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Grafts of BDNF-Producing Fibroblasts Rescue Axotomized Rubrospinal Neurons and Prevent Their Atrophy

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We have reported that intraspinal transplants of fibroblasts genetically modified to express brain-derived neurotrophic factor (BDNF) promote rubrospinal axon regeneration and functional recovery following subtotal cervical hemisection that completely ablated the rubrospinal tract. In the present study we examined whether these transplants could prevent cell loss and/or atrophy of axotomized Red nucleus neurons. Adult rats received a subtotal spinal cord cervical hemisection followed by a graft of unmodified fibroblasts or fibroblasts producing BDNF into the lesion cavity. One or 2 months later, fluorogold was injected several segments caudal to the lesion-transplant site to retrogradely label those Red nucleus neurons whose axons have regenerated. Unmodified fibroblasts failed to protect against either cell loss or atrophy. Neuron counts and soma-size measurements in Nissl-stained preparations showed a 45% loss of recognizable neurons and 40% atrophy of the surviving neurons in the injured Red nucleus. Grafts of BDNF-producing fibroblasts reduced neuron loss to less than 15% and surviving neurons showed only a 20% decrease in mean soma size. Soma size analysis of fluorogold-labeled Red nucleus neurons indicated that the Red nucleus neurons whose axons regenerated caudal to the graft did not atrophy. We conclude that fibroblasts engineered *ex vivo* to secrete BDNF and grafted into a partial cervical hemisection promote axon regeneration while reducing cell loss and atrophy of neurons in the Red nucleus. These results suggest that transplants of genetically engineered cells could be an important tool for delivery of therapeutic factors that contribute to the repair of spinal cord injury.

Key Words: **spinal cord injury; *ex vivo* gene therapy; retroviral vector; neurotrophins; retrograde cell death; Red nucleus.**

INTRODUCTION

Functional deficits following mammalian spinal cord injury (SCI) are usually attributed to the death of neurons, intrinsic neuronal changes (such as cell atrophy), and failure of axon regeneration (24, 55, 59, 61). One of the proposed mechanisms for these events is that axotomy deprives the injured neurons of target-derived trophic factors, particularly members of the neurotrophin family (3, 5, 12, 13, 33, 35, 46). Numerous studies have demonstrated that direct application of neurotrophic factors to injured axons prevents retrograde neuron death and atrophy of surviving axotomized neurons (2, 6, 10, 21, 25, 28, 57, 58, 69). Treatment with neurotrophic factors can also promote axon regeneration (7, 50, 54, 68).

Transplantation techniques have long been employed as a therapeutic strategy for experimental models of SCI (19, 59, 60). Transplants of fetal central nervous system (CNS) tissue have proven to be effective in preventing neuron death and promoting regeneration (4, 6–8, 11, 22, 45, 47). Fetal tissue has been proposed to act as a surrogate source of neurotrophic factors to support the axotomized neurons, and as a bridge or relay across the injury site (19, 59, 60). Fetal tissue transplants, however, are associated with ethical and logistical problems that hinder their clinical application. Techniques for grafting cells that have been genetically modified by *ex vivo* gene therapy have developed rapidly over the past decade and represent a very promising therapeutic strategy for spinal cord injury that avoids the problems associated with use of fetal tissue.

The basic concept of *ex vivo* gene therapy is to engineer cells under *in vitro* conditions using vectors encoding therapeutic genes (such as genes for neurotrophic factors) and then to graft the genetically altered cells as a vehicle to deliver the therapeutic gene product (15). *Ex vivo* gene delivery of neurotrophins has been demonstrated to enhance neuronal survival and regeneration (20, 23, 29, 40, 43, 53, 59). Retroviral vectors have been the vectors of choice in the spinal

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cord but adenoviral vectors can also be effective (39). Commonly used cells for this therapy include fibroblasts (20, 39, 40), Schwann cells (43), and neural stem-like cells (1, 23, 38, 41, 42).

We have reported that fibroblasts modified to express brain-derived neurotrophic factor (Fb/BDNF) grafted into a subtotal hemisection cavity in the cervical spinal cord promote rubrospinal tract (RST) regeneration and partial recovery of forelimb function in adult rats (40). Results from retrograde and anterograde tracing studies showed that about 7% of the neurons in the injured Red nucleus (RN) regenerated through or around the Fb/BDNF graft and continued growing for long distances in the white matter caudal to the lesion-transplant site. There is a 7- to 20-fold increase in the number of retrogradely labeled neurons over that found with unmodified fibroblasts or with gelfoam alone. We also observed that the regenerated axons projected branches with terminal bouton-like structures to laminae V–VII of the gray matter, the appropriate target regions of rubrospinal axons, suggesting target reinnervation. In behavioral tests that evaluated forelimb usage, recipients of Fb/BDNF showed significant functional recovery (40, 56) which was abolished by a second lesion that cut the regenerated RST axons, suggesting that the functional recovery could be at least partially attributed to RST regeneration (40). Additional motor and somatosensory tests confirmed the partial functional recovery (30). In the present study we examined whether transplants of Fb/BDNF also increased the survival of axotomized RN neurons and prevented the atrophy of the cell somata often associated with axotomy. Using stereological cell measurement and retrograde tracing techniques we analyzed the number and size of all RN neurons recognizable with Nissl staining and specifically the population able to regenerate axons several segments caudal to the injury site.

The Red nucleus is located bilaterally in the midbrain tegmentum and is composed of a caudal magnocellular and more rostral parvicellular region. Although neurons in both regions project to the spinal cord, the magnocellular projections are the major component of the RST (9, 52, 63, 65). More than 99% of RST axons cross in the ventral tegmental decussation and descend in the superficial dorsolateral funiculus (9, 63). Therefore, a left cervical hemisection axotomizes almost the entire right magnocellular neuron population, which results in the loss of approximately 45% of the RN neurons; the surviving RN neurons show a mean decrease in soma size (atrophy) of 40%. Similar findings have been previously reported (18, 47, 49), although Kwon *et al.* has recently contended that rubrospinal neurons do not die but become severely atrophied after cervical axotomy (34). Whether the cells have died or merely atrophied beyond recognition remains to be determined. In this study we are concerned with the effects of neurotrophin delivery on the neurons that are recognizable and potentially retain function. Fetal spinal cord transplants rescue about 50% of the cells that would have been lost but fail to prevent soma atrophy or to encourage RST regeneration (47). In the present study we demonstrate that intraspinal grafts of Fb/BDNF reduced loss of recognizable neurons to less than 15% and decreased atrophy of mean soma size to less than 20%. We were also able to show specifically that RN neurons that regenerated axons at least several segments caudal to the injury did not show measurable cell atrophy.

MATERIALS AND METHODS

Animal Groups

All procedures were approved by the institutional animal welfare committee and conformed to the National Institute of Health (NIH) guidelines for the care and use of laboratory animals. Fifty-one female Sprague–Dawley rats (250–300; Taconic, Germantown, NY) were studied. Forty-two rats received a right partial cervical hemisection and a transplant of either BDNF-producing fibroblasts (Fb/BDNF, $n = 18$), primary fibroblasts (Fb, $n = 12$), or gelfoam ($n = 12$) into the lesion cavity. Thirty-six of these animals received fluorogold (FG) injections 3 days before sacrifice to study RST regeneration. Animals were euthanized at 1 and 2 months postsurgery ($n = 6$ for each group at each time point). Six Fb/BDNF recipients were euthanized at 1 week without FG tracing to study transgene expression. Three normal animals received FG injections. Forty-five of these animals had been used in a previous study to demonstrate RST regeneration (40). In the current experiment, we examined the survival of axotomized RN neurons. A group of six sham-operated animals served as normal controls.

Immunosuppression with Cyclosporin A (CsA)

All animals received an identical immunosuppression protocol. Subcutaneous administration of CsA injection solution (Sandoz Pharmaceuticals Co., East Hanover, NJ) at a dose of 1 mg/100 g/24 h started 3–5 days before the transplantation procedures and continued for 2 weeks postoperation. After this, oral CsA solution (50 μg/ml, Sandoz) was administered through the drinking water until sacrifice.

Cell Culture

Primary fibroblasts (Fb) were isolated from adult rat abdominal skin. Fb/BDNF were obtained by infecting primary fibroblasts with a retroviral construct encoding the human-BDNF cDNA which was linked to a fusion reporter gene of lacZ and neomycin resistance by an encephalomyocarditis virus internal ribosomal

entry site (IRES). The IRES ensures a high coexpression of the gene of interest and the reporter genes (38, 40). This enabled us to isolate Fb/BDNF cells based on antibiotic resistance and to identify the donor cells using a histochemical stain for β-galactosidase (β-gal), which is highly specific for lacZ expression (37). Fb/BDNF, tested *in vitro*, secretes bioactive BDNF at a rate of 12.8 ng/10^6 cells/24 h (40). The procedures to culture Fb and Fb/BDNF have been described before (39). The cells were cultured on 100-mm uncoated dishes (Becton Dickinson Labware, Franklin Lakes, NJ) and split at 1:10 into fresh medium weekly. The day before surgery, cells were labeled with the nuclear dye bisbenzamide (Sigma, St. Louis, MO) as described by (43). On the day of surgery, confluent cultures of cells were trypsinized, counted, and resuspended in growth medium (Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum) at a final concentration of 10^5 cells/μl. The cells were kept on ice during surgery. After surgery, the remaining cells were either stained with trypan blue (Sigma) or replated and stained by β-gal histochemistry to verify viability and transgene expression. Culture supplies were purchased from Fisher Scientific (Pittsburgh, PA), unless specified.

Surgical Procedures

Rats were anesthetized by an intraperitoneal (ip) injection of a cocktail of acepromazine maleate (0.7 mg/kg, Fermenta Animal Health Co., Kansas City, MO), ketamine (95 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA), and xylazine (10 mg/kg, Bayer Co., Shawnee Mission, KS). A laminectomy was then carried out at the C3–4 level to expose one spinal cord segment. The dura over the right dorsal root entry zone was opened with a microscalpel and a shallow incision was made in the right dorsal spinal cord. A glass-pulled fine-tipped microaspiration device was then used to extend the lesion laterally and ventrally. This lesion completely disrupted the lateral funiculus (containing the entire RST), partially removed the ipsilateral ventral white matter and gray matter, but left the dorsal columns intact. The rostrocaudal extent of the lesion cavity was about 2–3 mm. After hemostasis was achieved, a piece of gelfoam soaked with growth medium alone, Fb, or Fb/BDNF cells was implanted into the cavity. In cell grafts this was followed by injection of another 10 μl of cells suspended in growth medium onto the gelfoam using a 10-μl Hamilton syringe attached to a glass pipette (tip diameter 50 μm). Cells were used at a concentration of 5×10^4 cells/μl. The dura was then closed with interrupted 10-O silk sutures, and the muscle and skin were closed in layers. Sham-operated animals received a laminectomy but the dura was not opened. Within 10 min of the spinal cord lesion, all rats received a bolus injection of methylprednisolone (30 mg/kg, Pharmacia & Upjohn Company, Kalamazoo, MI) through the tail vein. After the surgery, animals were kept on heating pads and closely observed until awake before returning to their home cages. The sham-operated animals received only a laminectomy at C3–4 without spinal cord lesion. For FG-retrograde tracing, animals received another laminectomy 3–4 segments (1–1.5 cm) caudal to the initial injury site, to avoid diffusion of FG into the transplant. One microliter of 2% FG (Fluorochrome Inc., Englewood, CO) was pressure injected into each side of the spinal cord and animals were euthanized 3 days later. Animals were subsequently screened for the presence of FG that had diffused into the graft and several animals were eliminated from the study to avoid misinterpretation of the regeneration data (40).

Tissue Preparation and Histology

Animals were anesthetized with an ip injection of sodium pentobarbital (100 mg/kg, Abbott Laboratories, North Chicago, IL) and perfused transcardially with 200 ml of physiological saline followed by 500 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and spinal cord were dissected out and immersed overnight in 0.1 M phosphate buffer (PB) at 4°C followed by cryoprotection in 30% sucrose for 3–5 days. Tissues were serially blocked, embedded in OCT compound (Fisher Scientific), and kept at −80°C before being cut into 20-μm (spinal cord) or 40-μm (brain) sections on a cryostat. Sections were mounted onto gelatin-coated slides. Spinal cord sections were processed for histological or immunocytochemical staining as described before (37). Brain sections containing FG-labeled RN neurons were first examined with a fluorescent microscope and images were captured with a digital camera (see below). The sections were then stained with cresyl violet for cell counting.

Image Analysis

Images were captured using a Photometric Sensys KAF-1400 CCD camera (Photometric Ltd., Tucson, AZ) and a DC-330 CCD color video camera (DAGE-MTI, Inc., Michigan City, IN) attached to a Leica DMRBE microscope (Wetzlar, Germany). They were then processed on a Macintosh Power PC 8500 with NIH Image, IP Lab (Scanalytics Inc., Fairfax, VA) and Photoshop (Adobe System Inc., San Jose, CA) image analysis software packages.

Cell Counting and Cell Size Analysis

Neurons in the magnocellular portion of RN at the level of the interpeduncular nucleus were counted. This region of RN spans about 480 μm from the caudal pole (47, 51, 52). Within 300 μm of the caudal pole, RN

neurons are uniformly distributed. Three hundred to 480 μm from the caudal pole, dorsomedial and ventrolateral subgroups can be distinguished in the magnocellular RN (52). Neuron numbers in RN were counted using stereological methods based on the optical fractionator technique (27, 66, 67). Sections containing the caudal pole of RN were identified and sections containing the caudalmost 480 μm of RN (a total of twelve 40-μm sections) were selected and serially labeled from 1 to 12. After processing and staining the sections have shrunk isotropically to a mean thickness of 22.4 $\pm$ 3.5 μm. Section thickness was measured at several sites in each section to be certain of uniform section thickness. Neurons in every other section were counted. The first section to be counted was chosen randomly so that the possibility of counting sections 1, 3, 5, 7, 9, and 11 equaled that of counting sections 2, 4, 6, 8, 10, and 12. The caudalmost 300 μm of RN was considered to be a single subnucleus, whereas the dorsomedial and ventrolateral subdivisions of RN located 300–480 μm from the caudal pole were considered as two individual subnuclei. Each subnucleus was subdivided into four quadrants and a random number between 1 and 4 was generated to determine the quadrant to be counted. An IP-Lab script was written to drive a Photometric Sensys KAF-1400 CCD camera and a *z*-axis stage attached to a Leica DMRBE microscope. A counting frame 39,235 μm^2 in area was used to sample RN neurons. A series of images was acquired (400×) through a section creating a counting box 15 μm thick. Our criteria for counting a cell within this counting box was a neuron that came into focus displaying both a clear neuronal morphology and a nucleus. Cells were not counted if their profile contacted either the top or left edge of the counting box. The area of RN in each section where cells were counted was then measured at 100× using NIH image software. An estimate of the total number of neurons in each RN was calculated (N) as the number of neurons sampled with the optical disectors (n) and the reciprocal of the fraction of sections sampled ($1/f$), the fraction of the sectional area sampled ($1/af$), and the fraction of the section thickness sampled ($1/st$). In summary total RN neuron counts $N = \Sigma\, n \cdot 1/f \cdot 1/af \cdot 1/st$. The cross-sectional area of FG-labeled or cresyl violet-stained RN neurons was measured using an NIH image macro under 400× magnification. A total of 150–200 neurons was measured for each RN. These cells were sampled from the region outlined by the original counting box. All neurons containing a nucleus were counted in each sampled counting box. In some experimental groups there were fewer than 200 FG-labeled neurons in the axotomized RN. In these cases all labeled cells from the sampled region that met our criteria were measured. The maximum cross-sectional area of the cell was measured. Neurons were subdivided into five groups according to cross-sectional area as being <100 μm^2 (very small), 100–300 μm^2 (small), 300–500 μm^2 (medium), 500–1,000 μm^2 (large), and >1000 μm^2 (giant).

Statistical Analysis

Statistical analyses of the numerical data (RN cell number and cross-sectional area) were performed using StatView software (SAS Institute Inc., Cary, NC). In all cases, one-way ANOVA analysis showed no statistical difference between the RN of unoperated control and the RN ipsilateral to the lesion in operated animals in any group for any of the variables measured. We therefore used the lesion/control ratio of each animal as the basis for comparison. Overall significance of the ratio data was determined using one-way ANOVA followed by Fisher's post hoc test.

RESULTS

Spinal Cord Lesion, Graft Survival, and Transgene Expression

Spinal cord tissue containing the lesion-transplant region was cut into cross sections and stained with cresyl violet and β-gal histochemistry to verify lesion extent, graft survival, and transgene expression. In all animals, the subtotal hemisection completely disrupted the lateral funiculus containing the entire right RST. The lesion also partially removed the ipsilateral gray matter and ventral white matter (Fig. 1). The morphology of the grafts in each treatment group was similar at 1 and 2 months. In animals receiving Fb/BDNF or Fb transplants, the grafts filled the entire lesion cavity with little or no cyst formation and restored the general contour of host spinal cord. The transplants were closely apposed to the host tissue without intervening scar or cysts (Fig. 1B). In animals receiving gelfoam alone, the implants were reabsorbed by 1 month leaving a partially collapsed cavity filled with CSF (data not shown). One week following transplantation, Fb/BDNF grafts were strongly and uniformly stained by β-gal histochemistry. The staining remained positive for at least 2 months, although it became less intense at later time points (data not shown). Our previous studies demonstrated that Fb/BDNF cells coexpress BDNF and β-gal at a high ratio and secrete bioactive BDNF at a rate of 10–20 ng/10^6 cell/24 h *in vitro* (40). Thus, the β-gal staining pattern suggested that the grafted cells expressed and secreted BDNF for at least 2 months.

Effects of Transplants on RN Neuron Survival

We studied the effects of gelfoam, Fb, and Fb/BDNF transplants on survival of axotomized RN neurons by comparing the number of neurons remaining in the injured RN to the number on the intact side. In sham-operated control animals, the left and right RN con-

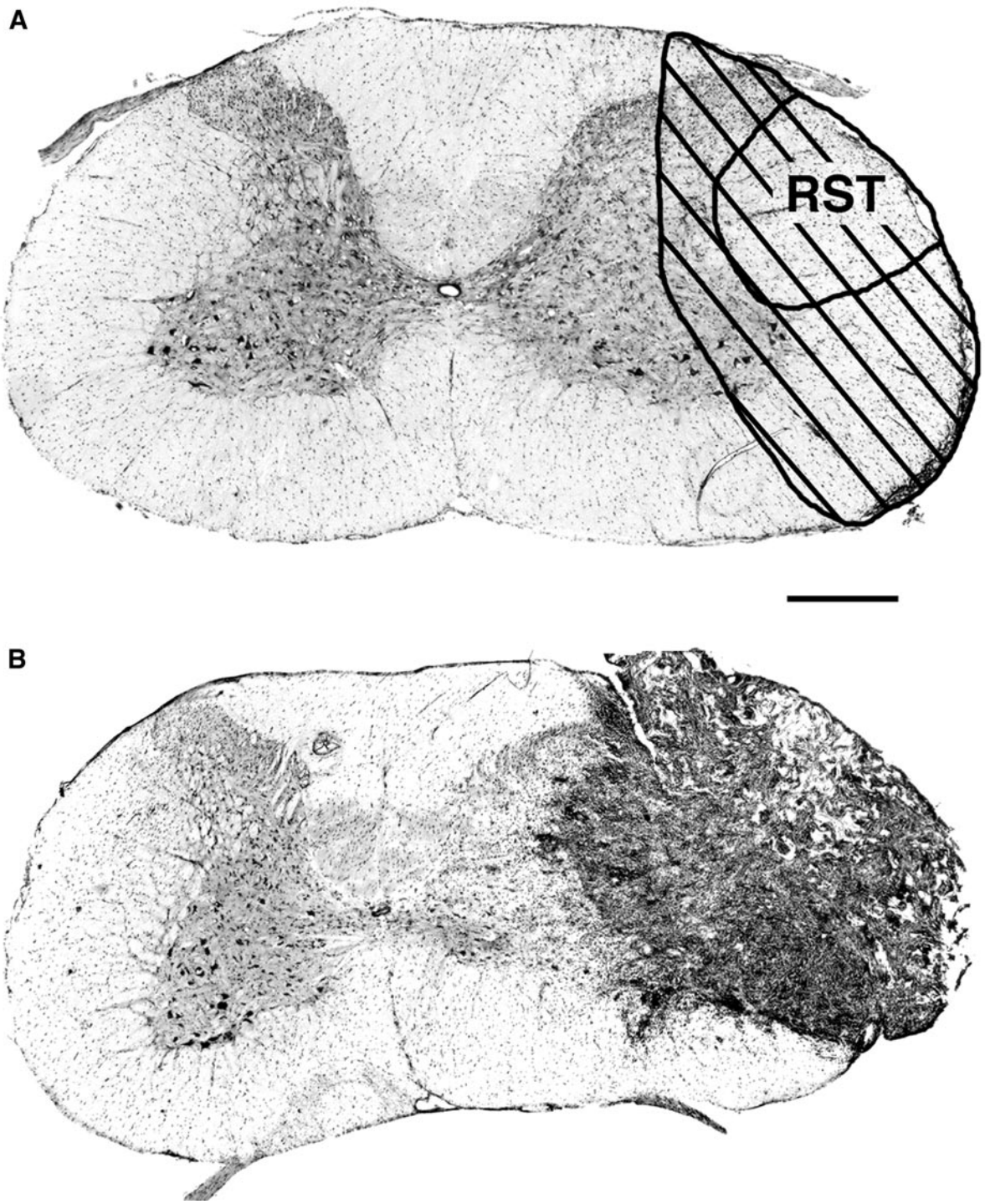

FIG. 1. Photomicrographs showing cross sections of cervical spinal cord from a normal rat (A) and an animal that received a Fb/BDNF transplant (B). The lesion completely disrupted the right lateral funiculus, partially lesioned the ipsilateral gray matter and ventral white matter, but left the dorsal columns intact. The fibroblast graft completely filled the lesion cavity and was closely apposed to the host tissue. Two month survival. Cresyl violet stain. Scale bar: 500μm. In A, the dashed line indicates the location of the normal rubrospinal tract (labeled as RST) and stippling indicates the extent of the lesion.

tained roughly equal numbers of neurons. There were more than 1200 neurons on each side and the left/right ratio was approximately 1.0 (Table 1, Figs. 2A, 2B, and 3). Similar results have been reported previously (47). One and 2 months after spinal cord hemisection, cell counts in animals receiving gelfoam implants alone

TABLE 1

RN Neuron Number Comparison

Group	Total neuron number		
	Left	Right	Ratio (L/R)
Normal	1245.505 ± 146.642	1267.846 ± 51.205	0.996 ± 0.031
Fb/BDNF	1091.557 ± 45.473	1273.159 ± 79.669	0.851 ± 0.016*
Fb	569.633 ± 31.529	1133.282 ± 132.523	0.535 ± 0.062*
Hx	633.134 ± 23.883	1144.198 ± 74.573	0.566 ± 0.052*

Abbreviations: Fb/BDNF, hemisection with BDNF-producing fibroblast transplants; Fb, hemisection with unmodified fibroblast transplants; Hx, hemisection with gelfoam implants. Two month survival. $n = 6$ for each group. Values are expressed as mean RN neuron number ± standard error.

* Significant difference from Normal (control), as determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P < 0.05$). Significant differences among groups: Normal > Fb/BDNF > Fb, Hx.

showed about 600 neurons remaining in the injured RN. This number represented approximately 55% of the neurons on the intact side, indicating a 45% cell loss in the injured RN (Table 1, Figs. 2G, 2H, and 3). Previous studies utilizing the modified Hendry method, rather than the stereological counting method employed in this study, showed a similar loss of 35–40% in the injured RN (18, 47). Counts of RN neurons, retrogradely labeled with true blue, provided comparable results (70). In animals receiving Fb/BDNF transplants, more than 1000 RN neurons remained on the injured side at 1 and 2 months, representing about 85% of the cell number found on the normal side. Fb/BDNF transplants therefore reduced RN neuron loss from 45 to 15% and provided a 60–70% rescue of RN neurons that would otherwise have been lost and presumably died (Table 1, Figs. 2C, 2D, and 3). In contrast, less than 600 neurons remained in the injured RN in animals receiving transplants of unmodified fibroblasts, representing about 45% cell loss (or severe atrophy to a size below the level of resolution) that was not significantly different from that of the gelfoam implants. Thus, unmodified fibroblasts offered no protection against axotomy-induced neuron loss in the RN (Table 1, Figs. 2E, 2F, and 3).

Effects of Transplants on RN Neuron Atrophy

The cross-sectional areas of RN neurons in sham-operated, lesion, and transplant animals were measured and compared to determine whether transplants prevented atrophy following axotomy. In sham-operated animals, the left and right RN contained equal numbers of small (100–300 μm^2), medium (300–500 μm^2) and large (>500 μm^2) neurons (Fig. 5A). Less than 5% of RN neurons were giant cells (>1000 μm^2) and no neurons smaller than 100 μm^2 could be recognized. The mean soma size was about 430 μm^2, with a left/right ratio of 1.0 (Table 2, Figs. 2 and 4). In animals receiving gelfoam implants that survived 1 or 2 months the mean soma area was reduced to about 220 μm^2 and the left/right ratio was reduced to 0.58, indicating more than a 40% decrease in mean cell size of the surviving RN neurons (Table 2, Figs. 2G and 4). The size distribution of the neurons was dramatically shifted toward the small size (Fig. 5D), with most of the neurons (nearly 80%) in the small size range. Neurons smaller than 100 μm^2, which were not present in the normal RN, composed about 10% of the total surviving RN population. Giant neurons were absent in the injured RN, and the number of medium- to large-sized neurons was also significantly reduced (Fig. 5D). In animals receiving Fb transplants, the soma size of neurons in the injured RN was reduced to about 55% of the intact side, representing 45% cell atrophy. The soma distribution was also dramatically shifted toward a small size, with 10% very small cells and no giant cells (Table 2, Figs. 2, 4, and 5). This cell-size distribution was very similar to that of the gelfoam implants and indicated that Fb transplants offered no protection against axotomy-induced neuron atrophy. In contrast, in recipients of Fb/BDNF transplants, the mean soma area was about 330 μm^2 and the left/right ratio was about 0.81, indicating a less than 20% decrease in mean soma size. Fb/BDNF transplants prevented more than 55% of the atrophy in surviving RN neurons (Table 2, Figs. 2 and 4). Their effect on reducing neuron atrophy was also evident in the soma size distribution (Fig. 5). The general distribution was restored almost to normal except for minor changes that included the lack of giant neurons, more cells in the small size category, and a minor fraction of very small cells (Fig. 5B).

Effects of Transplants on Retrogradely Labeled RN Neurons

We have reported the effects of gelfoam, Fb, and Fb/BDNF transplants on RN neuron regeneration following axotomy (40). FG was injected bilaterally into spinal cord 3–4 segments (1–1.5 cm) below the transplants 3 days before the animals were euthanized, and the number of retrogradely labeled RN neurons was

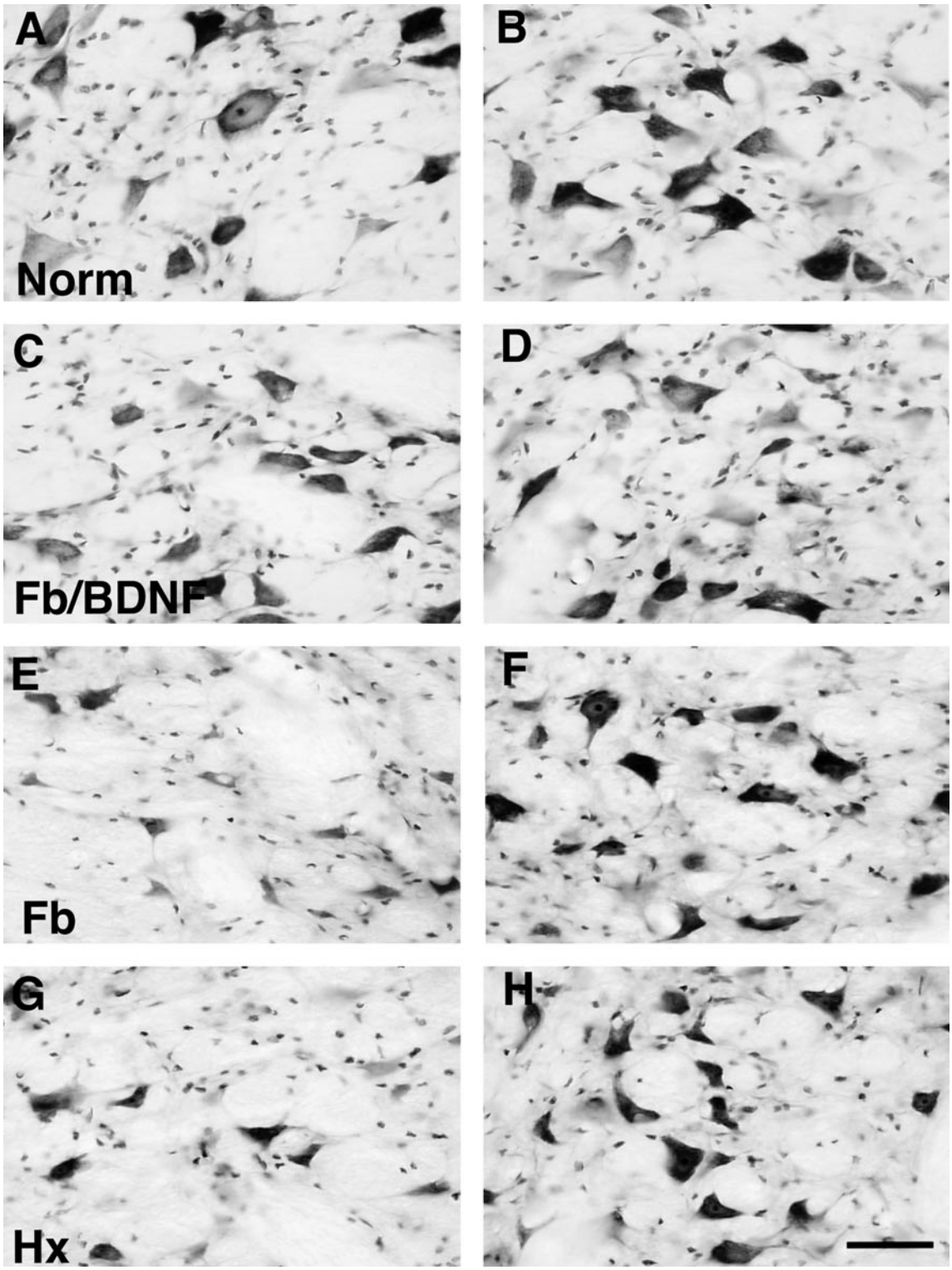

FIG. 2. Photomicrographs showing representative sections through the magnocellular portion of RN. (A, B) Normal. (C, D) Hemisection with Fb/BDNF transplant. (E, F) Hemisection with Fb transplant. (G, H) Hemisection with gelfoam implant. Panels A, C, E, and G are from the left or injured side. Panels B, D, F, and H are from the right or control side. Two month survival. Cresyl violet stain. Scale bar: 50 μm.

counted and compared across all treatment groups to evaluate RN axon regeneration. Animals in which FG had diffused into the grafts were eliminated from the study to avoid misinterpretation of the regeneration data. As reported previously about 7% of RN neurons were retrogradely labeled in Fb/BDNF recipients (40).

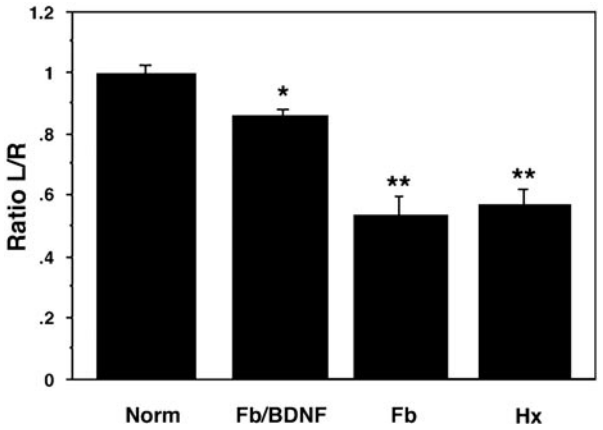

FIG. 3. Bar graph showing RN neuron survival as represented by the ratio of neuron numbers between the lesion side (L) and the control side (R). Each bar equals the mean ± SE of each group (n = 6). More than 40% RN neuron loss occurred in animals receiving Fb or gelfoam implants, whereas animals receiving Fb/BDNF transplants showed only 15% cell loss. Fb/BDNF transplants rescued significantly more RN neurons than Fb or gelfoam transplants, even though the rescue was not complete. *<Norm; **< Fb/BDNF < Norm.

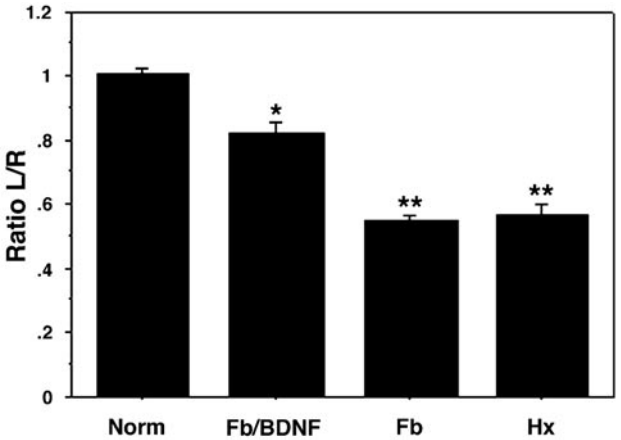

FIG. 4. Bar graph showing RN neuron size as represented by the ratio between the lesion side (L) and the control side (R). Each bar equals the mean ± SE of each group (n = 6). More than 40% RN neuron atrophy occurred in animals receiving Fb or gelfoam implants, whereas animals receiving Fb/BDNF transplants showed <20% cell atrophy. Fb/BDNF transplants significantly reduced the atrophy in RN neurons. *<Norm; **< Fb/BDNF < Norm.

In contrast less than 1% of RN neurons were retrogradely labeled in Fb or gelfoam recipients. About 1% of the rubrospinal neurons project axons ipsilaterally into spinal cord and these will not be axotomized by a spinal cord hemisection (9). Thus all contralaterally projecting rubrospinal axons were cut by the subtotal hemisection.

We measured the cross-sectional area of FG-labeled RN neurons in animals receiving gelfoam, Fb, or Fb/BDNF transplants and compared it to normal animals that had received only FG injections (Table 3, Fig. 6). The mean soma-size measurement of FG-labeled cells in intact RN of operated and normal animals did not differ significantly from that of FG-labeled cells in the injured RN of operated animals (Table 3). The ratio of sizes of FG-labeled neurons between injured RN and intact RN approximated 1.0 in all animal groups studied (Table 3, Fig. 6), and soma-size distribution showed a similar distribution pattern in each group (Fig. 7). These results indicated that all RN neurons that had been retrogradely labeled by FG maintained their soma size throughout the survival time, regardless of the treatment group to which they belonged. The 1% of FG-labeled RN neurons in Fb or gelfoam recipients therefore very likely did not atrophy because they escaped axotomy (9). In animals receiving Fb/BDNF transplants, however, about 7% of RN neurons that had been axotomized by the spinal cord hemisection presumably regenerated their severed axons. That these 7% of RN neurons also maintained their soma size indicates a strong relationship between the lack of atrophy and the ability to regenerate. The maintenance of normal soma size in the population of neurons

TABLE 2

Nissl-Stained RN Neuron Size Comparison

Group	Mean cell size (μm^2)		
	Left	Right	Ratio (L/R)
Normal	433.503 ± 19.531	434.920 ± 26.143	1.000 ± 0.017
Fb/BDNF	332.684 ± 18.583	407.793 ± 11.480	0.815 ± 0.038*
Fb	219.129 ± 4.389	401.174 ± 15.054	0.549 ± 0.018*
Hx	226.144 ± 5.670	413.121 ± 18.704	0.587 ± 0.031*

Abbreviations: Fb/BDNF, hemisection with BDNF-producing fibroblast transplants; Fb, hemisection with unmodified fibroblast transplants; Hx hemisection with gelfoam implants. Two month survival. n = 6 for each group. Values are expressed as mean cross-sectional areas of RN neurons ± standard error.

* Significant difference from Normal (control), as determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P < 0.05$). Significant differences among groups: Normal > Fb/BDNF > Fb, Hx.

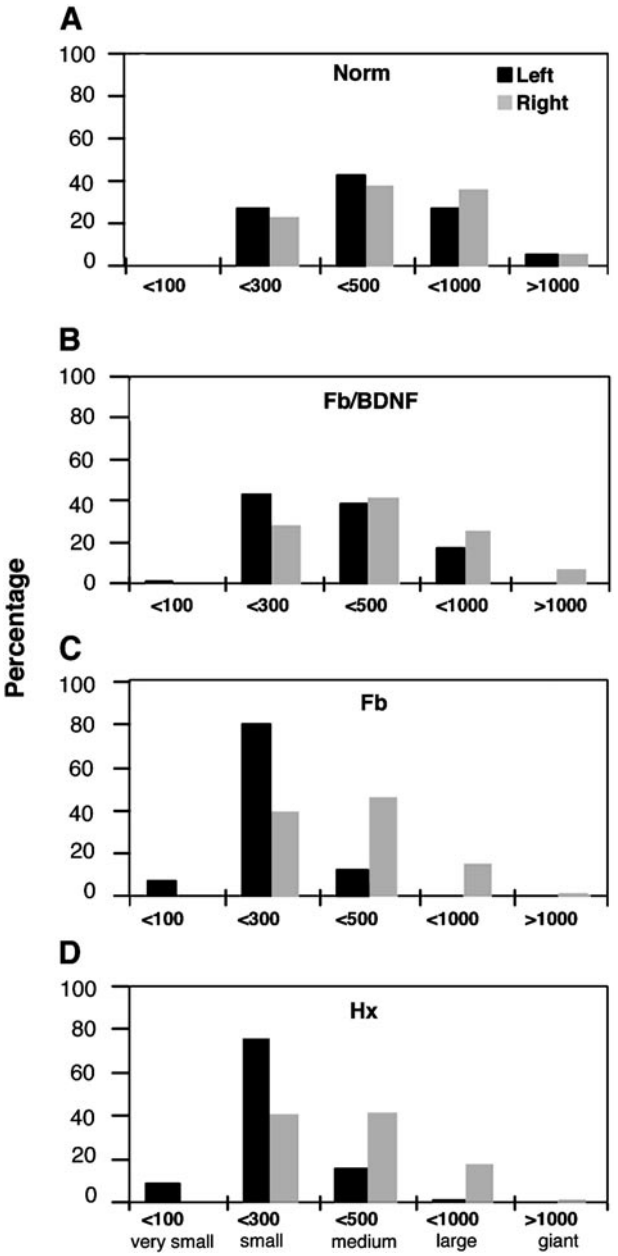

FIG. 5. Bar graphs showing RN neuron soma-size distribution in each group (n = 6). (A) Normal. (B) Hemisection with Fb/BDNF transplants. (C) Hemisection with Fb transplants. (D) Hemisection with gelfoam implants. In normal animals, RN neurons are relatively evenly distributed into small (100–300 μm^2), medium (300–500 μm^2), and large (500–1000 μm^2) ranges. A small percentage (<5%) of giant cells (>1000 μm^2) is also present in normal RN. Neurons smaller than (100 μm^2) are absent from normal RN (A). After cervical hemisection, in animals receiving Fb or gelfoam, the cell distribution is markedly skewed toward the small size range and includes very small neurons (<100 μm^2) but no giant neurons (C, D). In contrast, Fb/BDNF transplants maintain the soma distribution close to normal, despite the absence of giant cells and presence of very small neurons.

that regenerate contributes to but does not account for the overall effect of BDNF on prevention of atrophy of axotomized RN neurons.

DISCUSSION

The present report employed an *ex vivo* gene therapy paradigm to study RN neuron survival under conditions in which RST regeneration is promoted. The results demonstrated that cervical axotomy caused about 45% loss of rubrospinal neurons, by either retrograde cell death or severe shrinkage. Significant cell atrophy occurred among the surviving RN neurons, which on average decreased their mean soma size by more than 40%. BDNF-producing fibroblasts transplanted into the lesion cavity prevented 60–70% of the retrograde neuron loss and more than 55% of the neuron atrophy. Furthermore the Fb/BDNF transplants, which promoted axon regeneration in about 7% RN neurons (40), also completely prevented the atrophy of these neurons. In contrast, grafts of unmodified fibroblasts failed to prevent RN neuron loss and atrophy; they also failed to promote RST axon regeneration.

Transplants of BDNF-Producing Fibroblasts Partially Prevented RN Neuron Loss and Atrophy Following Cervical Axotomy

The responses of RN neurons to therapeutic interventions are well documented. For instance, fetal spinal cord transplants completely prevented RN neuron loss after a thoracic lesion in newborn rats (8). Fetal spinal cord transplants also partially prevented RN neuron loss following a cervical hemisection in adult rats, even though they failed to protect the surviving RN neurons against atrophy (47). The proposed mechanism by which fetal tissue transplants rescue axotomized RN neurons is that they act as a surrogate source of neurotrophins, especially BDNF (47, 59). Virtually all RN neurons express full-length TrkB (32), the specific receptor for BDNF. Several studies have shown that BDNF applied either at the spinal cord injury site or near RN neuron cell bodies offered protection against death and atrophy (10, 32). RN neurons respond to BDNF by up-regulation of two regeneration-associated genes, GAP-43 and Tα-1 tubulin, suggesting a regenerative response (32, 61). Based on these studies, we designed an *ex vivo* gene therapy strategy to study the effects of BDNF-producing fibroblasts on RN neuron survival and regeneration following cervical axotomy. Differences in findings of the present study and those of several previous reports revealed some of the strengths and weaknesses of this therapeutic strategy.

We observed that Fb/BDNF transplants rescued most (60–70%) RN neurons that would presumably have died, prevented much (55%) of the soma atrophy among the surviving cells, and promoted axon regeneration by 7% of RN neurons. In contrast, the report by Mori *et al.* (47) found that transplants of fetal spinal cord resulted in a 50% rescue from cell death but no

TABLE 3

FG-labeled RN Neuron Size Comparison

Group	Number of cells measured	Mean cell size on lesioned (left) side (μm^2)	Ratio L/R
Normal (n = 3)	2437	409.617 ± 18.539	1.000 ± 0.045
Fb/BDNF (n = 6)	1320	437.377 ± 21.403	1.068 ± 0.052
Fb (n = 6)	154	428.150 ± 27.498	1.045 ± 0.045
Hx (n = 5)	99	424.150 ± 22.272	1.035 ± 0.054

Abbreviations: Fb/BDNF hemisection with BDNF-producing fibroblast transplants; Fb, hemisection with unmodified fibroblast transplants; Hx, hemisection with gelfoam implants. All surgical groups survived for 2 months. Cell-size values are expressed as mean cross-sectional area of FG-labeled RN neurons ± standard error. The cell-size ratio was obtained by dividing the mean cross-sectional area of FG-labeled RN neurons on the left side of operated animals by the mean cross-sectional area of FG-labeled RN neurons pooled from the right side of all animals in an experimental group. There were no significant differences in cell size among groups.

prevention of atrophy and no RST axon regeneration. The differences between the two types of transplants support the suggestion that smaller quantities of neurotrophic factors provided by fetal tissue are sufficient for neuron survival but inadequate to maintain perikaryal size and to promote regeneration (47). We found that Fb/BDNF transplants expressed high levels of the transgene at 1 week and continued expression at reduced levels for at least 2 months. This is consistent with the idea that the engineered cells sustained the survival of axotomized RN neurons, prevented their atrophy, and promoted axon regeneration by continuously secreting bioactive BDNF for a prolonged period of time. While genetically engineered cells often downregulate their expression of the transgene and thus delivery of the active molecule may diminish over time, it appears that in this case the supply of BDNF was adequate to rescue axotomized neurons and initiate regeneration. Grafting cells genetically modified to express a therapeutic gene therefore has an advantage over fetal tissue transplantation because the engineered cells can provide the needed factors to the injured neurons in a more abundant, homogeneous, and sustained fashion.

Delivery of neurotrophic factors by transplanting genetically modified cells into the lesion site has several potential benefits for clinical application. For example, administration at the lesion site can provide neurotrophic factors that can be delivered by retrograde transport to the perikarya of injured and uninjured neurons whose axons possess the appropriate receptors. Focused delivery may induce both local sprouting by uninjured axons and regenerative growth by injured axons. In contrast, delivery to perikarya of functionally important neurons requires cannulation of multiple sites in the brain and spinal cord. In contrast to the local release of specific factors by indwelling catheters

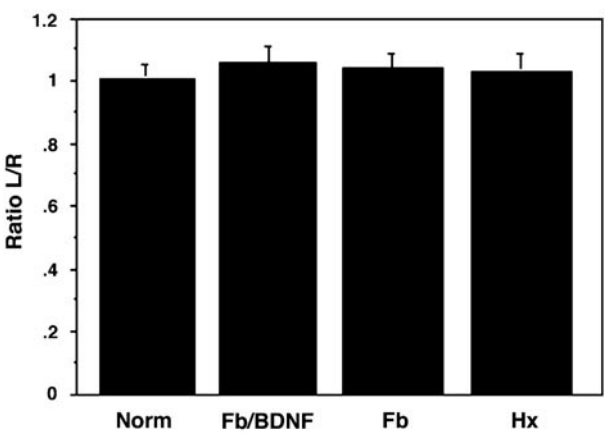

FIG. 6. Bar graph showing FG-labeled RN neuron size as represented by the ratio between the lesion (L) and the control RN (R). Each bar equals the mean ± SE of each group (n = 6). Soma-size measurement of FG-labeled RN neurons in Fb/BDNF, Fb, or gelfoam recipients does not differ from that of normal animals.

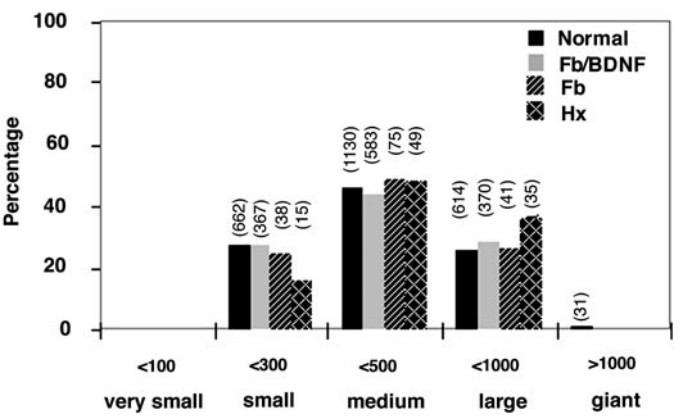

FIG. 7. Bar graphs showing FG-labeled neuron soma-size distribution in normal and operated RN for each group (n = 6). In normal animals, RN neurons are relatively evenly distributed into small (100–300 μm^2), medium (300–500 μm^2), and large (500–1000 μm^2) ranges. A small percentage (<1–2%) of giant cells (>1000 μm^2) is present, whereas very small neurons (<100 μm^2) are absent from normal RN. Following cervical axotomy and grafting of Fb/BDNF, Fb or gelfoam alone, a small percentage of RN neurons that were retrogradely labeled by FG maintain nearly normal soma distribution regardless of treatment. However, giant neurons are absent in the operated animals. Numbers above each bar represent the actual number of neurons measured in each group.

or implants such as gelfoam, genetically modified cells can provide growth factors in addition to those that they have been modified to express, as well as substrate molecules and a matrix that could minimize cyst and scar formation and provide a terrain for axon regrowth. Exogenously administered neurotrophic factors can prevent atrophy and rescue axotomized neurons (31, 34, 58), even if delivered only during the first few weeks following injury (58). Long-term delivery may therefore not be necessary for neuron survival and may even be detrimental to regeneration if an unfavorable concentration gradient of neurotrophic factors is established that keeps axons from leaving the transplant and entering host spinal cord.

Fb/BDNF transplants did not rescue all axotomized cells or prevent all soma atrophy. This is in contrast to findings of several studies using other approaches. For instance, fetal tissue transplants completely prevented RN cell death in newborn rats subjected to a midthoracic hemisection (8). The cervical lesion axotomizes more RST axons than the thoracic lesion and RN neuron survival may have more stringent requirements in adults than in neonates. Nevertheless BDNF, which is the only therapeutic factor produced by the Fb/BDNF transplants but is not by the unmodified fibroblast transplants, may be insufficient to prevent all RN neuron death. Several studies suggest that NT-3 is also a survival factor for RN neurons (10, 32). It has been reported that neither fetal transplants nor administration of BDNF or NT-3 completely prevented RN neuron atrophy following a midthoracic hemisection in adult rats, but a combination of transplants with either factor offered complete maintenance of neuron size (6). Multiple factors may therefore be necessary for maintaining perikaryal size of RN neurons. Furthermore, the infusion of BDNF or NT-4/5 directly into the vicinity of RN neuron cell bodies completely prevented atrophy induced by a cervical hemisection similar to that used in the present study (32). Therefore it is possible that even though all RN neurons express full-length trkB on their cell bodies (32), only a portion of them have the receptor on their axons. Alternatively axon receptors may have less access to BDNF because of the presence of truncated trkB receptors on reactive astrocytes at the lesion site (16, 17, 36). In addition, RN neurons may be a more heterogeneous population than some other CNS structures, such as Clarke's nucleus (CN), in their requirement for neurotrophic factors for survival and their expression of neurotrophin receptors. For example, fetal CNS tissues expressing high levels of NT-3 mRNA and cells genetically modified to produce NT-3 completely prevented axotomy-induced CN neuron death but did not prevent atrophy (22, 23, 59). However BDNF alone afforded no rescue of axotomized CN neurons and did not improve rescue when used in combination with NT-3 (58), indicating that CN neurons respond to NT-3 but not BDNF treatment. In contrast, BDNF in combination with NT-3 delivered at the appropriate time to the RN following injury provided even better protection than BDNF alone (49). Axotomized RN neurons may therefore respond even better to transplants of cells genetically modified to secrete multiple neurotrophic factors.

In our experimental control groups that received grafts of gelfoam alone or unmodified fibroblasts there was significant cell loss as well as atrophy of many surviving neurons. It is, of course, difficult to distinguish between a neuronal population that has undergone retrograde cell death and one that has atrophied below the level of microscopic resolution. Some of the apparent loss of axotomized RN neurons has been shown to represent extreme atrophy of these cells (44). In addition, Kwon *et al.* reported that all RN neurons survive cervical axotomy and can be stimulated by the application of BDNF to their perikarya to regenerate axons into a peripheral nerve graft even 1 year later (34). This study has encouraging clinical implications for recovery after chronic spinal cord injury but differs in several fundamental respects from other related investigations. For example, Kwon *et al.* do not detect a significant loss of axotomized RN neurons following vehicle treatment, whereas numerous previous studies have found at least a 30–35% loss (18, 26, 47, 49), suggesting that their counting method, based on immunocytochemistry rather than morphology and the presence of a nucleus, may overestimate neuronal survival. In the absence of a report of total cell numbers, rather than ratios, this possibility cannot be evaluated. Kwon *et al.* may also have based their cell counts on an atypical subset of RN neurons. The average cross-sectional area of the normal RN neurons which they counted was ~277 μm^2. Previous studies have consistently shown the mean cell area of neurons in the caudal portion of rat RN to be ~470 μm^2 (26, 32, 47, 49). The report by Kwon *et al.*, however, is consistent with the present findings that RN neurons that survive axotomy can be rescued from atrophy and encouraged to regenerate by the exogenous application of BDNF. We believe these results suggest that there are two populations of axotomized RN neurons, those that die and those that atrophy but survive. This second surviving population could retain the ability to respond to delayed administration of BDNF by an increase in soma size. Such an effect has also been described for axotomized adult dorsal root ganglia neurons (64). It would be interesting to determine whether the subsets of RN neurons that survive and those that disappear can be distinguished on the basis of their expression of calcium-binding proteins such as calbindin, perhaps in conjunction with their expression of trk B and trk C receptors.

Transplants of BDNF-Producing Fibroblasts Stimulated Some RN Neurons to Regenerate and Completely Prevented Soma Atrophy in These Cells

We reported previously that Fb/BDNF transplants promoted axon regeneration in 7% of RN neurons following cervical axotomy (40). This is a 7- to 20-fold increase in the number of retrogradely labeled neurons over that found with unmodified fibroblast grafts or with gelfoam alone. It may be argued that FB/BDNF did not induce regeneration but rather prevented cut RST axons from retracting from the lesion site, where they were retrogradely labeled by FG that diffused from the injection site several segments caudal. A second argument against regeneration is that the incomplete cervical hemisection spared RST axons in the anterolateral white matter. Several lines of evidence support our conclusion that the labeled neurons have regenerated their axons. Our lesions were complete, as demonstrated by our findings that 1% or less of the RN neurons are retrogradely labeled by FG in any of the 24 rats that received gelfoam alone or unmodified fibroblasts grafts. These labeled neurons are likely to represent the 1% of RN neurons whose projections are uncrossed (9, 63). The possibility of spurious labeling by diffusion was eliminated by applying FG at least 1–1.5 cm away from the lesion site and by removing from analysis those few animals in which we found evidence of FG diffusion into the injury/transplant site. Other investigators have reported that FG diffused less than 3 mm rostrally from the labeling site (62). In our previous study of fetal spinal cord transplants (47) we found only an occasional FG-labeled neuron in the transplant and no neurons rostral to the transplant retrogradely labeled by FG even though the FG was injected closer to the lesion using the same techniques as in the present study. Other investigators also found virtually no FG-labeled neurons in RN after a severe injury even when FG was applied in larger quantities and closer to the injury (48). Finally, anterograde labeling with BDA demonstrated regeneration in rats receiving Fb/BDNF, but in none of the 24 rats that received gelfoam alone or unmodified fibroblasts (40). These findings demonstrate that our lesion completely interrupts the ipsilateral RST and that Fb/BDNF transplants are responsible for the greater numbers of FG-labeled RN neurons seen in these experimental animals compared to animals that received grafts of unmodified fibroblasts.

In the present study, we examined the status of the regenerated RN neurons by comparing the soma size of FG-labeled neurons to that of intact (uninjured) RN neurons. We found no atrophy among these cells. Several mechanisms may account for this finding. First, these cells may represent a population of RN neurons that responds to BDNF treatment by metabolic changes that enable them to maintain their soma size and regenerate axons at least several segments caudal to the transplant. Other studies have demonstrated that exogenous BDNF can promote axon regeneration in a population of RN neurons (32, 43, 68, 70). The relationship between maintenance of soma size and axon regeneration was also suggested by studies using a combination of fetal tissue transplants and administration of neurotrophins (6, 7). Based on the results from fetal tissue transplants, Mori and colleagues proposed that larger quantities of neurotrophic factors are necessary to maintain the soma size of injured neurons than that required to rescue them (47). The ability of neurotrophic factors to promote axon regeneration may be more restricted than their ability to prevent cell death and atrophy, since we find that Fb/BDNF transplants rescue 60–70% of RN neurons and prevent 55% of cell atrophy among the survivors, but promote axon regeneration over several segments in only 7% of RN neurons. These cells may maintain soma size because they were able to reinnervate their original or new targets and thereby obtained trophic support from the targets, which may be more comprehensive and appropriate than the support from the Fb/BDNF transplants. Such innervation may provide activity-induced survival. It is also possible that the regenerating RN neurons represent a population of cells that do not die or atrophy after axotomy, perhaps because of sustaining collaterals to other targets. These cells may have a strong "intrinsic growth state" giving them a higher potential for successful axon regeneration (14). It is difficult to be certain whether these mechanisms are responsible together or alone for the normal soma size of regenerated RN neurons.

CONCLUSIONS

We have demonstrated that transplants of primary fibroblasts genetically modified by a retroviral vector encoding the human BDNF cDNA partially prevented axotomy-induced neuron loss and/or severe shrinkage in rubrospinal neurons following a subtotal cervical spinal cord hemisection. Enhanced survival of RN neurons that did not regenerate axons caudal to the transplants may also contribute to functional recovery by interacting with local segmental circuits. We found considerable RST axon sprouting rostral to a transplant in animals receiving Fb/BDNF but not Fb or gelfoam transplants (40). In contrast, unmodified fibroblasts or gelfoam alone failed to offer protection against neuron loss and atrophy or to promote axon regeneration and recovery of function. *Ex vivo* gene therapy may therefore prove to be an effective treatment of human SCI by both promoting axon regeneration and rescuing neurons from axotomy-induced cell death.

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