Activation of Expression of Brain-Derived Neurotrophic Factor at the Site of Implantation of Allogenic and Xenogenic Neural Stem (Progenitor) Cells in Rats with Ischemic Cortical Stroke

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Ischemic stroke was modeled in the sensorimotor zone of the brain cortex in adult rats. Rat embryonic nervous tissue, neural stem cells from human olfactory epithelium, and rat fibroblasts (cell control) were implanted into the peri-infarction area of rats of different groups immediately after stroke modeling. Expression of BDNF mRNA was analyzed 7 days after surgery by real-time PCR. BDNF expression in cell preparation before their implantation was minimum. The expression of BDNF mRNA increased by 5-6 times in the areas of implantation of rat fibroblasts and human olfactory epithelium and by 23 times in the area of implantation of rat embryonic nervous tissue compared to peri-infarction areas without cell implantation. These findings confirm the possibility of realization of the therapeutic effects of neural stem cells via expression of trophic factors.

Key Words: *neural stem cells from human olfactory epithelium; rat embryonic nervous tissue; rat fibroblasts; ischemic stroke; level of BDNF expression*

Among the hypothetic mechanisms of the therapeutic effects of neural stem (progenitor) cells (SC) implanted into the brain, the leading part is assigned to the production of neurotrophic and growth factor by these cells [11,13], *e.g.* brain-derived neurotrophic factor (BDNF). Its pronounced neuroprotective effect was shown on the models of ischemic stroke [8]. However, direct evidence of enhanced BDNF expression in the area of SC implantation is required. It is important to find out to what measure implantation of neurogenic cells of different origin increases the level of BDNF and whether this effect is specific for SC.

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Here we analyzed the expression of BDNF at the site of implantation of allogenic and xenogenic neural SC in rats with ischemic cortical stroke.

MATERIALS AND METHODS

Experiments were carried out on 26 albino outbred male rats weighing 300-350 g maintained under standard conditions with free access to food and water.

The rats were intraperitoneally narcotized with ketamine (120 mg/kg) and diazepam (5 mg/kg) [11] and ischemic stroke in the sensorimotor cortex was modeled by the method of Kolb *et al*. [6]. Surface cortical vessels were removed together with the pia matter within a rectangular bone opening with coordinates +3 and -1 mm from bregma and 1.5 and

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4.5 mm from the midline [9]. The open brain surface was coated with a dura matter flap and the operation wound was sutured. Antibiotics were not administered. Preparations of neurogenic SC: rat embryonic nerve tissue (rENT; *n*=4), neural stem cells from human olfactory epithelium (NSChoe; *n*=8), and rat fibroblasts (rF; *n*=8) were implanted (1 mil cell/12 μl PBS solution) into the brain cortex at the boundary of the devascularization zone immediately after removals of blood vessels. Injection coordinates were: +3 mm from bregma, 2.3 mm from the midline, and 2.2 mm from the surface of the brain for neurogenic SC and -1 mm from bregma, 2.7 mm from midline, 2.2 mm from the brain surface for rF [9].

Seven days after stroke modeling, the rats were narcotized with ketamine (200 mg/kg intraperitoneally). For analysis of BDNF mRNA expression within areas of cell implantation and the corresponding brain areas without cell implantation (*n*=4), brain areas 1.5× 1.5×2.4 were isolated and frozen in liquid nitrogen.

rENT were prepared as described previously [10], NSChoe were isolated according to the previously described protocol [1]. Rat fibroblasts Rat2, allogenic cells with minimum progenitor properties, served as cell control. Characteristics of rENT and NSChoe were reported previously [2,7]. The viability of cells before implantation was ≥90% (determined by trypan blue staining).

The viability of implanted cells was studied in a group of rats with ischemic cortical stroke (*n*=6). Before implantation, the cells were labeled with CFDA-SE vital dye (carboxyfluorescein diacetate succinimidyl ester, Invitrogen) according to manufacturer's recommendations. After 7 days the animals were narcotized and transcardially perfused with 4% paraformaldehyde in PBS. The brain with membranes was isolated and placed in 4% paraformaldehyde for 24 h and then in 30% sucrose for 24 h. The cells labeled with CFDA-SE on brain sections were visualized by bright green fluorescence using a Leica DMLB fluorescent microscope (emission and excitation peaks at 517 and 492 nm, respectively) and by the absence of fluorescence in other bands.

Total RNA was isolated from cell cultures and biopsy specimens using Illustra RNA Spin mini kits (GE Healthcare) according to manufacturer's instructions. During RNA isolation, DNase I was used for DNA elimination from samples. RNA was eluted with 100 μl water. The purity and concentration of the isolated RNA preparation were evaluated on a Biophotometer Plus biophotometer (Eppendorf) and the degree of RNA degradation was assessed by agarose gel electrophoresis. Aliquots of isolated RNA were stored overnight at -20°C or in liquid nitrogen.

For reverse transcription (RT), 1 μg isolated DNA was used. The reaction was performed using Superscript II kit (Invitrogen) according to manufacturer's recommendations. Oligo-dT18-primer was used as the primer. The concentration of obtained complimentary DNA (cDNA) was measured fluorometrically on a Qbit fluorometer (Invitrogen). RT efficiency was evaluated by PCR with primers to GAPDH (Table 1) followed by visualization of the reaction products in 2% agarose gel.

Plasmids carrying the studied genes were prepared by ligation of PCR products with linearized pAL-TA vector. The strains transformed with the corresponding plasmids were isolated by chemical transformation of *E. coli* (TOP10, Invitrogen) followed by selection on agar (LB, ampicillin). Screening was performed by PCR; plasmids were isolated from positive strains using a kit for isolation of plasmid DNA (Invitrogen). Nucleotide sequence in the insertion site was determined on an ABI Prism 310 sequenator (ABI) using Plac primer.

For primer selection *in silica*, Vector NTI software (Invitrogen) was used. Uniqueness of PCR primers was verified by searching their sequences in GenBank database using BlastN algorithm. Real-time PCR was performed on a iQ5 amplifier (Bio-Rad) using Eva Green dye (Evagreen Supermix, Bio-Rad) according to manufacturer's instructions. GAPDH was chosen as a housekeeping gene. After optimization of PCR, the following conditions for real-time RT-PCR were chosen for our genes: 1) denaturation at 95°C for 2 sec; 2) annealing at 60°C for 20 sec; 3) elongation at 72°C for 20 sec. The program consisted of 40 cycles.

At stage I of the experiment, quantitative analysis of a series of 10-fold plasmid dilutions (TE buffer) containing GAPDH and BDNF cDNA was performed.

TABLE 1. Characteristics of Primers for PCR

Primer	Nucleotide sequence	Length, b.p.
BDNF_q_f	TCATACTTCGGTTGCATGAAGG	137
BDNF_q_r	AGACCTCTCGAACCTGCCC	
GAPDH_q_f	CTTTGACGCTGGGGCTGGCATT	161
GAPDH_q_r	TTGTGCTCTTGCTGGGGCTGGT	

We determined a dilution range where mean square deviation of the linear approximation of a dependence of the threshold cycle value on the amount of initial template DNA was ≥0.99. At stage II, qualitative analysis of BDNF cDNA in cell preparations was performed; at stage III, qualitative analysis of BDNF cDNA in brain specimens was carried out. For minimization of pipetting errors, each reaction was performed in triplicates.

The results were evaluated by the standard method [4] characterizing multiplicity of enhancement of the analyzed gene expression (BDNF) relative to the expression of the housekeeping gene (GAPDH). The expression was calculated by the formula:

$$\text{BDNF}=\frac{E_{(\text{GAPDH})}^{\Delta Ct\ (\text{control}-\text{experiment})}}{E_{(\text{BDNF})}^{\Delta Ct\ (\text{control}-\text{experiment})}},$$

where E is the efficiency of reaction and Ct is the threshold cycle in the experiment and control. Averaged Ct value for peri-infarction area of rat brain isolated on day 7 after stroke modeling was used as the control. For analysis of cell preparations, the averaged Ct value for normal rat fibroblasts was used as the control.

Significance of differences between the groups by the level of BDNF expression was evaluated using the Kruskal–Wallis test.

RESULTS

Analysis of BDNF content in cell preparations before and after their implantation into rat brain detected minimum expression of this factor. The cells implanted into the brain remained viable for at least 7 days: CFDA-SE-labeled cells of all types were found at the site of implantation (Fig. 1).

The total RNA yield from cell and tissues was 30-50 μg from one sample. A260/280 values varied from 1.97 to 2.07. Electrophoresis in formalin-agarose gel (1.2%) revealed two clear-cut bands corresponding by their molecular weight to 18S and 28S ribosomal RNA. After PCR with gene-specific primers and with 1 μl RT-PCR reaction mixture as the template, electro-

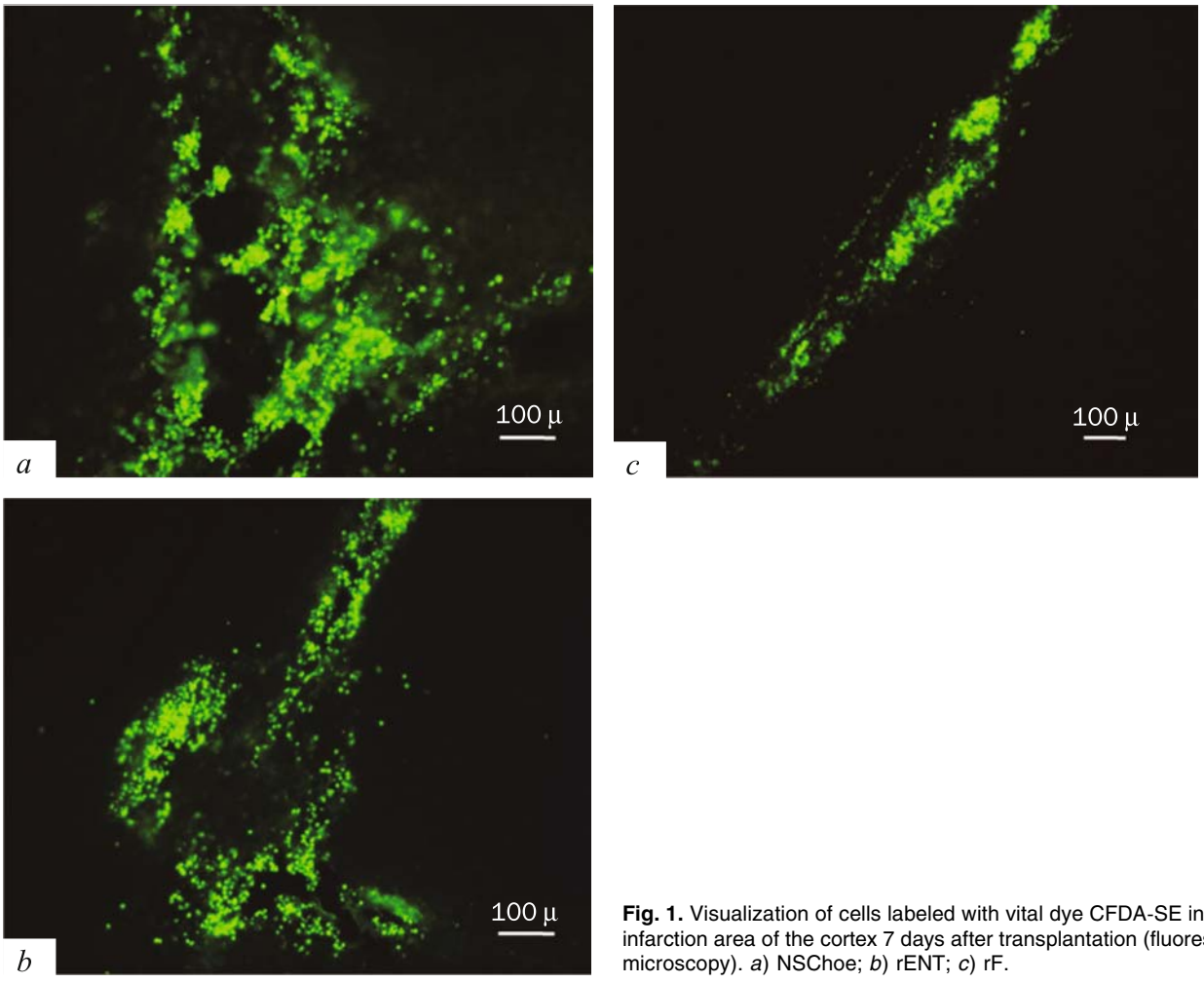

Fig. 1. Visualization of cells labeled with vital dye CFDA-SE in peri-infarction area of the cortex 7 days after transplantation (fluorescent microscopy). *a*) NSChoe; *b*) rENT; *c*) rF.

phoresis in 2% agarose gel visualized solitary clear-cut bands with expected length of 161 and 137 b.p. for GAPDH and BDNF, respectively.

The level of BDNF mRNA in samples of the peri-infarction area of brain cortex 7 days after implantation of rF, NSChoe, and rENT increased by 5.9±0.7, 5.0±0.9, 22.9±0.9 times, respectively, compared to the corresponding parameter without cell implantation. The values in the group receiving rENT significantly differed from those in other rat groups ($p<0.01$ the Kruskal−Wallis test).

Thus, we observed increased expression of BDNF mRNA at the site of implantation of all studied cell preparations. The most intensive expression of BDNF was stably recorded in rats receiving rENT. Accumulation of BDNF in the brain can be directly related to the mechanisms of the therapeutic effect of rENT previously demonstrated in numerous preclinical studies [3]. In our experiments, BDNF expression in cell preparation *in vitro* was minimum. Intensive expression of BDNF in brain samples from rats receiving implantation of rENT can be explained by induction of the synthesis of mRNA for this factor under the effect of microenvironment or the same reaction of brain parenchyma to the presence of these cells.

In sites of rF and NSChoe implantation, BDNF expression was similar and surpassed the corresponding parameter in intact tissue, but remained significantly below the level observed in sites of rENT implantation. It cannot be excluded that this is determined by the nature of transplanted cells. Thus, evaluation of the expression of BDNF mRNA in cell preparations before and after implantation helps to define more precisely the functional characteristics of these cells and to plan studies of the role of growth factors in mechanisms of the therapeutic effect of stem cells.

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