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Research report

Intranigral transplants of a GABAergic cell line produce long-term alleviation of established motor seizures

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ABSTRACT

We have previously shown that intranigral transplants of immortalized GABAergic cells decrease the number of kainic acid-induced seizures [Castillo CG, Mendoza S, Freed WJ, Giordano M. Intranigral transplants of immortalized GABAergic cells decrease the expression of kainic acid-induced seizures in the rat. Behav Brain Res 2006;171:109–15] in an animal model. In the present study, recurrent spontaneous behavioral seizures were established by repeated systemic injections of this excitotoxin into male Sprague–Dawley rats. After the seizures had been established, cells were transplanted into the substantia nigra. Animals with transplants of control cells (without hGAD67 expression) or with sham transplants showed a death rate of more than 40% over the 12 weeks of observation, whereas in animals with M213-2O CL-4 transplants, the death rate was reduced to less than 20%. The M213-2O CL-4 transplants significantly reduced the percentage of animals showing behavioral seizures; animals with these transplants also showed a lower occurrence of stage V seizures than animals in the other groups. *In vivo* and *in vitro* analyses provided evidence that the GABAergic cells show sustained expression of both GAD67 and hGAD67 cDNA, as well as increased gamma-aminobutyric acid (GABA) levels in the ventral mesencephalon of transplanted animals. Therefore, transplantation of GABA-producing cells can produce long-term alleviation of behavioral seizures in an animal model.

1. Introduction

The earliest studies demonstrating functional effects of fetal tissue transplants employed animal models of Parkinson's disease [4,14,35] and diabetes insipidus [15] and involved transplantation of fetal tissue known to produce neurochemicals that can alleviate these conditions, i.e. dopamine or vasopressin. It has reasonably been presumed that the influence of intracerebral transplants would be spatially limited, and these disorders were targeted because of their known localized pathophysiology. Other disorders, for which the underlying defect is similarly localized, might also be good candidates for neural transplantation-based therapeutic approaches.

Of all disorders that might conceivably be treated by neural transplantation, epilepsy would seem to be ideal: some forms of epilepsy are focal in nature, and in many cases epilepsy is not adequately controlled by drugs. In animal models, generalized or kindled seizures can be suppressed by local injections of GABAergic drugs into the substantia nigra or pyriform cortex [21,27]. Thus, it seems reasonable that local delivery of an inhibitory neurotransmitter, e.g. gamma-aminobutyric acid (GABA), by transplanted cells might be used to reduce excess neuronal excitability in brain regions from which seizures are generated.

The principle of employing localized release of GABA to inhibit seizures was demonstrated in a study by Kokaia et al. [28] through

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the use of implanted GABA-releasing polymers. These polymers produced a localized release of GABA into the brain and suppressed seizures, at least for the few days during which high-level GABA release was sustained. This study suggests that fairly high concentrations of GABA released non-synaptically would be required to obtain functional effects in epilepsy, perhaps in order to obtain high local concentrations comparable to those achieved at synapses [7]. Nevertheless, there has been little progress in developing a transplantation-based therapeutic approach for the treatment of epilepsy, since neural transplantation has not been demonstrated to produce long-term suppression of established seizures in animal models.

Many studies have shown that grafts of fetal noradrenergic, cholinergic, and GABAergic neurons and cells can suppress subsequently induced seizures, in some cases with considerable intervals between the transplantation and seizure induction [1,3,12,13,29,30]. Another group of studies of transplantation of fetal brain regions containing GABAergic or noradrenergic neurons indicated only minor effects on established seizures [25,26,40,44], or a short lasting effect [31]. One recent study has shown that intrahippocampal grafts of hippocampal fetal cells treated with neurotrophic factors and a caspase inhibitor survive robustly and reduce the frequency of spontaneous motor seizures measured 2 months after grafting [37].

Lately, attention has turned to the possibility of employing cell lines engineered to produce high levels of GABA in epilepsy models. For example, Gernert et al. [17] found that transplantation of cells modified to express the GABA-synthesizing enzyme GAD65, elevated initial seizure thresholds. Seizure thresholds subsequent to kindling were not significantly elevated, although the transplanted cells continued to express GAD65 at least for the duration of the experiment, i.e. 3 weeks. Ross et al. [38] found that cells engineered to express human GAD67 decreased audiogenic seizures for 2 weeks. Thompson [42] found that transplantation of cells engineered to express GAD65 into the dentate gyrus increased the threshold and reduced the duration of the afterdischarge induced by granule cell stimulation and also slowed the appearance of stage V seizures after stimulation of the entorhinal cortex. Using transplants of the same cell line into the substantia nigra, Thompson and Suchomelova [43] were able to produce a short-term (1 week) suppression of previously established seizures. Castillo et al. [5] also demonstrated an effect of cells expressing human GAD67 on seizures induced subsequently by kainic acid (KA)-induced kindling. None of these studies using engineered cell lines have demonstrated a long-term seizure-suppressant effect.

To develop cells for transplantation in epilepsy, we have taken the approach of first, employing immortalized cells which exhibit some properties of GABAergic cells, similar to the primary cells employed by Loscher et al. [31], on the theory that such cells would have the inherent machinery to produce and release GABA [18,19]. Second, GABA production was enhanced by introducing the GAD67 gene, the presumed non-synaptic GAD isoform, using an episomal Epstein-Barr virus-based plasmid. These cells were found to produce relatively high levels of GABA [8] and to suppress audiogenic seizures on a short-term basis [38] and kainic acid-induced seizures 8 weeks after transplantation [5]. In this study we sought to determine whether grafts of M213-2O/CL-4 cells could suppress established kainic acid-induced behavioral seizures on a long-term basis.

The kainic acid animal model of epilepsy produces spontaneous seizures that do not disappear over time. Repeated systemic administration of low doses of KA, results in a pattern of hippocampal degeneration similar to that observed after a single, high dose of kainate, with improved survival, and an increased percentage of animals showing spontaneous seizures [20,23,24,33]. After KA administration, during the acute phase that may last up to 10 h, the animal presents immobility, facial clonus, sniffing, wet dog shakes, and stage III–V seizures (rated according to Racine's scale), all leading to status epilepticus (SE). Then, there is an active phase that lasts 10–30 days, during which seizures of brief duration are observed. This phase is followed by a latent phase of 30–90 days, during which no seizures occur. It is during this phase that synaptical reorganization takes place, as evidenced by neurogenesis, synaptogenesis, and up-regulation of transcription factors, among other significant cellular and molecular changes. Finally, there is the chronic phase that lasts from 90 days to several months. Seizures similar to those observed during the acute phase reappear, and they increase in frequency and duration [2,24,32].

The aims of the present study were: first, to characterize the effects of the systemic administration of low doses of kainate by evaluating the occurrence of behavioral seizures during the light/dark cycle and observing the duration of the seizures over several months; second, to evaluate, over a 3-month period, the effect of intranigral transplants of a GABAergic cell line on already established motor seizures induced by systemic kainate administration; and third, to evaluate the changes in GABA content of cells grown *in vitro*, and in tissue homogenates obtained from the area of the transplant.

2. Material and methods

2.1. In vitro experiments

2.1.1. Cell cultures

Briefly, cell lines were cultured in DMEM-F12 (1:1 Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal calf serum (FCS, Gibco) and 1% penicillin–streptomycin (Gibco) in 75-cm^2 culture flasks at 33 °C and 5% CO_2. Only M213-2O GAD 67 (M213-2O CL-4) cells were grown in selection medium containing Hygromycin B (200 μg/μl) (Sigma–Aldrich, St. Louis, MO, USA). Some cultures were used to verify the expression of various proteins, glutamate decarboxylase-67 (mouse anti-GAD67, Chemicon MAB5401; 1:1000, 1:3000; Chemicon, Temecula, CA, USA), glial acidic fibrillary protein (rabbit anti-GFAP, DAKO Z0334; 1:1000, 1:3000; DAKO, Hamburg, Germany), doublecortin (guinea pig anti-doublecortin Chemicon AB5910; 1:1000, 1:3000), and β-tubulin (mouse anti-beta tubulin E7 Developmental Studies Hybridoma Bank, University of Iowa[5]; 1:1000, 1:10,000). The secondary antibodies (1:200) were either a Cy Trade Mark 3-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (H + L) for GAD67 (Jackson ImmunoResearch, West Grove, PA, USA); a goat anti-rabbit IgG (H + L) FITC conjugate for GFAP (Zymed, Carlsbad, CA, USA); a Cy Trade Mark 2-conjugated Affini-Pure goat anti-guinea pig IgG (H + L) for doublecortin (Jackson ImmunoResearch), or a goat anti-mouse IgG FITC conjugate for beta tubulin (Zymed). Briefly, culture medium was aspirated, cells were incubated in 4% paraformaldehyde for 10 min, washed (3×) with phosphate buffered saline (PBS, 0.1 M, pH 7.4), permeabilized with EtOH:acetic acid (95:5, v/v), washed (3×) with PBS, incubated in 10% horse serum (10–40 min), incubated with the first antibody overnight, washed with PBS (3×), and incubated in the secondary antibody for 120 min. The coverslips with the cells attached were carefully retrieved and mounted onto glass slides using a Vectashield/DABCO Mounting medium (Vector Laboratories).

2.1.2. GABA determination by high performance liquid chromatography (HPLC) of cells grown in vitro

In order to test if cells responded to a depolarizing solution, the M213-2O CL-4 cell line and the control cell line without the hGAD67 transgene, M213-2O (CONTROL) were cultured in 6-well plates until they reached 90% confluence. Media was then aspirated, and cells were washed with sterile saline solution (NaCl 0.9%); 500 μl of a buffer solution (1.0 mM $MgCl_2$; 1.8 mM $CaCl_2$; 3.0 mM NaH_2PO_4; 140 mM NaCl; 10 mM HEPES: 5 mM glucose, pH 7.4) was added and retrieved after 5 min in order to obtain basal GABA levels; then a similar buffer solution except that it contained 50 mM KCl and 94 mM NaCl was added and retrieved after 15, 30, or 45 min. The samples were stored at −70 °C until chromatographic analysis. Cells from each well were resuspended in saline, and an aliquot was used to obtain the total cell count and percent viability using the trypan blue dye-exclusion test. The remaining cells were stored at −20 °C for total protein determination with the Bradford method.

[5] Developed by Klymkowsky, M.; Developmental Studies Hybridoma Bank under the auspices of NICHD and University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.