Basic Fibroblast Growth Factor Ameliorates Rotational Behavior of Substantia Nigral-Transplanted Rats with Lesions of the Dopaminergic Nigrostriatal Neurons

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ABSTRACT — Basic fibroblast growth factor (bFGF) was injected with dissociated substantia nigral cells into the striatum of rats prepared by unilateral lesion of the nigrostriatal pathway with 6-hydroxydopamine. The transplanted cells with 5 and 50 ng bFGF reduced the apomorphine-induced rotations by 40 and 30%, respectively, while the decrement of rotations was only 15% in the grafted control without bFGF. Immunohistochemical staining with anti-tyrosine hydroxylase antibody showed that bFGF also tended to increase the number of grafted catecholaminergic neurons along the tracts. In the case of 50 ng bFGF treatment but not 0 or 5 ng bFGF treatment, however, severe gliosis was detected along the grafted region by staining of anti-glial fibrillary acidic protein antibody. These immunohistochemical studies suggested that high-dose bFGF induced extensive gliosis, which might affect the survival of the grafted neurons.

Keywords: Basic fibroblast growth factor (bFGF), Cell transplantation, Rotational behavior, Apomorphine

Basic fibroblast growth factor (bFGF), a polypeptide found in adult brain tissue, is a potent mitogenic protein capable of inducing cell division in a wide variety of cell types (1, 2). Recently, bFGF has been demonstrated to be a neurotrophic factor inducing an increase of CNS neuron survival in vitro (3–10). In addition, some cultural studies showed that bFGF improved the neuronal functions of several subpopulations, such as choline acetyltransferase activity, dopamine uptake activity and GABA uptake activity (3, 11). This wide selectivity of bFGF for improving the survival and the activity of several neuronal subpopulations is a remarkable feature; in contrast, the effect of nerve growth factor (NGF), the best characterized factor, is limited to cholinergic CNS neurons. Thus it is of much interest to investigate the potency of bFGF in various types of CNS neurons in vivo. Several studies have been conducted to examine the effects of bFGF on neuronal survival in vivo. For example some studies in fimbria forni transected rats showed that bFGF increased both the survival of cholinergic neurons and the choline acetyltransferase activity in the lesion site (12–15). On the other hand, little is known about the potency of bFGF in other neuronal subpopulations in vivo.

A rat prepared by unilateral lesion of the nigrostriatal pathway with 6-hydroxydopamine (6-OHDA) is well-known to demonstrate asymmetric rotational behavior. This abnormal behavior is reduced by grafting dopaminergic neurons derived from substantia nigral cells or superior cervical ganglia cells, or dopamine-releasing cells such as adrenal medulla cells or PC12 cells (15–20). Hence the 6-OHDA lesioned rat is suitable for studies designed to elucidate the potency of the neurotrophic effect on the transplanted dopaminergic neuronal cells. In this report, we deal with the effect of bFGF on substantia nigral transplantation in the 6-OHDA lesioned rat in vivo.

MATERIALS AND METHODS

Materials

Recombinant bovine bFGF (more than 95% purity) was obtained from Amersham, USA. Anti-tyrosine
hydroxylase (TH) mouse monoclonal antibody was generously supplied by Dr. Hiroshi Hatanaka (Osaka University, Japan). Anti-glial fibrillary acidic protein (GFAP) was obtained from SANVIO BV (Uden, Netherlands). Avidin-biotin peroxidase complex was obtained from Vector Laboratories (CA, USA).

Preparation of Parkinson’s disease model rat
Eight micrograms of 6-OHDA in 4 μl of saline containing 0.8 μg of ascorbic acid was unilaterally injected into the nigrostriatal pathway of 5-week-old female Wistar rats (SLC, Shizuoka, Japan) under xylasin (100 mg/kg) and ketamin (20 mg/kg) anesthesia (i.p.). The stereotaxic coordinates were: 4.2 mm posterior to the bregma, 1.1 mm lateral to the midline and 7.5 mm below the dura (the atlas of Paxinos and Watson) (21). After the lesion, the rats were tested for apomorphine (250 μg/kg, s.c.)-induced rotational behavior, twice a week for 2 weeks. The numbers of rotations (as full right turns) were counted by direct observation (by the naked eye) during the interval between 15 and 25 min after the injection of apomorphine in a square acrylic box (35 X 40 X 20 cm). Twenty-three rats showing stable apomorphine-induced rotation were selected for further studies.

Preparation of dissociated neuronal cells
The procedure of dissociation of substantia nigral cells was previously reported (5). Briefly, the ventral midbrain dissected from 16- to 17-day-old embryonic Wistar rats was cut with a knife and dissociated with 0.25% trypsin and 0.01% DNase I at 37°C for 30 min, followed by pipetting with a plastic pipette. The dissociated cells were centrifugally concentrated and then suspended in saline containing 0.6% glucose with 1 or 10 μg/ml of bFGF at the density of 6.3 × 10^6 – 1.2 × 10^7 viable cells/ml.

Transplantation
Five microliters of the cell suspension (3.15 × 10^4 – 6.0 × 10^4 cells) was injected stereotaxically into the striatum (1.0 mm anterior to bregma, 2.5 mm lateral to the midline and 4.5 mm below the dura) of the lesion side at the injection rate of 1 μl/min under xylasin (100 mg/kg) and ketamin (20 mg/kg) anesthesia (i.p.). Eight rats were transplanted with the dissociated cells without bFGF, as controls. Seven and 8 other rats were grafted with the cells together with 5 and 50 ng bFGF, respectively. From one week after the grafting, the rotations induced by injection of apomorphine (250 μg/kg, s.c.) were checked every week for 8 weeks.

Immunohistochemistry
Twelve weeks after the transplantation, anesthetized rats were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4. Brains were removed, trimmed to the striatum area, dehydrated with graded ethanol solutions and embedded in paraffin. Eight-micron paraffin sections were serially cut, and every third section was stained with anti-TH mouse monoclonal antibody or anti-GFAP mouse monoclonal antibody by using the avidin-biotin-peroxidase complex. About 10 slices, including the grafted region, stained with anti-TH antibody were used for counting all of the grafted TH-positive neurons along the tracts.

RESULTS
The transplantation of the substantia nigral cells with 5 and 50 ng of bFGF reduced the apomorphine-induced rotations gradually for 3 weeks and after that the decrement of rotations, became stable at 40% and 30%, respectively. The decrement in the grafted control, not treated with bFGF, was only 15%. The reduction of the rotations was statistically significant (P < 0.01) in the 5 ng-treated group versus the control. The lessened rotations continued for at least 8 weeks in all groups (Fig. 1). Although observation was continued for 4 more weeks, essentially no further changes of the rotation number were observed at 12 weeks after the transplantation (data not shown).

After 12 weeks, 9 brains (2 brains from control rats, 3 from the 5 ng bFGF-treated rats, and 4 from the 50 ng bFGF-treated rats) were stained immunohistochemically with anti-TH antibody (Fig. 2). There were 5 and 8 TH-positive cells along the tracts in the grafted control rats (mean: 6.5 cells per rat). In the 5 ng bFGF-treated rats, 4, 26 and 32 TH-positive cells were detected, respectively (mean: 20.6 cells per rat). In bFGF (50 ng)-treated rats, 6, 6, 17 and 17 TH-positive cells were detected (mean: 11.5 cells per rat). The immunohistochemical studies suggested that bFGF tended to increase grafted TH-positive neuron survival as compared with the control.

Other immunohistochemical studies with anti-GFAP antibody using 3 brains of each group showed severe gliosis along the tracts in all of the stained brains in the 50 ng bFGF-treated group. The other two groups (0 and 5 ng bFGF treatment) showed little gliosis at the grafted regions (Fig. 3).
Fig. 1. Effect of bFGF on amelioration of rotational behavior after substantia nigral transplantation. Basic FGF at the dose of 5 (•: n = 7) or 50 (○: n = 8) ng was injected with dissociated substantia nigral cells. Controls (●: n = 8) received dissociated cells without bFGF. The numbers of abnormal rotations induced by apomorphine (250 µg/kg, s.c.) were gradually decreased for 3 weeks after transplantation. The behavioral amelioration continued for at least 8 weeks. *P < 0.05, **P < 0.01 versus control (t-test). *P < 0.05, **P < 0.01 versus the pre-transplantation average (t-test).

Fig. 2. Immunohistochemical observation of the grafted region in the striatum stained with anti-tyrosine hydroxylase antibody using avidin-biotin-peroxidase complex. The grafted dopaminergic neurons in the striatum of the bFGF-treated rat were stained. A: Bar = 200 µm. B: Bar = 50 µm.
DISCUSSION

In this paper, we have, for the first time, demonstrated that co-injected bFGF improved the behavioral amelioration in 6-OHDA lesioned rats which were transplanted with fetal substantia nigral cells in the striatum. Although some authors have reported that bFGF increased the survival and outgrowth of grafted neurons and dopamine release in the brain of lesioned rats, there have been no observations of behavioral amelioration induced by bFGF administration (22). Although we did not measure the content of dopamine released in the grafted regions, the increase of grafted TH-positive cells in the bFGF treated rats presumably caused the behavioral improvement.

Many previous reports have shown that lesioned rats induced by nigrostriatal injection of 6-OHDA are useful for transplantation research. Catecholaminergic cells derived from various tissues and cells such as the substantia nigra (14–20, 23), adrenal medulla (20, 24, 25), superior cervical ganglion (26), and PC-12 (27) were implanted and ameliorated abnormal rotational behavior. The substantia nigra was the most commonly used and most effective; in particular, two-site grafting of dissociated substantia nigral cells, derived from embryonic day 14–15 rats into the striatum, resulted in nearly 100% recovery from abnormal behavior (24). However, such a successful protocol is not applicable for investigating the potency of bFGF as a neurotrophic factor which improves the survival of grafted neurons and ameliorates the abnormal behavior. So, we adopted suboptimal conditions, that is, a lower level of grafting (one site of transplantation) of older embryonic neurons (derived from 16- to 17-day-old fetus), which
gave a less advantageous outcome in comparison with the above procedure. We have ascertained that only 10–20% of the dissociated cells from 16- to 17-day-old embryonic ventral midbrain were viable, as assessed by nigrosine staining, after dissociation as described under Materials and Methods; in contrast, 70–80% of cells from 14- to 15-day-old embryo were viable. The vulnerability of the older cells was compatible with the weak amelioration (only 15%) of the asymmetric rotation of the control rats which received nigral transplants without bFGF. Despite such a low level of amelioration in the control, the addition of 5 ng of bFGF reduced the abnormal rotations by 40% (3 times more than the control) and tended to increase survival of TH-positive cells in the brain. These results indicated that bFGF was able to decrease the degree of abnormal behavior under suboptimal conditions; i.e., the transplantation of less viable neurons from an older fetus (16–17 days-old).

There was one possibility that bFGF alone could ameliorate the abnormal behavior without the transplantation. We detected many TH-stained fibers in the non-lesioned side of the striatum, but no TH-positive fibers in the lesioned side of the striatum by the anti-TH staining. This complete disappearance of the TH-positive fibers in the lesioned side was observed in both bFGF-treated and bFGF-non-treated rats (data not shown). These results suggest that the nigro-striatal pathway was completely destroyed by 6-OHDA injection, and the terminals of dopaminergic neurons were also injured, no matter how bFGF was treated; and bFGF treatment without a neuron graft probably causes no amelioration of rotational behavior. Thus the graft of dopaminergic neurons is necessary for amelioration of the abnormal behavior, and the improvement of the amelioration of behavior was attributable to the increase of the survival of the grafted neurons induced by bFGF.

Reports have indicated that bFGF induces morphological changes in astrocyte cells both in vitro and in vivo (14, 28). In this study, staining with anti-GFAP antibody showed stronger gliosis in the grafted regions in the 50 ng bFGF-treated group as compared with the control and 5 ng bFGF-treated rats (Fig. 3). Although the relation between induction of gliosis and improvement of neuronal survival has not been clarified exactly, the dense gliosis induced by a high dose of bFGF (50 ng) presumably results in less grafted neuron survival and less amelioration of abnormal rotations.

Besides the morphological alteration of glial cells, bFGF induced other responses in glial cells, such as proliferation (29), glial fibrillary protein synthesis (28), nerve growth factor release (30), glutamine synthase synthesis (31) and protein kinase activation (32). Among these bFGF-induced multiple responses, there may be an ability to stimulate dopaminergic neurons, e.g., by release of a dopaminotrophic factor, but this effect has not yet been identified.

The cardinal signs of Parkinson's disease, akinesia, resting tremor, cogwheel rigidity, and postural reflex impairment, result primarily from the loss of dopaminergic neurons in the nigrostriatal pathway (33). In contrast to our study the sign of the 6-OHDA lesioned rat induced by apomorphine, rotational behavior, is completely different from that of a Parkinsonian, despite the fact that both signs result from the same deletion in the brain (loss of dopaminergic neurons in the nigrostriatal pathway). Thus it may be difficult to regard a 6-OHDA lesioned rat as a Parkinson's disease model rat. However, the usefulness of the 6-OHDA lesioned rat for research on dopaminergic neuron graft remains unaltered and important. Although some clinical trials of brain grafting have been performed for Parkinson's disease patients, there are still many difficulties owing to various limiting factors such as donors (source, age, etc.), region of the brain for grafting, and so on (34, 35). From these points of view, we think that our present research, which indicated that bFGF has the potency for supporting brain grafts under suboptimal conditions, is relevant to the clinical situation.

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