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Transplantation of In Vitro-Expanded Fetal Neural Progenitor Cells Results in Neurogenesis and Functional Recovery After Spinal Cord Contusion Injury in Adult Rats

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Neural progenitor cells, including neural stem cells, are a potential expandable source of graft material for transplantation aimed at repairing the damaged CNS. Here we present the first evidence that in vitro-expanded fetus-derived neurosphere cells were able to generate neurons in vivo and improve motor function upon transplantation into an adult rat spinal-cord-contusion injury model. As the source of graft material, we used a neural stem cell-enriched population that was derived from rat embryonic spinal cord (E14.5) and expanded in vitro by neurosphere formation. Nine days after contusion injury, these neurosphere cells were transplanted into adult rat spinal cord at the injury site. Histological analysis 5 weeks after the transplantation showed that mitotic neurogenesis occurred from the transplanted donor progenitor cells within the adult rat spinal cord, a nonneurogenic region; that these donor-derived neurons extended their processes into the host tissues; and that the neurites formed synaptic structures. Furthermore, analysis of motor behavior using a skilled reaching task indicated that the treated rats showed functional recovery. These results indicate that in vitro-expanded neurosphere cells derived from the fetal spinal cord are a potential source for transplantable material for treatment of spinal cord injury.

Key words: therapeutic use; neural stem cells; regeneration

Traumatic spinal cord injury affects many people, including young people, and can result in severe damage, leading to paraplegia, tetraplegia, or worse. Many strategies, including surgical, pharmacological, neurophysiological, and technological approaches, have been used in attempts to develop new therapies that will allow patients to regain use of their paralyzed limbs. One such strategy is the transplantation of fetal spinal cord tissue into damaged spinal cords, which has been performed in rats and cats over the past several decades (Reier et al., 1983; Anderson et al., 1995; Diener and Bregman, 1998). Such transplants survive and integrate with the host tissue and may be associated with functional improvement. In fact, the transplantation of fetal CNS tissue has already been performed in human patients with Parkinson's disease, resulting in some clinical improvement (Lindvall et al., 1990; Freed et al., 1992; Freeman et al., 1995). For spinal cord injury, however, such treatment has not yet been established. One underlying reason for this lack of research is that large

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number of fetuses is required to obtain enough tissue to treat even one patient (for Parkinson's disease, four to eight fetuses are required), a requirement that generates both practical and ethical problems. On the other hand, recent progress in the biology of neural stem cells has made it possible to routinely expand neural progenitor cells obtained from a small amount of fetal CNS tissue in vitro as floating cell aggregates called *neurospheres* (Reynolds and Weiss, 1992). Therefore, expansion of neural progenitor cells in vitro may overcome the practical and ethical problems associated with fetal tissue transplantation and provide a potential source for the graft material needed to attempt the regeneration of injured spinal cord. However, these in vitro-expanded neural progenitor cells have some potential problems as a source for graft material. One possibility is that neuronal differentiation from transplanted cells might not occur when these are transplanted into a nonneurogenic region for endogenous progenitor/stem cells, including adult spinal cord. In this study, we addressed this issue and demonstrated that transplanted neurosphere cells prepared from the rat embryonic day 14.5 (E14.5) spinal cord, expanded and prelabeled with bromodeoxyuridine (BrdU) in culture, can introduce neurons, astrocytes, and oligodendrocytes into the injured adult rat spinal cord. Furthermore, we observed that the transplantation resulted in mitotic production of new neurons in vivo, and these neurons extended their processes into the host tissue, where they formed synaptic structures upon transplantation at an appropriate time. In addition, we observed behavioral improvement in rats with transplanted neurosphere cells compared with control rats.

MATERIALS AND METHODS

Animals

Adult (~200–230 g) female Sprague-Dawley rats (Japan SLC Inc.) were used in all experimental groups. All the experiments were performed in compliance with relevant laws and institutional guidelines.

Spinal Cord Contusion Lesions

Spinal contusion lesions were made in accordance with the weight compression method (Holtz et al., 1989). Briefly, under a dissecting microscope, a laminectomy was performed at the C_4 and C_5 level, and the spinal cord was compressed by placing a 35 g weight on the dura for 15 min. The tip of the weight had an area of 6.6 mm^2 (3.0 × 2.2 mm) and a concave shape that ensured equal distribution of the pressure on the cord. The head and T_2 spinous process were immobilized to minimize movement of the cervical spine during the compression. The body temperature was kept at 37°C ± 0.3°C during the operation.

Primary Cultures and Passaging Procedures

With sterile technique, the spinal cords from E14.5 Sprague-Dawley rats or Tα1-enhanced yellow fluorescent protein (EYFP) transgenic rats (described below) were dissected and prepared as described elsewhere (Reynolds and Weiss, 1992). The culture medium was DMEM/F12 supplemented with the hormone mixture used by Reynolds and Weiss (1992) and human recombinant fibroblast growth factor-2 (FGF-2; 20 ng/ml; Prepro Tech, Inc., Rocky Hill, NJ). Fresh FGF-2 was added every 2 days. Passages were performed once per week.

Transplantation

Transplantation was performed 9 days after the spinal cord injury (SCI + TP, n = 15). For transplantation, spheres passaged two to five times were resuspended in the medium without FGF-2 at 5–10 × 10^6 cells/ml. Spheres from wild-type rats were prelabeled with BrdU (1 μM; Sigma, St. Louis, MO), which was added to the medium 48 hr before the transplantation. The injury site was reexposed, a glass micropipette attached to a Hamilton syringe was inserted into the spinal cord, and 20–40 μl of the cell suspension were injected manually into the injury-induced intraspinal cavity. Rats with induced spinal cord injury alone (SCI, n = 13) and rats that had been received the injection of the culture medium without FGF-2 into the cavity (SCI + med, n = 17) were used as controls.

Analysis of Donor-Derived Cell Differentiation Into Neurons In Vivo

To examine whether the transplanted progenitor cells differentiated into neurons in vivo, we prepared neural progenitor cells from transgenic rats carrying the Tα1-EYFP transgene (Sawamoto et al., 2001). Earlier reports (Gloster et al., 1994; Wang et al., 1998; Roy et al., 2000a,b; Sawamoto et al., 2001) suggested that, in this transgene, EYFP cDNA is placed under the control of the 1.1-kb promoter element of the Tα1 tubulin gene, which is expressed in the neuronal lineage. We then transplanted neurospheres derived from the spinal cord of the Tα1-EYFP transgenic rat embryos (E14.5) into the injured spinal cord of rats. To examine whether mitotic neurogenesis occurred within the spinal cord of the host animals, we also injected BrdU (50 μg/g body weight) into the transplanted rats intraperitoneally, once every day, from days 3 to 14 after the transplantation.

Immunohistochemistry

After behavioral assessment was performed, anesthetized animals underwent intracardiac perfusion with 4% paraformaldehyde for 15 min. Spinal cords were removed and postfixed in the same fixative for an additional 1 hr at 4°C. Tissues were transferred to 25% sucrose in phosphate-buffered saline (PBS) and immersed overnight at 4°C. They were then frozen in liquid nitrogen, and transverse (12 μm thick) or sagittal (14 or 20 μm thick) sections were cut with a cryostat; then, were the sections immunostained. To detect BrdU, sections were incubated in 2 N HCl for 30 min at 60°C before the staining procedure. To detect EYFP expression, a TSA-Direct kit (NEN Life Science Products, Boston, MA) was used to amplify the signal. For immunostaining, sections were visualized with diaminobenzidine solution. For double staining with BrdU and other antigens, the other antigens were stained and visualized with dianinobenzidine or Vector SG (Vector, Burlingame, CA) first, and then BrdU was stained and visualized with Vector SG or diaminobenzidine.

Primary antibodies were as follows: for BrdU, a mouse monoclonal, used at 1:100 (Sigma), or a sheep polyclonal, used at 1:1,000 (Fitzgerald, Concord, MA); for EYFP, an antigreen fluorescent protein (GFP) rabbit polyclonal that also reacts with

EYFP, 1:3,000 (MBL Japan); for Hu, a mouse monoclonal, 1:500 (16A11 Hybridoma Bank, University of Oregon, Eugene, OR); for 2'3'-cyclic nucleotide 3'-phosphohydrolase (CNP), a mouse monoclonal, 1:1,000 (Sigma); and for GFAP, a rabbit polyclonal, 1:20 (Dako, Glostrup, Denmark). Primary antibodies were diluted in PBS containing 10% normal goat serum and 0.01% Triton X-100. The secondary antibodies were as follows: anti-mouse IgG Cy-3, 1:2,000 (Chemicon, Temecula, CA); anti-mouse IgG biotin, 1:500 (Vector); anti-rabbit IgG biotin, 1:500 (Vector); anti-sheep IgG biotin, 1:300 (Chemicon). Secondary antibodies were diluted in PBS containing 10% normal goat serum, 10% normal rat serum, and 0.01% Triton X-100.

Quantitative Analyses

Quantitative analyses of the differentiation of donor-derived progenitor cells and of donor-derived neurons generated after the transplantation were performed by counting every fourth transverse section (12 μm thick) from the injury epicenter in both the cranial and the caudal directions. Four rats were analyzed for each experiment. For the quantitative analysis of the differentiation of donor-derived progenitor cells, 20 sections (10 cranial and 10 caudal to the injury epicenter) were taken from spinal cords that had received neurospheres derived from wild-type rat embryos and prelabeled with BrdU. The sections were immunostained with antibodies to markers for each cell lineage (neurons, Hu; oligodendrocytes, CNP; astrocytes, GFAP) and BrdU. All the double-positive cells (i.e., cells that were positive for both BrdU and a lineage-specific marker) and BrdU-positive cells were counted in each section with a light microscope (Axiophoto 2; Carl Zeiss, Jena, Germany) at a magnification of ×400. The numbers of double-positive cells and BrdU-positive cells in 20 sections were summed, and the proportion of BrdU-positive cells that were doubly positive was calculated.

For the quantitative analysis of donor-derived neurons that were generated after the transplantation, 20 sections (10 cranial and 10 caudal to the injury epicenter) were taken from spinal cords that had received neurospheres derived from Tα1-EYFP transgenic rat embryos. The sections were immunostained with antibodies to GFP (to detect EYFP) and BrdU. Cells were counted as EYFP positive only when the maximum cell body diameter was longer than 4 μm. All double-positive cells and EYFP-positive cells were counted in each section. The numbers of double-positive cells and EYFP-positive cells were summed, and the proportion of EYFP-positive cells that were doubly positive was calculated.

Electron Microscopic Analysis

For electron microscopy, transverse free-floating sections (30 μm thick) were prepared. After reacting with an anti-GFP antibody, sections were incubated in 1% glutaraldehyde for 10 min. They were then treated as described above, and the peroxidase reaction was developed in diaminobenzidine. Immunostained sections were further treated with OsO_4, dehydrated, and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome (Reichert Ultracut, Germany) and observed using a Hitachi electron microscope (H7100, Japan).

Behavioral Analysis

To assess whether motor functions were improved by the transplantation of neural progenitor cells, we examined the ability of treated and control rats to retrieve food pellets. The pellet retrieval test was a modification of Diener and Bregman's method (1998). Before testing, rats were trained on the task for 1 week before the spinal cord injury and were retrained for an additional 1 week before the test was administered. Tests were performed on 2 days in succession, 5 weeks after transplantation, and the final score was calculated as the sum of the number of pellets that a rat could eat on both days. The examiner performing the pellet retrieval tests did not know the experimental group to which the rats had been assigned.

Pellet Retrieval Test

The design of the reaching apparatus for the pellet retrieval test was the same as described elsewhere (Diener and Bregman, 1998; see Fig. 5A). Five pellets were placed in each cubbyhole. Using this apparatus, rats could eat pellets only if they accurately reached their forelimb to a target (pellet) and maintained their grasp around the pellet while they moved it to their mouth. For training, on the first day, after a 48 hr fast, the rat was placed in the apparatus with five pellets in each cubbyhole (total 60 pellets) for 3 hr or more, until it ate some pellets. Rats that could not eat any pellets on the first day of training were placed back in the apparatus on the night of the first day and water was provided, to give them additional time to learn the task. This procedure allowed almost all of the rats to learn how to eat pellets from the cubbyholes. From the second day onward, each rat was placed in the apparatus for 1 hr of additional training. Rats were given 15 g of food after the training every day. Water was provided freely. Rats that never learned to eat pellets in the week of training before surgery were excluded from the experiment. During the training and the test, the apparatuses were placed in a large box, and a loud noise was generated in it so that the rats would not fear the change in their surrounding environment. The testing sessions were performed after a 48 hr fast. Each rat was placed in the apparatus with five pellets in each cubby for 15 min, and the number of pellets that the rat ate was counted. The effects of transplantation on this behavior were analyzed using a Mann-Whitney U-test.

RESULTS

Transplantation of Neurosphere Cells Into the Injured Spinal Cord of Rats

We used the contusion model as our rat model of spinal cord injury, because it has many similarities to actual spinal cord injury, which have been reported previously (Balentine, 1978; Hughes, 1998). In this model, cavities replaced mainly central and dorsal gray matter and the dorsal column of white matter, including the corticospinal tract, fasciculus gracilis, and fasciculus cuneatus, a type of lesion that is frequently seen in human spinal cord injury (Balentine, 1978; Hughes, 1998). In addition, the remaining white matter often exhibited areas of demyelination, especially in the dorsal column and areas in which multiple microcysts were observed (Fig. 1A-1,2,B-1,2). In the ventral horn, the number of motor neurons was reduced, but some healthy motor neurons remained (Fig. 1A-3,B-3). Collectively, the losses of both neurons and glia are obvious in this contusion model and cause a functional deficit. Thus, it is reasonable to expect that the replacement of lost

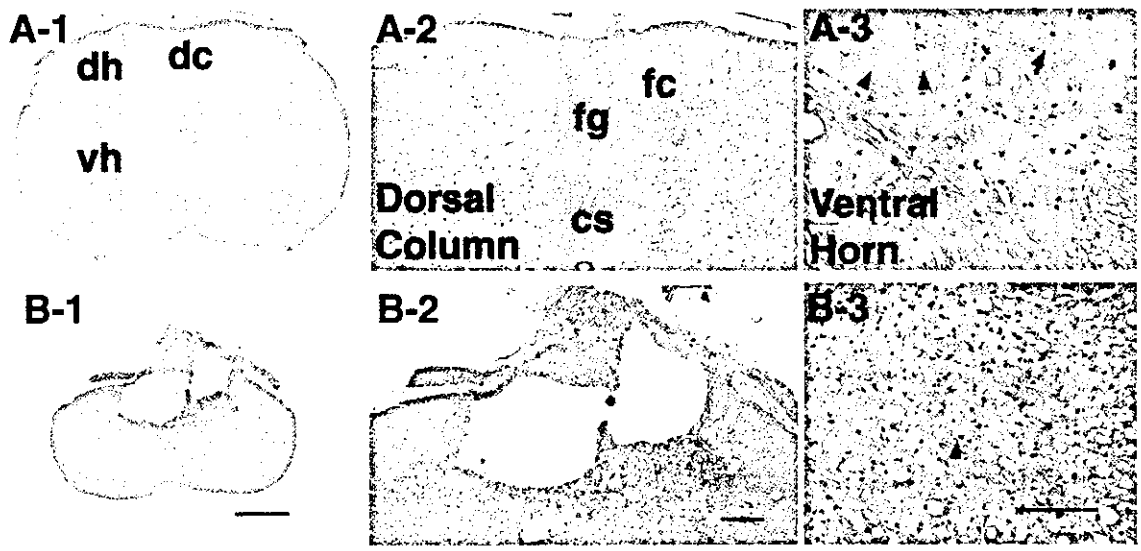

Fig. 1. Normal and injured spinal cords 44 days after the injury. **A-1–3:** A normal adult rat spinal cord (C4/5 level). vh, Ventral horn; dh, dorsal horn; dc, dorsal column; cs, corticospinal tract; fg, fasciculus gracilis; fc, fasciculus cuneatus; arrowhead, motor neuron. **B-1–3:** An injured spinal cord (C 4/5 level, epicenter of the injury site). Cavities replaced mainly central and dorsal gray matter and the dorsal column of the white matter, including the corticospinal tract, fasciculus gracilis, and fasciculus cuneatus (B-1,2). The remaining white matter often exhibited areas of demyelination that stained with eosin, especially in the dorsal column and areas in which multiple microcysts were observed (B-1,2). In the ventral horn, the number of motor neurons was reduced, but some healthy motor neurons (arrowhead) remained (B-3). H&E and LFB staining. Formalin-fixed and paraffin-embedded transverse section (4 μm thick). Scale bars = 500 μm in A-1,B-1; 250 μm in A-2,B-2; 125 μm in A-3,B-3.

neurons and glia by the transplantation of neural progenitor cells would result in functional recovery.

For the transplant group, cells that had been prelabeled with BrdU in vitro were placed in the cavity (Fig. 2A,B-1,2) but did not completely fill it. Among the BrdU-prelabeled cells (i.e., cells that were derived from the transplanted cells), neurons (5.9% ± 1.3%, ±1 SEM), astrocytes (32.6% ± 1.7%), and oligodendrocytes (4.4% ± 0.5%) were observed in the transplantation site 5 weeks after the transplantation (Fig. 2C–E). Neurons were identified with an anti-Hu antibody, which has been used by Barami et al. (1995); astrocytes with an anti-GFAP antibody; and oligodendrocytes with an anti-CNP antibody. These results indicate that donor-derived neurons, astrocytes, and oligodendrocytes exist in the host spinal cord upon transplantation 9 days after spinal cord injury.

Some Transplanted Cells Differentiated Into Neurons In Vivo and Were Integrated Into the Host Tissue

To examine whether the mitotic neurogenesis had occurred from the transplanted donor cells within the host spinal cord, we took advantage of the fact that the 1.1-kb promoter element of the Tα1 tubulin gene is active only in cells of the neuronal lineage (including neuronal progenitors and postmitotic neurons) and not in those of the glial lineage (Gloster et al., 1994; Wang et al., 1998; Roy et al., 2000a,b). Here, we used rats that had been transplanted with neurospheres derived from Tα1-EYFP transgenic rats. The host rats received a BrdU injection intraperitoneally after the transplantation. By injecting BrdU intraperitoneally, we could label cells that had divided during the period of BrdU injection. Thus, the presence of postmitotic neurons that were doubly positive for BrdU labeling and EYFP expression would demonstrate that donor-derived progenitor cells underwent mitotic neurogenesis within the host spinal cord.

Five weeks after the transplantation, we characterized the antigenic phenotype of the donor-derived cells within the host spinal cord. First, we performed double immunostaining using an anti-GFP antibody (to detect EYFP) and an anti-Hu antibody as a neuron-specific marker. We found that all the EYFP-positive cells were Hu-positive (Fig. 3A-1–3), indicating that the donor-derived cells that retained EYFP expression were exclusively postmitotic neurons 5 weeks after the transplantation. We next performed immunostaining with anti-GFP and anti-BrdU antibodies. We observed that a substantial fraction (64.07% ± 2.38%, ±1 SEM) of EYFP-positive cells was BrdU-positive (Fig. 3B). Because all the EYFP-positive cells expressed the postmitotic neuronal marker (Hu), the existence of EYFP and BrdU double-positive cells suggested that mitotic neurogenesis from donor-derived neural progenitor cells occurred in vivo, after the transplantation.

In addition, donor-derived EYFP-positive neurons extended a process longitudinally (Fig. 3C). After performing immunostaining with the anti-GFP antibody, we also observed the transplanted tissue by electron microscopy. We found that some EYFP-positive axons were myelinated (Fig. 4A). Furthermore, we observed that EYFP-positive presynaptic structures that had presynaptic vesicles were connected with EYFP-negative postsynaptic structures that had postsynaptic densities. We also found

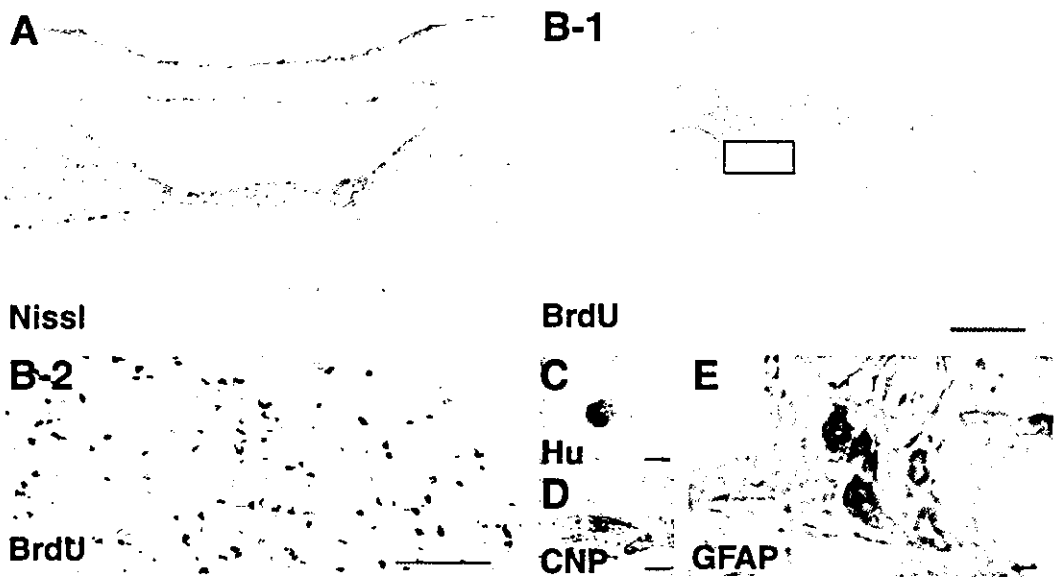

Fig. 2. BrdU-prelabeled transplanted cells occupying the cavity at the lesioned site of a spinal cord 5 weeks after transplantation. **A:** Sagittal section (14 μm thick) of the lesioned site of a spinal cord transplanted with only culture medium. Nissl staining. **B-1:** Sagittal section (14 μm thick) of the lesioned site of a spinal cord transplanted with BrdU-prelabeled neurospheres. BrdU-positive cells occupied the cavity at the lesion site. BrdU immunostaining (black). **B-2:** Higher magnification of B-1. **C–E:** BrdU-prelabeled transplanted cells differentiated into neurons, oligodendrocytes, and astrocytes. C: Hu and BrdU double-positive cell (brown; Hu, blue; BrdU). D: CNP and BrdU double-positive cell (brown; CNP, blue; BrdU). E: GFAP and BrdU double-positive cell (brown; GFAP, blue; BrdU). Scale bars = 250 μm in B-1; 100 μm in B-2; 5 μm in C–E.

EYFP-negative presynaptic structures connected with EYFP-positive postsynaptic structures (Fig. 4B,C). Interestingly, EYFP-positive neurons were, in some cases, found to form a synapse with host motor neurons at the injury site (Fig. 4D).

Behavioral Analysis

Rats in the control groups ate fewer pellets than rats in the transplanted group in the pellet retrieval test (Table I). In the control groups, only 8 of 13 SCI rats and 9 of 17 SCI + med rats were able to eat more than five pellets during the test period, although in the transplanted group 13 of 15 rats could do so. These data showed a statistically significant difference between the transplanted and the control groups (Fig. 5B; $*P < 0.01$). Naive, unoperated rats [ope(−), n = 10] ate more pellets than did the transplanted group, and this difference was also statistically significant (Fig. 5B; $*P < 0.01$). Rats that ate fewer than five pellets showed motor deficits in targeting the pellet. They could barely reach the pellet, as a result of insufficient shoulder and elbow extension. Even the rats in the control groups that ate more than five pellets showed abnormalities in limb movement, including the frequent misdirection of forelimb extension beyond the pellet. In addition, lesioned (SCI, SCI + med) rats also frequently brought their paw to their mouth, even if it did not contain a pellet. Although similar behaviors were also observed in the transplantation group, they occurred at a much lower frequency.

DISCUSSION

Grafting of cells or tissue derived from the embryonic spinal cord has been performed in rat and cat spinal cord injury models over the past several decades (Reier et al., 1983; Anderson et al., 1995; Diener and Bregman, 1998), and recently McDonald et al. (1999) reported the grafting of progenitor cells derived from embryonic stem cells. Grafting of neural progenitor cells derived from the embryonic spinal cord to the total resection model of spinal cord has also been reported (Vacanti et al., 2001). However, in these reports the authors have transplanted heterogeneous population of neural cells, including differentiated neurons, and do not prove in vivo neurogenesis from transplanted cells in an adult spinal cord, which proof should be based on lineage study using BrdU labeling. Here, we present the first evidence that grafting a neural stem cell-enriched population, prepared by neurosphere formation, into the injured adult rat spinal cord results in the generation of new neurons in vivo from transplanted cells by BrdU labeling and results in behavioral improvement. The major advantage of the present method, which uses cultured neural progenitor cells, is that we can expand neural progenitor cells to the desired quantity from a small amount of fetal CNS tissue. By passaging the cells obtained from one pregnant rat (12–15 fetuses) three times, we could obtain enough immature neural progenitor cells for the transplantation of at least 450 rats. Thus, in the future, fetus-derived expanded neural progenitor cells may be a useful source for transplantable material with which to treat spinal cord injury.

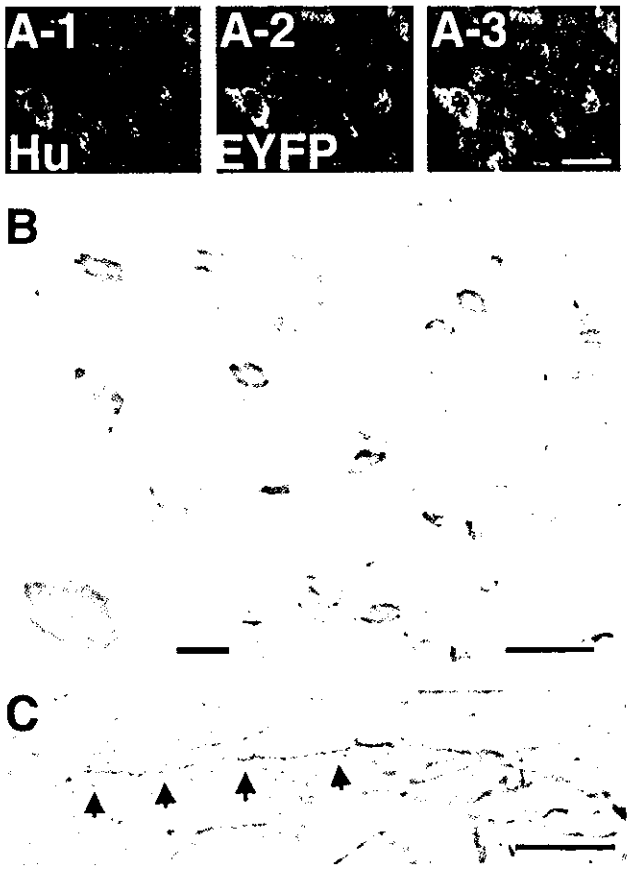

Fig. 3. Transplanted cells differentiated into neurons in vivo. **A:** EYFP-positive cells (green) were Hu-positive (red). **B:** Double-positive cells for EYFP (blue) and BrdU (brown) were found. **C:** EYFP-positive donor-derived neurons (black) extended their processes longitudinally (arrow). Sagittal section (20 μm thick). Scale bars = 5 μm in A; 10 μm in B; 2.5 μm in inset, 25 μm in C.

In Vivo Neurogenesis From Donor Progenitor Cells in the Host Spinal Cord: Its Putative Regulatory Mechanisms

Recently, it was reported that endogenous neural stem cells, which are present in the adult rat spinal cord, proliferate and differentiate exclusively into astrocytes, and not into neurons, upon injury (Johansson et al., 1999; Namiki and Tator, 1999). In the present study, however, transplanted neural progenitor cells differentiated into neurons in vivo (Fig. 3A,B). This apparent difference could be explained as follows. First, because we transplanted a large number of immature neural progenitor cells, enough autocrine and/or paracrine factors may have been supplied by the donor cells to facilitate their own survival and neuronal differentiation. Second, because we transplanted neural progenitor cells 9 days after the injury, the microenvironment around the injury site may no longer have been in the acute inflammatory phase and therefore might have been permissive for the neuronal differentiation of donor progenitor cells. During the acute phase after injury, severe inflammation occurs around the injured site. In fact, Nakamura et al. (2002) have reported that, immediately after injury, the levels of many inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, which have neurotoxic effects, increased and then declined sharply within 24 hr. Consistent with this, when we transplanted neurosphere-derived neural progenitor cells 24 hr after the injury, almost none of the grafted cells survived, or, in some cases, a small number of cells survived that formed a small mass (n = 8, data not shown). These observations indicated that the microenvironment of the acute phase was not suitable for grafted cells to survive. In addition, the increased production of astrocytes, but not neurons, in this acute inflammatory phase (Johansson et al., 1999) may be due to a microenvironment that favors the generation of astrocytes to prevent the inflammation from spreading to the surrounding area. However, by 9 days after the injury, the acute inflammatory phase is over. At this time, the microenvironment around the injured site is likely to have changed to the reparative or remodeling phase. In fact, expression of some growth and trophic factors and microvascular regeneration were shown to be induced, responding to brain or spinal cord injury at this time (Koshinaga et al., 1993; Miyoshi et al., 1995), which could correspond to the benefits of "delayed" transplantation for functional recovery. Hammang et al. (1997) and Park et al. (1999) suggested that new microenvironments created by degenerative conditions can facilitate the differentiation of grafted cells into cell types that had been lost in the spinal cord to repair the damaged tissue. In spinal cord injury, many neurons and oligodendrocytes are lost at the injured site. In our rat model, therefore, new microenvironments might have been suitable for the grafted cells to survive and differentiate into neurons and oligodendrocytes by 9 days after the injury. Accordingly, in this study, donor-derived neurons and oligodendrocytes were observed in the host spinal cord.

Shihabuddin et al. (2000) reported that adult spinal cord stem cells generated neurons after transplantation in the neurogenic region of adult central nervous system but did not do so in the nonneurogenic region, including adult spinal cord. The authors concluded that an appropriate microenvironment was needed for neuronal differentiation from neural stem cells. In tour study, donor-derived neural progenitor cells transplanted 9 days after the injury were observed to differentiate into neurons in vivo. Furthermore, we could demonstrate new synapse formation between donor-derived neurons and host neurons (including motor neurons; Fig. 4B–D). This also might have been the result of a postinjury microenvironment that favored the neuronal generation and integration of donor-derived neurons into the host tissue.

These findings suggest that a time window for the successful transplantation of neural progenitor cells exists. It also must be determined, however, whether the observed in vivo neurogenesis from the transplanted progenitor cells is responsible for the functional recovery.

Putative Neural Mechanisms for Functional Recovery

In the behavioral analysis, there was a significant difference in the pellet retrieval test between the transplanted and the control groups 44 days after injury. In

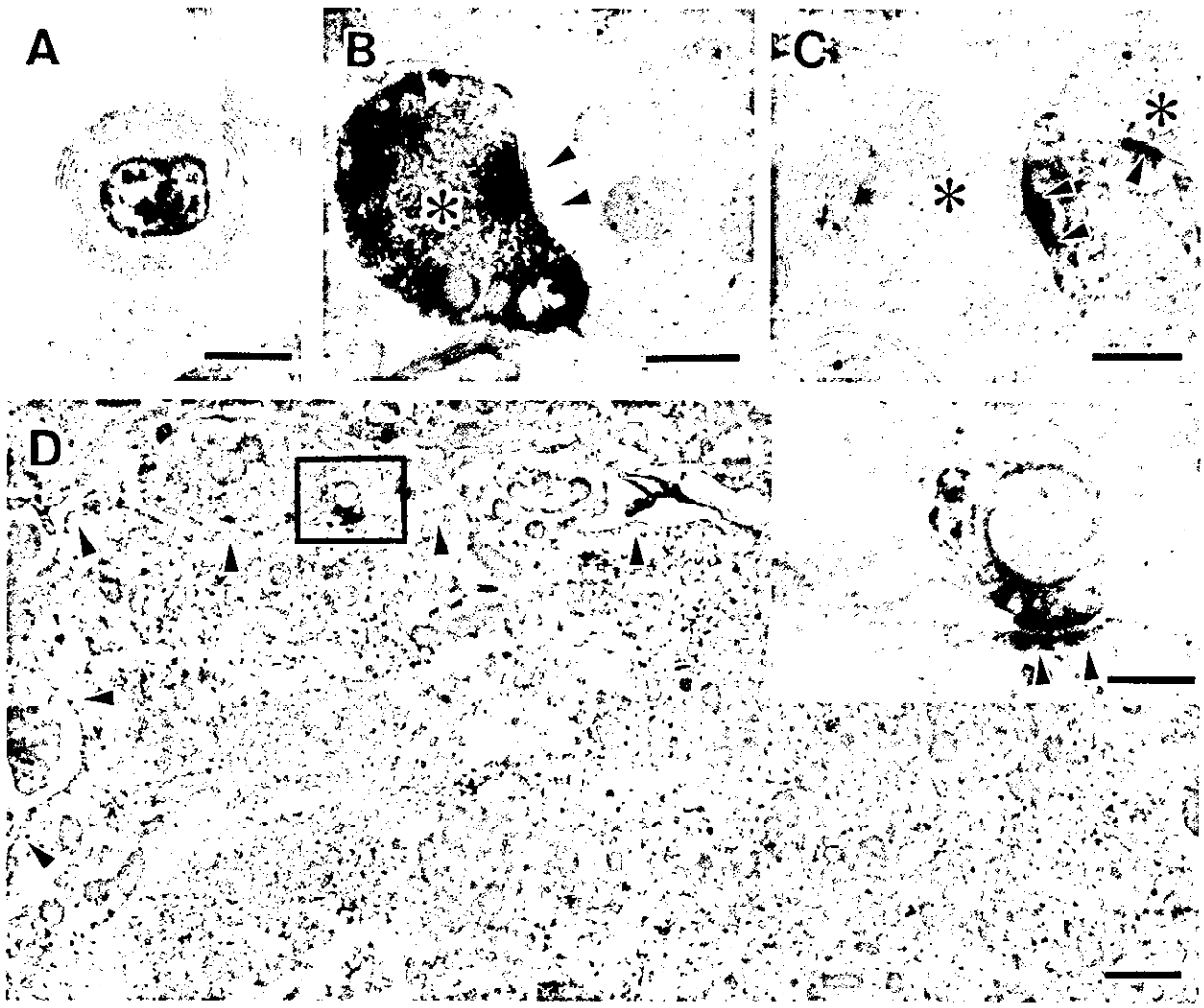

Fig. 4. Neurons derived from transplanted cells were integrated into the host spinal cord. By electron microscopy, EYFP-positive (black) myelinated axon (**A**), presynaptic structure (**B**), and postsynaptic structure (**C**) were observed. Interestingly, synapse formation was observed between a host motor neuron (surrounded by arrowheads in D) at the injured site and an EYFP-positive (black) neuron (**D**). The motor neuron was identified by its appearance (i.e., a large cell body, more than 15 μm in diameter, and the presence of many synaptic boutons in the surface of the cell body) and its location in the spinal cord (i.e., the ventral horn). Asterisks, presynaptic vesicles; arrowheads, postsynaptic densities. Scale bars = 0.2 μm in A,C; 0.5 μm in B; 1 μm in D; 0.3 μm in inset.

the spinal cord injury model we used, the regions that sustain the most injury are the dorsal columns, the dorsal horn, and the central gray matter. Schrimsher and Reier (1992, 1993) examined changes in forelimb motor function following various spinal cord lesions in rats. In their papers, they suggest that the ascending sensory fiber components of the dorsal columns may play an important role in mediating the performance of skilled forelimb reaching movements, such as pellet retrieval, and thus that the damage of these components may be responsible for the severe reaching hypometria in rat cervical spinal cord contusion injury. The present results, showing a decrease in the number of pellets obtained by rats in the control group, can be explained largely by these behavioral deficits produced by the lesion of the dorsal columns.

We demonstrated that grafted progenitor cells generated neurons, which survived in the grafted site. Furthermore, some new donor-derived neurons extended myelinated axons and formed synapses with host neurons. One possible explanation for the behavioral improvement in the transplanted rats is that, by making new synapses with host neurons, neurons derived from grafted cells relayed signals from the disrupted fibers in the host, including ascending fibers that existed in the dorsal column, as proposed by Bregman et al. (1993). In this scenario, signals from disrupted ascending fibers that existed in the dorsal column would be relayed by donor-derived neurons to the cuneate and/or gracile nucleus, where the ascending fibers in the dorsal column terminate.

Another possible explanation is the contribution of glial cells derived from grafted cells. Oligodendrocytes derived

TABLE I. Numbers of Pellets Eaten by Rats in Each Group

No. of pellets	SCI + TP (n = 15)	SCI (n = 13)	SCI + med (n = 17)	Ope (−) (n = 10)
1	75	51	52	82
2	0	64	60	77
3	75	0	0	83
4	51	0	0	76
5	62	22	0	83
6	0	59	49	82
7	75	34	0	78
8	67	0	2	83
9	70	30	37	79
10	57	0	64	80
11	71	2	51	
12	68	53	0	
13	70	64	0	
14	68		24	
15	73		0	
16			54	
17			60	

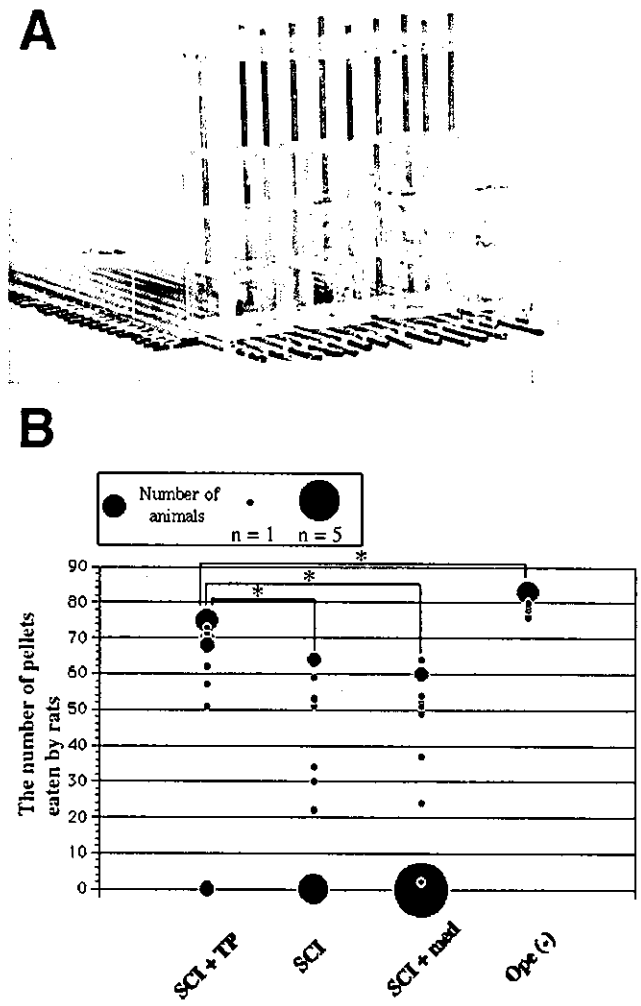

Fig. 5. Transplantation of neural progenitor cells improved behavioral recovery. **A:** Pellet retrieval test. Rats could obtain pellets only with their forelimbs (arrow). **B:** Results of pellet retrieval test. The number of pellets that could be eaten by rats from each group is marked with a dot. The diameter of the dots increases in proportion with the number of rats that ate the same number of pellets. $*P < 0.01$. The P value was determined using a Mann-Whitney U-test.

from grafted cells remyelinated fibers that had been demyelinated as a result of injury and restored the connectivity. Crowe et al. (1997) reported from a study of traumatic spinal cord injury in rats and monkeys that oligodendrocytes underwent apoptosis during Wallerian degeneration of fiber tracts and that the number of apoptotic cells peaked 8 days after the injury. Because a single oligodendrocyte myelinates multiple axons in the CNS, loss of these cells from apoptosis could be responsible for the demyelination of axons spared by the injury. We transplanted graft cells 9 days after the injury and demonstrated the presence of donor-derived oligodendrocytes in the injured site. These donor-derived oligodendrocytes could have remyelinated the demyelinated axons that had been spared by the injury and prevented the delayed functional loss caused by the demyelination. In addition to the oligodendrocytes, astrocytes derived from donor neural progenitor cells might have played active roles for the generation of neuronal cells, axonal regeneration of host neuronal axons, enhancement of axonal extension of donor-derived neurons, syanapse formation, and/or physiological maturation of neuronal cells. In the present study, the largest population differentiated from the grafted neural progenitor cells was astrocytes. Because the donor cells are in vitro-expanded fetal-derived neural progenitor cells in the present case, donor-derived astrocytes might have functions similar to those of fetal CNS-derived astrocytes rather than reactive astrocytes that had been induced after the CNS injury. Notably, fetal brain-derived astrocytes were shown to regulate the precise growth of neuronal axons (Garcia-Abreu et al., 2000) and promote the naturation of neuronal cells physiologically (Blondel et al., 2000).

CONCLUSIONS

Neural stem cells have been defined as self-renewing, multipotent cells, whose characteristics are based mainly on their behavior in culture. Generally, however, the ability to repair the activity of injured organs is also known to be an important criterion for identifying stem cells. Here we have shown that in vitro-expanded neural stem cells could be involved in repairing the injured central nervous system, upon their transplantation at an appropriate time point, although we cannot exclude the possibility progenitor or differentiated cells present in the neurosphere contributed to the functional recovery.

In the present study, we found that successful transplantation (neurogenesis and functional recovery) is likely to depend on timing of the transplantation (i.e., the microenvironment of host tissue). Therefore, the introduction of expression vectors of key molecules that control cell fate into the donor cells ex vivo could improve the therapeutic efficiency of this technique.

Note added in proof:

In this present paper, potential functions of astrocytes derived from donor neural progenitor cells are discussed, which

might have played active roles for functional recovery of the injured spinal cord. Notably, after this paper had been accepted for publication, the active roles of astrocytes to induce neurogenesis were published from Dr. Fred Gage's group (Song et al., 2002).

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Expression of brain-derived neurotrophic factor in the central nervous system of mice using a poliovirus-based vector

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Brain-derived neurotrophic factor (BDNF) is a promising candidate for the gene therapy of neurological disease. To deliver BDNF to neurons of the central nervous system (CNS), a nucleotide sequence encoding the mature peptide of BDNF was inserted into the genome of poliovirus, a neurotropic virus that is known to replicate mainly in motor neurons of the spinal cord of the CNS. Thus, the recombinant poliovirus constructed was replication-competent. The expression of BDNF in cultured cells infected with the recombinant poliovirus was evident when the cells were analyzed using an immunofluorescence assay and Western blotting. When the recombinant viruses were injected intramuscularly into transgenic mice that carry the human poliovirus receptor gene, the antigens of poliovirus and BDNF were detected in the motor neurons of the spinal cord at 3 days postinfection, and had disappeared by 7 days postinfection. This study suggests that poliovirus can be used as a virus vector for the delivery of neurotrophic factors to the motor neurons of the central nervous system and may provide a new approach for the treatment of motor neuron diseases. *Journal of NeuroVirology* (2002) **8**, 14–23.

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Introduction

In mammals, the neurotrophin family consists of nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 (Leibrock *et al*, 1989; Hohn *et al*, 1990; Maisonpierre *et al*, 1990; Berkemeier *et al*, 1991; Ip *et al*, 1992; Lewin and Barde, 1996). Neurotrophin genes are candidate therapeutic genes for neurodegenerative diseases, because they regulate neuronal development and promote the survival of peripheral sensory neurons, sympathetic neurons, and various types of central nervous system (CNS) neurons in cell culture systems and animal injury models (Eide *et al*, 1993; Korsching, 1993). Much effort has been made to develop virus vectors that could deliver neurotrophins into the CNS. Adenovirus vectors have been used as tools of gene therapy for neurodegenerative diseases (Barkats *et al*, 1998; Di Polo *et al*, 1998; Haase *et al*, 1998). Adeno-associated virus and herpes simplex virus vectors have also been explored for gene therapy in neurodegenerative disease (Battleman *et al*, 1993; Goodman *et al*, 1996; Klein *et al*, 1998; Goins *et al*, 1999; Mandel *et al*, 1999). In addition, transplants of fibroblasts modified by retrovirus-mediated

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expression of BDNF have also shown promising effects on the promoting the regeneration of severed neurons (Liu *et al*, 1999).

Because neurotrophic factors influence various types of CNS neurons, the broad delivery of neurotrophic factors to the CNS may affect multiple nontarget populations. Thus, for gene therapy of a particular type of neurological disease it would be more desirable to use vectors that display specificity to the neurons of interests. Poliovirus is known to have a distinct neuron tropism in the CNS, especially to the motor neurons in the anterior horn of the spinal cord. In addition, intramuscularly inoculated poliovirus can be transported to the cell body of projection neurons. Therefore, a poliovirus-based vector may offer promising applications for the specific targeting of selected neuronal populations such as the motor neurons in the spinal cord, which are not easily accessible by noninvasive approaches.

The genome of poliovirus is a single-stranded RNA molecule of positive polarity, composed of approximately 7,500 nucleotides. A unique open reading frame (ORF) encodes a large polyprotein precursor that is processed by two viral proteinases, $3C^{pro}$ and $2A^{pro}$. The major proteinase, $3C^{pro}$, recognizes and cleaves at specific amino acid sequences (AXXQ/G) within the exposed polyprotein domains. Many studies have been performed to engineer poliovirus for the delivery of foreign proteins (Burke *et al*, 1988; Choi *et al*, 1991; Alexander *et al*, 1994; Andino *et al*, 1994; Ansardi *et al*, 1994; Lu *et al*, 1995; Porter *et al*, 1995). These are classified into two strategies.

One of these two strategies is to generate replication-competent recombinant viruses in which foreign ORFs are directly fused to the ORF of the poliovirus polyprotein. In this case, foreign peptides are designed to be separated from poliovirus polyprotein by artificial cleavage sites of $3C^{pro}$ or $2A^{pro}$ (Andino *et al*, 1994; Mattion *et al*, 1994; Yim *et al*, 1996; Mandl *et al*, 1998; Crotty *et al*, 1999). Another strategy using poliovirus as an expression vector is to replace the coding sequence for the capsid protein region (P1) with a foreign ORF. Cleavage of the foreign polyprotein from the P2-P3 regions is then carried out by the viral proteinase $2A^{pro}$ at an endogenous cleavage site (Percy *et al*, 1992; Ansardi *et al*, 1994; Morrow *et al*, 1994; Porter *et al*, 1995). In the latter case, progeny virions cannot be generated because no viral capsid protein is produced in the infected or transfected cells.

By using the poliovirus replicon strategy (the latter case), foreign proteins were expressed in motor neurons of the spinal cord in transgenic (Tg) mice carrying the gene for the human poliovirus receptor (PVR; CD155) (Bledsoe *et al*, 2000a, 2000b). We report here on the construction of a replication-competent poliovirus recombinant that is manipulated to express a neurotrophin BDNF and the characterization of its nature in the cultured cell lines and CNS neurons of PVR-Tg mice, mice transgenic for the human PVR gene.

Results

Construction and replication characteristics of recombinant polioviruses expressing BDNF As the first step in construction of a replication-competent poliovirus vector to deliver a neurotrophin to the CNS, a nucleotide sequence encoding the mature peptide of BDNF was inserted at the 5′ terminus of the ORF in poliovirus type 1 Mahoney strain (PV1/Mahoney) (Figure 1). This recombinant cDNA, designated pMah/BDNF, yielded a replication-competent virus after transfection of African green monkey kidney (AGMK) cells with the *in vitro* synthesized RNA from pMah/BDNF. The virus (Mah/BDNF) harvested displayed smaller plaques and replicated more slowly than the parental

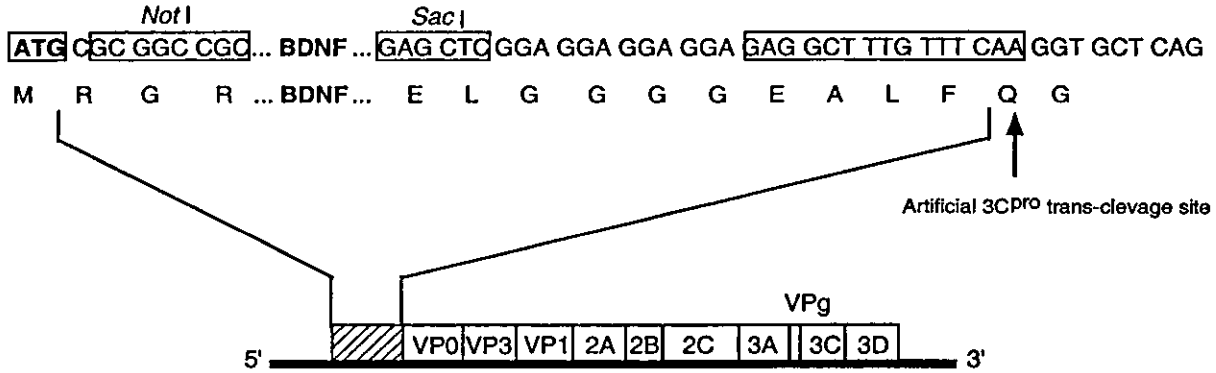

Figure 1 Genome structure of recombinant poliovirus. Schematic diagram of recombinant poliovirus expressing BDNF is shown. The virus genome is indicated by the bold line, and the organization is within the corresponding boxes. VPg at the 5′ end and poly A at the 3′ end are omitted in this figure. A linker sequence carrying restriction enzyme sites for *Sac*I and *Not*I, and a nucleotide sequence for an artificial $3C^{pro}$ cleavage site, were inserted at the 5′ end of the poliovirus ORF. The sequence encoding the mature peptide of BDNF was inserted into the linker. The start codon for the poliovirus polyprotein, the restriction cleavage sites, and a sequence for the artificial $3C^{pro}$ cleavage site are squared.