining with propidium iodide solution (PI, 5 µg/ml in PBS, Sigma, USA) or with bi-/benzamide solution (Hoechst 33258 dye, 5 µg/ml in PBS; Sigma-Aldrich, USA). Finally, sections were picked up on SuperFrost® Plus glass slides, mounted in anti-quenching medium and examined with a laser scanning microscope (LSM 510-Meta, Carl Zeiss, Germany).

**RESULTS**

**In vitro Therapeutic Gene Expression Study**

**Fluorescent microscopy**

Five days after hUCBMCs transduction with Ad5-EGFP, EGFP expression was studied using fluorescent microscopy. All visualized cells had intensive green fluorescence. The results are shown at the Fig. (1).

**Real-Time PCR TaqMan Reaction**

Relative quantitation of mRNA expression of NCAM1, VEGF, EGFP and GDNF was performed by normalizing gene expression data against 18S ribosomal RNA expression level in corresponding sample. The results are demonstrated on Fig. (2). mRNA expression in hUCBMCs simultaneously treated with Ad5-NCAM1+Ad5-GDNF and Ad5-VEGF165+Ad5-GDNF 5 days after transduction was 3000-6000 times higher relatively to non-transduced cells. Efficiency of mRNA expression in single virus treated hUCBMCs was 3-5 times less effective than in case of double treatment.

Obtained data present an evidence of effective therapeutic gene mRNA expression in genetically modified hUCBMCs. Simultaneous transduction with adenoviruses encoding NCAM1 and VEGF165 seems to be more effective than with single adenovirus Ad5-VEGF165.

**Western Blotting**

Expression of target recombinant proteins in genetically modified hUCBMCs was confirmed by Western blot analysis 5 days after cell adenoviral transduction (Fig. 3). Analysis of appearing bands revealed a positive reaction of genetically modified cells with Ab against GDNF and EGFP proteins. Staining with mouse monoclonal Ab against β-actin demonstrated comparable amount of total protein loaded onto the gel (Fig. 3).

**Behavioral Tests**

The efficacy of gene-cell therapy of ALS mice was evaluated employing two behavioral tests: (1) open field and (2) grip strength.

The first two days before the experiment mice of the control group showed following results: horizontal activity 70.9±2.7, vertical activity 10.2±2.1, exploration activity 6.3±1.4, test grip strength 56.6±4.2 seconds. ALS mice from other experimental groups did not show significant difference (P>0.05). Following the start of experiment, gradual decline of horizontal activity in open field test was observed.
Fig. (1). Visualization of EGFP in hUCBMCs. A-B, hUCBMCs treated with recombinant adenovirus Ad5-EGFP (MOI 10) are shown 5 days post infection in green channel (A) and bright field channel (B). C-D, Wild-type hUCBMCs (NTC) are shown in green channel (C) and bright field channel (D). Scale bar 100 µm.

Fig. (2). Levels of VEGF, NCAM1, GDNF mRNA expression in gene-cell constructs. Levels of VEGF, NCAM1 and GDNF mRNA expression in hUCBMCs modified with recombinant adenoviruses are shown: Ad5-NCAM1+Ad5-VEGF165 (NCAM+VEGF), Ad5-GDNF+Ad5-VEGF165 (GDNF+VEGF), Ad5-VEGF165 (VEGF), Ad5-GDNF (GDNF) and wild-type hUCBMCs (NTC). Data presented as mean±S.D; the differences between NTC and other experimental groups are statistically significant (n=3, P<0.01).

in all experimental groups except group VEGF-GDNF, in which marked biphasic dynamics - initial increase to the week 3 and the subsequent decline was found. In general, more rapid decrease of horizontal activity was observed in the control group, EGFP and VEGF groups, whereas slower decline was detected in GDNF, GDNF-NCAM and VEGF-GDNF groups (Fig. 4A).

Vertical activity decreased faster in the control group, EGFP and VEGF groups, whereas GDNF-NCAM, VEGF-NCAM and VEGF-GDNF groups showed slower decline (GDNF-NCAM and VEGF-GDNF groups even showed initial increase in vertical activity, although insignificant) (Fig. 4B).

The exploration activity of the animals in the open field markedly fluctuated during the experimental period, reflecting the variability of this parameter and impeding evaluation of results.

Fig. (3). Western blotting analysis of recombinant protein expression in hUCBMCs. Expression of target recombinant proteins in hUCBMCs was detected using specific Ab listed in Table 2. β-actin was used as a reference (house-keeping protein). 1, wild-type hUCBMCs (NTC); 2, hUCBMCs+Ad5-EGFP; 3, hUCBMCs+Ad5-VEGF165; 4, hUCBMCs+Ad5-GDNF; 5, hUCBMCs+Ad5-GDNF+Ad5-VEGF165; 6, hUCBMCs+Ad5-NCAM1+Ad5-VEGF165.
Fig. (4). Behavioral tests performance and survival rates of ALS mice after genetically modified hUCBMCs transplantation. A-C. Performance of ALS mice in open field (A - horizontal activity, B - vertical activity) and grip strength (C) tests is shown at 3, 6, 9, 12 weeks after hUCBMCs transplantation. Average meanings with standard error are shown, (*) represents statistically significant differences between mice groups. D. Survival rates of ALS mice (percentage of alive mice to initial amount of animals in group) is shown. X-axis - weeks after hUCBMCs transplantation, y-axis - parameters as percentage of initial. Experimental groups: control (black), EGFP (red), VEGF (blue), GDNF (magenta), VEGF-NCAM (green), GDNF-NCAM (orange), VEGF-GDNF (violet).

In the grip strength test experimental groups showed either gradual reduction (control, EGFP, GDNF) or initial growth and subsequent decline of strength (VEGF, VEGF-NCAM, GDNF-NCAM, VEGF-GDNF). In general, overall decline of grip strength was detected earlier in control, EGFP, VEGF-NCAM and VEGF-GDNF groups than in other groups (Fig. 4C).

Survival rates of ALS mice varied significantly between experimental groups (Fig. 4D). On week 11 after transplantation 27.3% of the mice in the control group, 60% in the VEGF, 50% in the GDNF and 87.5% in the GDNF-NCAM group were alive, and only the latter group was significantly different from control. The survival of mice from GDNF-NCAM group was also significantly higher compared to VEGF-NCAM, VEGF-GDNF and EGFP groups. The worst survival rates were observed in the VEGF-GDNF group - only 20% of the mice survived on this term. On week 15 after transplantation, there were no alive mice in the control, EGFP and VEGF-GDNF groups left. 50%, 28.6% and 16.7% of mice remained alive in GDNF-NCAM, VEGF-NCAM and GDNF groups, respectively, which was significantly higher in comparison with the control (Fig. 4D).

**Immunofluorescent Study**

Microscopic analysis of lumbar spinal cord was performed 2-20 weeks after hUCBMCs transplantation. We used anti-HNA Ab to reveal grafted hUCBMCs in spinal cord. HNA+ cells were observed in gray and white matter of spinal cord in all experimental mice except control group (Fig. 5). The fact that HNA+ cells were observed in the lumbar spinal cord of mice sacrificed at terminal stage of the disease demonstrated an incredible survivability of grafted cells without immunosuppression. For hUCBMCs phenotyping anti-HNA Ab was used in combination with one of the following markers: neuronal β-III tubulin, oligodendrocyte specific protein (OSP), S-100, Iba1 and CD34. We did not observe co-localization of HNA with β-III tubulin (neuronal marker), OSP (oligodendrocyte marker), and S-100 (astrocyte marker) (Fig. 5A), although we found that hUCBMCs may differentiate into microglia-like cells (Iba1+) and endothelial cells (CD34+) (Fig. 5B-D). In other words, hUCBMCs utilized their endogenous potential to differentiate into macrophages and endothelial cells.

To verify recombinant genes expression in genetically modified hUCBMCs quadruple fluorescent staining was performed (Fig. 6). Lumbar spinal cord sections of mice treated with hUCBMCs expressing recombinant GDNF (VEGF) and NCAM were stained with PI and Ab to HNA, human NCAM, and human GDNF (VEGF). Immunofluorescent study revealed HNA+/NCAM+/GDNF+ cells in ALS mice spinal cords 9, 15, 17 weeks after hUCBMCs transplantation (Fig. 5A,C). HNA+/NCAM+/VEGF+ cells were observed in
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Fig. (5). Visualization of grafted cells in the ALS mice spinal cord 2 weeks after hUCBMCs transplantation. A, panoramic images of anterior horns of ALS spinal cord. HNA expression (red) indicate grafted cells, nuclei are stained with Hoechst 33258 dye (blue). Upper left panel shows few HNA+ (grafted) cells. Upper right panel represents βIII-tubulin+ cells (green) - motoneurons. HNA+ cells are βIII-tubulin-negative. Lower panels represent HNA+ cells, which neither express astrocytes marker S100 (green staining, shown at lower left panel), nor oligodendrocytes marker OSP (green staining, shown at lower right panel). B, C, D, triple labeling of cells with Ab against HNA (red) and CD34 (B and C, green) or Iba1 (D, green) gives an evidence that grafted cells may differentiate in endothelial (CD34+) and microglia-like (Iba1+) cells. Nuclei are stained with Hoechst 33258 dye (blue). Triple-labeled cells are indicated with arrows. 2 triple-labeled cells on B panel are located within blood vessel wall. Scale bar: 20 µm.

mice 7, 9, 12 weeks after hUCBMCs transplantation (Fig. 5B). The finding of human NCAM+ grafted cells in spinal cord even 17 weeks after transplantation points to the important role of recombinant NCAM expression in hUCBMCs for their survival.

Our attempts to measure tissue or serum level of human VEGF and GDNF were not successful (data not shown), which may be explained by very low abundance of human proteins comparing to total mouse proteins due to low number of survived grafted cells expressing recombinant human proteins compared to total number of cells in mouse tissues.

DISCUSSION

Apoptosis of spinal motoneurons at ALS is induced by multiple cellular disorders including glutamate excitotoxicity, mitochondrial dysfunction, oxidative stress, axonal transport defects, accumulation of toxic intracellular aggregates, aberrant glial reactivity, etc. [21]. Replacement of lost neurons, stimulation of neurite growth and reestablishment of neuronal circuits are anticipated results of ALS treatment. There are three well-known approaches for delivery of the therapeutic molecules to pathological foci: (1) injection of recombinant proteins; (2) treatment with plasmid or viral
expression vectors; (3) transplantation of genetically modified auto- or allo-harvested cells. Nowadays cell types which may be genetically modified and used as bioreactors for neurodegenerative diseases treatment include neural stem cells, neural progenitor cells, mesenchymal stem cells, and hUCBMCs. However, in spite of simplicity of apparent bioengineering technology only a few clinical trials are going on and there are not any completed to be used for effective treatment of neurodegenerative diseases.

The current clinical protocols for umbilical cord blood cells transplantation strictly require HLA matching and immune suppression. These procedures are obliging for treatment of hematopoietic disorders to replace hematopoietic stem cell and to prevent graft versus host disease. However, for the past few years it was proved in growing up clinical trials that hUCBMCs use for the treatment of non-hematopoietic disorders, such as neurodegenerative and cardio-vascular diseases may not require matching or immunosuppression [22, 23].

Rationality to employ genetically modified hUCBMCs for the treatment of neurodegenerative diseases includes: (1) cell viability and their possibility to cross brain-blood barrier after intravenous injection; (2) migration potential in nervous tissue toward to degeneration loci; (3) high level of adenoviral cell transduction in these cells; (4) validation of recombinant gene expression in the cells; (5) production of recombinant therapeutic molecules as well as endogenous growth and neurotrophic factors, cytokines and chemokines; (6) ability to differentiate into various cell types; (7) ability to use hUCBMCs without HLA matching and immunosuppression.

The attempts to separate from hUCBMCs specific cell population for transplantation may have some positive therapeutic effect. However in our view, less you interfere with native hUCBMCs then less side effects you may expect. Moreover, using only one cell population you may loose the other cells which could have higher contribution in stimulation of neuroregeneration. Nowadays it is established that hUCBMCs contain hematopoietic stem cells, mesenchymal stem cells, unrestricted somatic stem cells, cord blood-derived embryonic stem cells, side population cells and endothelial progenitor cells [24]. Thus, if you take one pool of cells from hUCBMCs you may loose many others. Moreover, hUCBMCs secrete cytokines (IL-6 and IL-10), chemokines (IL-8, MCP-1, SDF-1), growth factors (ANG, hepatocyte growth factor, VEGF, platelet-derived growth factor, epidermal growth factor), neurotrophic factors (NGF, GDNF, BDNF, neurophins-3 and neurophin-5) [24-26]. The source of specific factor is unclear, so choosing one cell population from hUCBMCs you may rid of some valuable molecular factors.

In our previous studies we presented VEGF-L1 and VEGF-FGF2 gene-modified hUCBMCs for ALS treatment [12, 13]. We found that hUCBMCs transacted by plasmid vectors carrying individually or in combination different therapeutic genes (VEGF, GDNF, FGF2) can successfully home in gray and white matter of ALS mouse spinal cord, survive there for a long period without immunosuppression and differentiate into endothelial cells, microglial cell or astrocytes.

Analysis of these results and considering short therapeutic effect of plasmids we have aimed to viral vectors for hUCBMCs gene modification. Our main interest was prolonged and combined therapeutic genes expression for stimulation of neuroregeneration in ALS mice. According to obtained results the neuroprotective factor alone (VEGF or GDNF) had approximately the same therapeutic effect. These factors as compared with hUCBMCs transduced with EGFP, showed better results in behavioral tests and had longer lifespan. Intriguing results we obtained after trans-
planted with VEGF and NCAM or GDNF and NCAM. The lifespan of these mice was more and performance in behavioral tests was better comparing to VEGF or GDNF alone, with the best result found after transplantation of hUCBMCs expressing GDNF and NCAM genes. Our results are in line with data demonstrating that simultaneous injection of human mesenchymal stem cells modified to secrete VEGF and GDNF showed synergistic effect on survival ALS rats and protecting neuromuscular junctions [27]. Although we did not show synergistic effect of VEGF and GDNF on ALS mice which may be explained by animal model and type of cells chosen for VEGF and GDNF delivery we believe that hUCBMCs co-transduced with Ad5-VEGF-GDNF had higher effect than with Ad5-VEGF, or with Ad5- GDNF alone at earlier stages of disease development when survival of hUCBMCs was equal in all groups. In our view Ad5-NCAM helps to survive hUCBMCs which in turn may be responsible for the beneficial effect in treatment of ALS mice.

The possible role of NCAM in hUCBMCs modification is to increase their homing into CNS and survival. High survival level of hUCBMCs in spinal cord parenchyma partially may be explained by CNS immune privilege [19]. CNS isolation from the immune system is believed to be based on blood-brain barrier, lacking of lymphatics drainage, and the apparent immunoincompetence of microglial cells. Although it is discussed that blood-brain barrier integrity is insufficient to prevent leucocyte trafficking into the CNS. Bypass the blood-brain barrier by leukocytes via trans-endothelial migration and choroid plexuses from blood to cerebro-spinal fluid was shown [19]. These evidences suggest the possible way of hUCBMCs homing into CNS and defense from the host immune system.

In our view, the improvement of ALS mice performance in behavioral tests after hUCBMCs transplantation is a local effect of the hUCBMCs at the sites of neurodegeneration. HNA+ cells observed in the sections of spinal cords and secreting therapeutic proteins (VEGF or GDNF, NCAM) few months after hUCBMCs transplantation are survived descendants of hUCBMCs, since the leucocytes have a much shorter period of life. Phenotyping revealed that hUCBMCs under current experimental conditions can differentiate into endothelial (CD34+) and microglia-like (Iba1+) cells. Here in it is worth to mention that these types of cells a priori are involved both in neurodegeneration and neuroregeneration, but their participation is transitory, the cells will be rejected. Thus the main positive action of hUCBMCs is the addressed delivery of therapeutic molecules to motor neurons to prolong their survival. Based on the obtained results in this study the therapeutic effect of the hUCBMCs may be explained by paracrine action of the transplanted cells. Their co-localization with affected motor neurons, ability to synthesize and secrete recombinant molecules as well as other trophic and growth factors may give more positive effects comparing with endocrine action when hUCBMCs are located far away from affected neural cells.

Thus, we found that transplantation of gene-cell constructs based on hUCBMCs co-transduced with adeno-viral vectors expressing GDNF and NCAM or VEGF and NCAM genes into ALS mice induce most prominent increase of mice lifespan and performance in behavioral tests comparing to other constructs tested. Shown results can serve as a base for development of effective gene-cell treatment of ALS.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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