The Effect of Basic Fibroblast Growth Factor (bFGF) and Nerve Growth Factor (NGF) on the Survival of Septal Neurons Transplanted into the Third Ventricle in Rats

Yoshitsugu Shitaka and Hiroshi Saito

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan

ABSTRACT—Effects of short-term pretreatment with basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) on the survival of neurons transplanted into the third ventricle in rats were studied. Septa from 16-day-old rat embryos were pretreated with growth factor containing medium for 30 min and transplanted into the third ventricle of adult rats. Twenty-one days after the operation, the recipient rats were perfused with 4% paraformaldehyde. Paraffin sections of the removed brains were made, and the sections were stained with cresylviolet. The number of neurons in the grafts was counted under a light microscope. Although pretreatment with NGF was not effective, bFGF at the concentration of 1 and 10 μg/ml enhanced the survival of transplanted septal neurons. These results suggest that short-term pretreatment with bFGF could increase the efficiency of neuronal transplantation.

Keywords: Basic fibroblast growth factor (bFGF), Nerve growth factor (NGF), Transplantation, Septum
MATERIALS AND METHODS

Growth factors

CS23 and human recombinant NGF were generously supplied by Takeda Chemical Industries, Ltd. (Osaka). CS23 is an acid-resistant mutein of recombinant human bFGF (23). We have already confirmed that the neurotrophic activities of CS23 in cultured brain neurons are equivalent to those of the wild type of human recombinant bFGF in cultured brain neurons (24).

Animals and grafting procedures

Male Wistar rats weighing 220–250 g served as recipients. Surgery was performed under combined ketamine and xylazine anesthesia. The fetal septal tissue was dissected out from 16-day-old Wistar rat fetuses under microscopic observation and preincubated with L-15 medium containing 0.1, 1 and 10 μg/ml CS23; 0.1, 1, 10 and 100 μg/ml NGF; or growth factors-free L-15 medium for 30 min on ice. Then, it was rinsed by growth factor-free medium and aspirated into a stainless steel catheter.

Fig. 1. Transplantation of the septum from 16-day-old rat embryo. (A) Schematic drawing of transplanted region. 3V: third ventricle, LV: lateral ventricle, G: graft. (B) The septum from 16-day-old rat embryo transplanted into the third ventricle of a male adult rat for 21 days, stained with cresylviolet. An arrowhead shows the transplanted tissue. Bar = 300 μm.
(O.D. = 1.2, I.D. = 0.8 mm). The catheter was stereotaxically inserted into the third ventricle of a recipient rat according to the atlas of Paxions and Watson, A = −3.0 (behind bregma), L = 0.0 (from the midline), V = 8.5 mm (ventral to dura); and then the septal tissue was slowly pushed out into the ventricle (one unilateral septal tissue per recipient; Fig. 1A). After the operation, the rats were housed in wire-mesh cages in a temperature- and humidity-controlled room (25 ± 1°C, 55 ± 10%) four or five per cage, under a normal day and night cycle, and free access to food and water. Twenty-one days later, the recipient rats were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, and then the brains were removed.

**Histological processing**

Removed brains were fixed in the same solution for 2 hr and then placed in 0.1 M phosphate buffer at 4°C. To make paraffin sections, the brains were dehydrated with graded ethanol solutions and embedded in paraffin. Then 8-μm paraffin coronal sections were serially cut and stained with cresylviolet. In order to confirm the presence and the growth of neurons in the graft with the immunohistochemical method and acetylcholine esterase (AChE) histochemistry, we also made frozen sections. Surgery and fixation were performed as described above. Before sectioning, the brains were immersed in 15% and 25% buffered sucrose solutions. Sections of 16-μm thickness were cut on a cryostat. Immunohistochemical identification was made by using monoclonal antibodies directed against microtubule-associated protein 2 (MAP2; Amersham, Amersham, UK) and choline acetyltransferase (ChAT; IBL, Tokyo). The frozen sections were incubated with anti-MAP2 antibody (at a final concentration of 1:2000) and anti-ChAT antibody (1:400), processed with avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Labs, Burlingame, CA, USA) and reacted with diaminobenzidine (DAB). The staining for AChE was performed using ethopropazine as the inhibitor of non-specific esterases (20).

![Fig. 2](image-url)  
**Fig. 2.** Nissl preparations of the septum from (A) 16-day-old rat embryo, (B) 16-day-old rat embryo transplanted into the third ventricle of a male adult rat for 21 days and (C) postnatal 16-day-old rat. Big arrows show septal neurons and small arrows show non-neuronal cells. (D) Septal neurons in a graft stained immunohistochemically with anti-MAP2 antibody. Arrowheads show the transplanted septal tissue and arrows show septal neurons. Bar = 30 μm.
Evaluation of effect

It is known that there are large size of cholinergic neurons in the septum. Thus, the number of large septal neurons was counted under a light microscope. Abercrombie’s formula was employed for the correction of overcount due to split cell bodies. The area and volume of each graft were calculated with an image analysis apparatus (MGV-1300; Mutoh Kogyou, Tokyo). Analysis of variance (ANOVA) followed by Duncan’s test was used for statistics.

RESULTS

Twenty-one days later, most of the transplanted septal tissues were present in the third ventricle of recipient rats (Fig. 1B). Since the third ventricle is covered with ependymal cells, it was easy to distinguish the graft from the host brain. The septum from 16-day-old rat embryo before transplantation consisted of a large number of small cells and the transplanted neurons had grown larger morphologically in the third ventricle (Fig. 2, A and B). Thus, we could easily differentiate the large septal neurons from non-neuronal cells. Compared to the septal neurons of postnatal 16-day-old rat, transplanted neurons had grown into the same size (Fig. 2C). Moreover, septal neurons were confirmed by MAP2 immunohistochemistry both in the growth factor pretreated and control groups (Fig. 2D). We made a criterion of the size of neurons, and the large septal neurons were counted on a Nissl preparation under a light microscope. There was no difference in the size of neurons between the control and growth factor pretreated groups. The results are shown in Figs. 3 and 4. The ordinates show the density of the surviving septal neurons in the grafts, which is normalized to the control.

Fig. 3. Effect of short-term pretreatment with CS23 on the survival of neurons transplanted into the third ventricle. The septum from 16-day-old rat embryos were preincubated with CS23 containing or not containing medium for 30 min and then transplanted into the third ventricle. Twenty-one days later, the number of surviving neurons/mm³ graft volume was obtained and normalized to the control. The number at the bottom of each column represents the number of animals studied. *, ** Significant difference compared to the control at P<0.05 and P<0.01, respectively (Duncan’s method).

Fig. 4. Effect of short-term pretreatment with NGF on the survival of neurons transplanted into the third ventricle. The septum from 16-day-old rat embryos were preincubated with NGF containing or non-containing medium for 30 min and then transplanted into the third ventricle. Twenty-one days later, the number of surviving neurons/mm³ graft volume was obtained and normalized to the control. (A) Pretreatment with a lower concentration of NGF and (B) pretreatment with a higher concentration of NGF. The number at the bottom of each column represents the number of animals studied.
Short-term pretreatment with 1 and 10 \( \mu \text{g/ml} \) CS23 exhibited a significant increase in the survival neurons by 62% and 77%, respectively, as compared to the control (Figs. 3 and 5). The number of non-neuronal cells showed a tendency to increase in the groups pretreated with a higher dose of CS23. Preincubation with 0.1, 1, 10 and 100 \( \mu \text{g/ml} \) NGF had no effect on the survival of neurons and the number of non-neuronal cells (Fig. 4). Several clusters of neurons were observed in some grafts both in the control and growth factor-pretreated groups. The clusters were not observed in the septum of postnatal 16-day-old rats.

DISCUSSION

In present study, we demonstrated that short-term pretreatment with CS23 enhanced the neuronal survival of the septum transplanted into the third ventricle. The reasons for this may be as follows: 1) CS23 acts directly on septal neurons and maintained their survival: Immediately after the operation, it was considered that there was no blood supply in the grafts and that the grafts were under a temporal ischemic condition. This initial period is supposed to be critical for the survival of neurons in the grafts. Indeed, it was reported that the majority of the transplanted peripheral SCG neurons were necrotic within 24 hr after transplantation (25). Several observations have shown that bFGF maintains the survival of neurons from various regions of embryonic brain, including the septum, in culture (14, 24). Thus, it could be considered that CS23 acted on neurons in the grafts, which were under an ischemic condition, and prevented the initial rapid cell loss. 2) CS23 has indirect actions through glial cells: According to the study of the developing rat CNS, it was considered that the septum from 16-day-old rat embryo consisted of type-1 astrocytes and oligodendrocyte-type2 astrocyte progenitor (O-2A) cells, except for neurons (26). In vitro studies have revealed that bFGF has a variety of effects on glial cells. Basic FGF has a mitogenic activity, causes a morphological change, and increases the synthesis of many kinds of proteins in astrocytes (27). Basic FGF can also act as a mitogen for O-2A cells (28). In this study, the number of non-neuronal cells showed a tendency to increase in the groups pretreated with a higher dose of CS23. Thus, it was possible that CS23 acted on glial cells in the grafts and showed the trophic effect on transplanted neurons, through some kinds of changes on the glial cells, for example, quantitative or qualitative changes in neurotrophic factors secreted by these cells. The identification of the type of those increased cells remains to be elucidated in the future. 3) CS23 acts on vascular endothelial cells: Basic FGF is known to be a mitogenic factor for vascular endothelial and smooth muscle cells in vitro (29), and intraventricular injection of bFGF promotes angiogenesis in vivo (30). Rapid blood supply from the surrounding host brain to grafts is supposed to be critical for neuronal survival. Thus, it could also be the case in our experiments that a rapid or rich vascular...
supply enhanced by pretreatment with bFGF prevented neuronal death in the grafts. However, the enhancement of neovascularization in CS23-pretreated grafts was not observed, at least under a light microscope. Ohta et al. reported that pretreatment with the combination of NGF and bFGF enhanced neovascularization in SCG tissues transplanted into the third ventricle and prevented the death of SCG neurons (22). The combined effect of bFGF and NGF on the survival of septal neurons needs to be examined. 4) CS23 promoted the proliferation of neuroepithelial stem cells: Several clusters of neurons were observed in some grafts both in the control and growth factors-pretreated groups. It is known that the time of final mitotic activity of the cholinergic neurons in the septum is by day E17 in the rat (31). In our study, it was considered that a small part of the neurons in E16 septal tissues are neuronal stem cells and have a mitotic activity. Previous studies revealed that bFGF promoted the proliferation of neuroepithelial stem cells derived from early embryonic brain in culture, and when NGF was added together with bFGF, the proliferation increased in comparison to the bFGF-treated cultures (32). Thus, there might be a possibility that CS23 acted on the neuronal stem cells and enhanced their proliferation rather than maintaining neuronal survival in transplants.

In our previous report, a high concentration of NGF (10 μg/ml) promoted the survival of SCG neurons transplanted into the third ventricle (21). In this study, however, NGF did not enhance the survival of septal neurons, even in higher concentration groups. Several reports have demonstrated that NGF enhances the survival of mature septal neurons, although NGF has no effect on the survival of fetal septal neurons in culture (19, 20). Our present result is consistent with these reports in this point. The survival of neonatal or older central tissues may need to be examined with the same pretreatment. It was reported that chronic administration of NGF increased the size of fetal basal forebrain tissue transplanted into the anterior chamber of the eye (33). Therefore, there is a possibility that NGF requires chronic administration to express its effects.

In order to confirm the survival of cholinergic neurons in the grafts, we preliminarily performed both immunohistochemical identification using monoclonal antibody against ChAT and histochemical identification for AChE. With the histochemical staining for AChE, the survival of cholinergic neurons was confirmed in both control and CS23-pretreated grafts (data not shown). With the immunohistochemical staining using anti-ChAT antibody, although the control grafts were stained strongly and the survival of ChAT-immunoreactive neurons was confirmed, the CS23-pretreated grafts were stained only weakly. The effects of pretreatment with growth factors on the survival of cholinergic neurons and ChAT activity in grafts remain to be elucidated in the future.

In conclusion, we demonstrated that pretreatment with CS23 enhanced the survival of neurons transplanted into the third ventricle, and NGF had no effect on the survival of neurons. Our results suggested that pretreatment with CS23 could increase the efficiency of neuronal transplantation. Further studies are required to investigate the effects of CS23 on the septal neurons transplanted intraparenchymally.

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