Immunoenzyme Assay of Glial Fibrillary Acidic Protein For Evaluation of Functional Activity of Cell Grafts from Embryonic Ventral Mesencephalon in Rats with Experimental Hemiparkinsonism

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The relationship between the release of glial fibrillary acidic protein (GFAP) into systemic circulation and the efficacy of transplantation of embryonic nervous tissue was studied on rats with 6-OHDA-induced hemiparkinsonism. It was found that intrastriatal transplantation of cell preparations from embryonic ventral mesencephalon significantly attenuated apomorphine-induced rotation, which points to functional recovery of the dopaminergic nigrostriatal system. The degree of this recovery depends on reactive astrogliosis around the graft and survival of dopaminergic neurons. Analysis of GFAP concentration revealed significant elimination of this antigen into the circulation 7 and 14 days after transplantation. In rats with good consolidation of the graft without pronounced reactive gliosis, the concentration of GFAP reached 253.99±79.30 ng/ml on week 4 after transplantation and decreased to 8.2±3.3 ng/ml 8-12 weeks after transplantation. In rats with poor graft consolidation associated with death of transplanted neurons and gliosis in the graft and surrounding tissue the concentration GFAP increased to 476.4±111.0 ng/ml within 4 weeks after transplantation and remained elevated (235.0±44.8 ng/ml) for 12 weeks. Thus, monitoring of serum GFAP concentrations allows *in vivo* evaluation of the functional state of intracerebral graft and the level of reactive gliosis. This test can be used for the prognosis of transplantation efficacy.

Key Words: *experimental parkinsonism; transplantation of embryonic nervous tissue; GFAP*

Transplantation of embryonic nervous tissue (ENT) from the ventral mesencephalon (VM) is an alternative to pathogenetic therapy of Parkinson's disease. However, clinical application of this method is limited, because regression of neurological symptoms is often incomplete and the probability of recurrence of motor disturbances 10-12 months after transplantation is high [1,8]. The failure in most cases is associated with significant loss of transplanted dopaminergic neurons and insufficient reinnervation of the striatum [7,9,10]. Ttransplanted neurons die via apoptosis triggered by hypoxia and oxidative stress during the first days after transplantation [5,12]. Moreover, local damage to the parenchyma associated with reactive astro- and microglia activation [5,11] enhances permeability of the blood brain barrier (BBB) for neurospecific proteins after transplantation. At the same time, autosensibilization with neurospecific proteins, in particular, astrocyte intermediate filament protein GFAP [4], considerably impairs survival of transplanted dopaminergic neurons. In light of this, studies of BBB permeability for neurospecific proteins after ENT transplantation are of great interest. Elaboration of methods for *in vivo* evaluation of the degree of graft consolidation and

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glial reaction of the brain parenchyma are also actual. This is important for choosing additional neuroprotective therapy preventing death of transplanted neurons and stimulating striatum reinnervation. Previous studies were aimed at the evaluation of dopamine-producing function of the graft, rather than evaluation of the degree of graft consolidation [9,10].

Here we studied the possibility of using immunoenzyme assay of the main astrocyte marker GFAP in blood serum for characterization of the functional state and consolidation of intracerebral graft and evaluated the dependence of the efficacy of ENT transplantation in hemiparkinsonism on BBB permeability in the brain-blood direction.

MATERIALS AND METHODS

The study was carried out on 40 adult male Wistar rats weighing 250-300 g. Hemiparkinsonism was induced by stereotaxic injections of 6-hydroxydopamine (6-OHDA; 4 μg/μl, Sigma) into the right ascending mesostriatal dopaminergic tract. The injections were performed under ketamine narcosis (80 mg/kg) in a Narishige stereotaxis apparatus. The coordinates were: injection 1 (2.5 μl) — upper incisor plane 2.3 mm below the interaural line (Ap 4.4, L 1.2, V 7.6); injection 2 (2 μl) — upper incisor plane 3.4 mm above the interaural line (Ap 4.0, L 1.0, V 7.8). Motor defect was evaluated quantitatively by asymmetry of apomorphine-induced rotation.

VM suspension containing dopaminergic neurons was prepared from 13-day-old rat embryos as described elsewhere [6]. Cell viability in the suspension estimated by trypan blue exclusion was above 95%, cell preparations contained 10^6 cells in 7 μl medium. Transplantation was performed on day 28 after 6-ODHA injection under ketamine narcosis into the dorsolateral striatum (coordinates Ap 1, L 3.0, V 4.5-4.1) [14]. Freshly isolated cells were injected with a Hamilton microsyringe and dosator (Sage Instruments) attached to a stereotaxis (1 μl/min).

Experimental group consisted of 20 rats subjected to intrastriatal transplantation of embryonic VM. Control group 1 (10 rats) received stereotaxic injection of cell-free Hanks solution (placebo) into the caudoputamen. To exclude the effect of local brain trauma on BBB permeability for GFAP, 10 rats (control group 2) received injections of an equivalent volume of 6-OHDA solvent (0.25% ascorbic acid) into the caudoputamen.

One and 2 weeks after 6-OHDA injection and 2, 4, 8, and 12 weeks after ENT transplantation the animals were injected subcutaneously with 0.05 mg/kg apomorphine (nonselective D-receptor agonist) and rotation was monitored for 40 min in an automatic rotometer (APO-test). The rotometer and the software for automatic registration of rotation were elaborated on the base of U. Ungerstedt rotometer [15]. In the experiments with transplantations, 30 rats with APO-test parameters equal or above 7.5 rpm were used, which points to pronounced damage of the dopamine nigrostriatal system (DNS) corresponding to 90-95% decrease of dopamine concentration in the striatum [13].

Twelve weeks after transplantation brain sections of the striatum and substantia nigra from experimental animals were analyzed morphologically. The rats were deeply anesthesized and perfused with 400 ml 4% neutral cold paraformaldehyde via the ascending aorta. Serial 40-μ sections were prepared on a freezing microtome. Dopaminergic neurons were visualized immunocytochemically using anti-tyrosine hydroxylase monoclonal antibodies (Sigma) followed by the reaction with Vectstain ABC kit (Vector Laboratories) according to the protocol supplied with the kit. Total morphological analysis was performed on parallel sections stained with cresyl violet.

GFAP in the blood serum was detected by solid-phase sandwich immunoenzyme assay [4,16] using test-systems based on anti-GFAP monoclonal antibodies produced at the Laboratory of Immunochemistry of V. P. Serbskii Institute.

Rat blood (1 ml) was obtained from the caudal vein 1, 2, and 4 weeks after 6-ODHA denervation and 1, 2, 4, 8, and 12 weeks after transplantation. Basal GFAP level was determined in the same rats before 6-ODHA injection. The blood was centrifuged at 1500 rpm, the serum was frosen and stored at -80°C.

Quantitative data were processed by paired and two-sample Student's *t* test and Pierson correlation analysis using Excel 98 and SPSS 8.0 for Windows Software.

RESULTS

Apomorphine-induced rotation during hemiparkinsonism depends on denervation hypersensitivity of D-receptors on the lesion side and reflects the decrease of dopamine content in the striatum [1,6,13]. This test was used for the estimation of DNS recovery after transplantation. Mean values ($M \pm m$) of APO-test parameters were 9.1±0.6 and 8.9±70.7 rpm in the experimental and control groups, respectively. The rats receiving Hanks medium (control group 1) showed progressive increase in APO-test parameters. Three months after surgery the mean APO-test values surpassed the initial levels and reached 9.8±1.0 rpm. This points to high level of denervation hypersensitivity of D-receptors resulting from critical decrease of dopamine concentration in homolateral striatum and the absence of spontaneous recovery in controls.

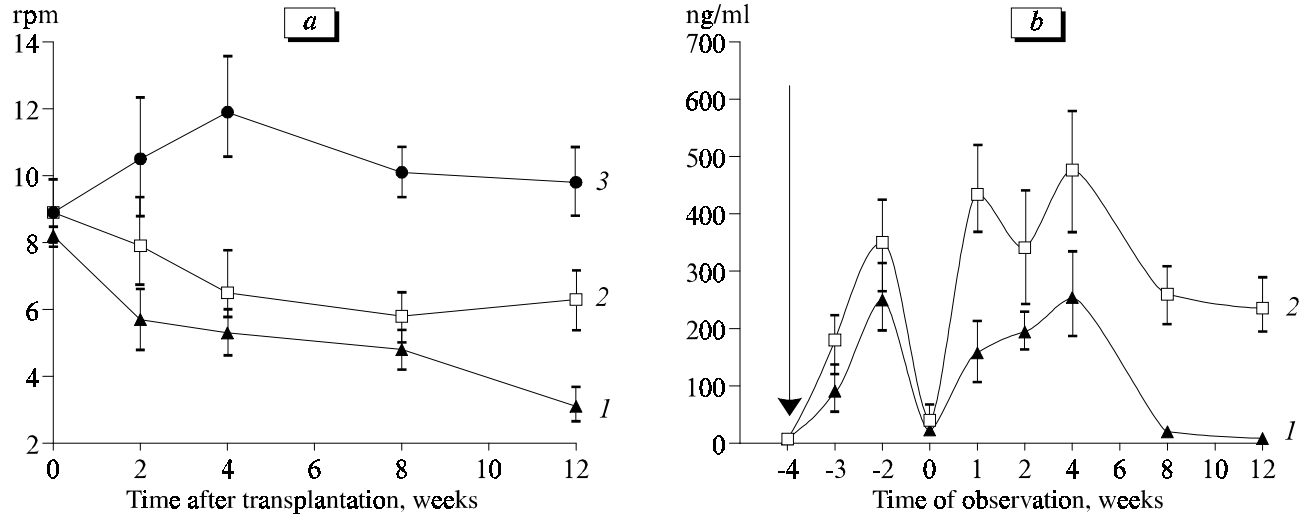

Fig. 1. Dynamics of apomorphine-induced rotation (*a*) and serum GFAP concentration (*b*) after transplantation of embryonic nervous tissue in rats with good consolidation (*1*), gliosis (*2*), control animals (Hanks medium, *3*). Arrow indicates 6-OHDA injection; day of transplantation corresponds to zero point on the abscissa.

In experimental group (after 1 month), significant regression of apomorphine-induced asymmetry ($p<0.05$) was observed. At the end of the experiment the mean APO-test value decreased to 4.3±0.5 rpm ($p<0.01$ compared to control), *i.e.* by more than 50% compared to the initial values. However, analysis of individual curves showed that the efficacy of ENT transplantation was different. In 11 rats APO-test values decreased by more than 65%, while in 9 rats these values decreased by 30-40%, which allowed to divide experimental rats into 2 subgroups with high (1) and low (2) transplantation efficacy (Fig. 1).

Morphological analysis of the grafts showed different degree of their consolidation in these subgroups. In subgroup 1 animals showing significant regression in APO-test parameters, the graft consisted of polymorphic neurons without pronounced glial reaction in the graft and surrounding recipient tissue, which indicated good consolidation (Fig. 2, *a*). Tyrosine hydroxylase immunoreaction revealed grafts containing differentiated dopaminergic neurons with processes penetrating to the surrounding tissue (Fig. 2, *b*). Ineffective transplantation (subgroup 2) was associated with poor consolidation characterized by enhanced proliferation of astrocytes and microglia in the grafts and surrounding tissues. The neurons formed groups separated from the recipient striatum by a glial scar. The reaction for tyrosine hydroxylase revealed only solitary weakly stained cells.

Irreversible changes in *s. nigra pars compacta* similar to those described earlier [2,3] were revealed in all experimental and control rats.

Serum concentration of GFAP was measured using a calibration curve of the immunoenzyme test system (Fig. 3). Working segment of the calibration curve (*i.e.* a region with close to linear dependence) was localized in a concentration range of 1.5-700 ng/ml. Parallel testing of this system after serial dilutions of human brain extract allowed to obtain a curve parallel to the standard one. The test system was precise, reliable, and reproducible, its sensitivity was about 0.5 ng/ml.

Immunoenzyme assay of serum GFAP revealed a significant 6-OHDA-induced increase in BBB permeability compared to insignificant increase in this antigen elimination in control rats after injection of the solvent. Thus, increased BBB permeability for GFAP could results from 6-OHDA-induced neurodegeneration in *s. nigra pars compacta*, rather from local trauma during surgery. The maximum increase in GFAP concentration (290.4±59.9 ng/ml) was observed on day 14 after denervation. After 1 month GFAP concentration decreased to 41.2±717.7 ng/ml but 6- and 10-fold surpassed the corresponding values in control group 2 and basal GFAP level (7.6±3.3 and 4.0±71.5 ng/ml, respectively).

Examination of BBB permeability for GFAP after ENT transplantation also showed a significant increase in GFAP elimination to the circulation. However, serum GFAP concentrations differed significantly in the subgroups with high and low transplantation efficacy (Fig. 1, *b*). Both subgroups showed a significant increase in GFAP concentrations 1, 2, and 4 weeks after transplantation, however this increase was more pronounced in the subgroup with reactive gliosis. On week 4 post-transplantation maximum GFAP concentrations in subgroups with poor and good consolidation reached 476.4±111.0 and 253.0±99.3 ng/ml ($p<0.05$), respectively. It should be noted that 8-12 weeks after effective transplantation GFAP concentration returned to normal (8.2±3.3 ng/ml). In the subgroup with low transplantation efficacy, neuronal death,

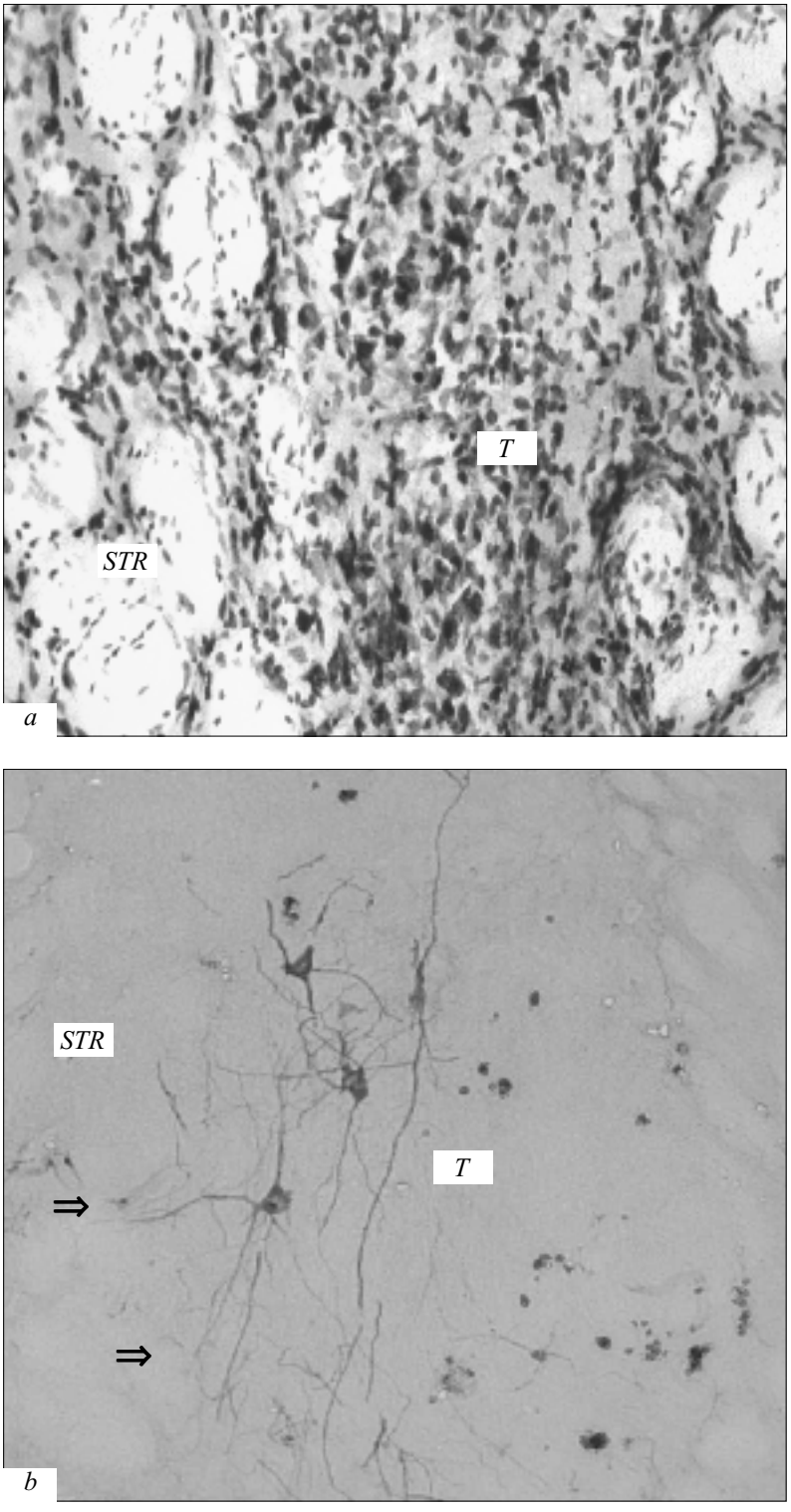

Fig. 2. Morphological characteristics of good consolidation of intrastriatal grafts of embryonic ventral mesencephalon, ×100. *a*) cresyl violet staining. Absence of gliosis in the graft and surrounding tissue; *b*) immunocytochemical visualization of tyrosine hydroxylase-positive neurons. Arrows indicate tyrosine hydroxylase-positive processes growing outside the graft. *T*: transplant; *STR*: striatum.

and gliosis in the surrounding striatum tissue, GFAP concentration surpassed the normal level (235.0±44.8 ng/ml, $p<0.001$ compared to subgroup 1) even 12 weeks post-transplantation.

In control group 1 BBB permeability for GFAP did not differ significantly from that in experimental subgroup 1.

The dependence between the levels of GFAP elimination and 6-OHDA denervation was confirmed by correlation analysis. Maximum correlation dependence was found between GFAP concentration and APO-test parameters before ENT transplantation and 2 and 4 weeks after transplantation (correlation coefficients 0.52, 0.57 and 0.49, respectively, $p<0.01$). Thus, the

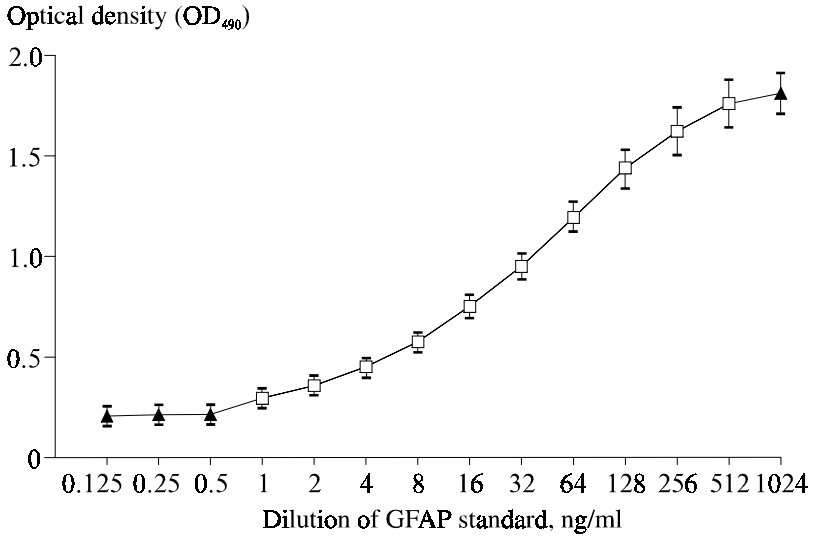

Fig. 3. Calibration curve of immunoenzyme test system on the base of monoclonal anti-GFAP antibodies. Working segment (1.5-700 ng/ml) is shown with a continuous line.

animals with high serum GFAP concentration before transplantation showed intensive apomorphine-induced rotation. In these animals regression of rotation was decelerated 4 weeks after transplantation and transplantation efficacy was low.

The results obtained on the present experimental model confirm increased BBB permeability during intracerebral transplantation in rats with hemiparkinsonism. This phenomenon could not depend only on local brain trauma, since it was not observed after intrastriatal placebo injections, and could be underlied by some pathological factors associated with neurodegeneration and reactive astrogliosis in response to transplantation. Pronounced reactive astrogliosis accompanying low survival of transplanted neurons results in more pronounced and prolonged (12 weeks after transplantation) BBB damage. Since it is known that immune tolerance to neurospecific proteins including GFAP is absent, the increase in GFAP concentration above the threshold value can induce immune response and production of cytotoxic autoantibodies [4]. Therefore, it can be assumed that increased BBB permeability for neurospecific proteins can serve as a pathogenic component in the death of transplanted neurons due to penetration of cytotoxic autoantibodies through BBB. The dependence between the development of gliosis in intrastriatal grafts and GFAP release into the circulation allows to conclude that quantitative analysis of this antigen in the blood serum is important for vital evaluation of the state of intracerebral grafts.

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