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GDNF-Enhanced Axonal Regeneration and Myelination Following Spinal Cord Injury is Mediated by Primary Effects on Neurons

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Abstract

We previously demonstrated that coadministration of glial cell line-derived neurotrophic factor (GDNF) with grafts of Schwann cells (SCs) enhanced axonal regeneration and remyelination following spinal cord injury (SCI). However, the cellular target through which GDNF mediates such actions was unclear. Here, we report that GDNF enhanced both the number and caliber of regenerated axons *in vivo* and increased neurite outgrowth of dorsal root ganglion neurons (DRGN) *in vitro*, suggesting that GDNF has a direct effect on neurons. In SC-DRGN coculture, GDNF significantly increased the number of myelin sheaths produced by SCs. GDNF treatment had no effect on the proliferation of isolated SCs but enhanced the proliferation of SCs already in contact with axons. GDNF increased the expression of the 140 kDa neural cell adhesion molecule (NCAM) in isolated SCs but not their expression of the adhesion molecule L1 or the secretion of the neurotrophins NGF, NT3, or BDNF. Overall, these results support the hypothesis that GDNF-enhanced axonal regeneration and SC myelination is mediated mainly through a direct effect of GDNF on neurons. They also suggest that the combination of GDNF administration and SC transplantation may represent an effective strategy to promote axonal regeneration and myelin formation after injury in the spinal cord.

Keywords

GDNF; axon; myelination; regeneration; Schwann cell; spinal cord injury

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INTRODUCTION

GDNF, a member of the transforming growth factor β (TGF-β) superfamily, was originally identified as a trophic factor for the survival and morphological differentiation of dopaminergic nigrostriatal neurons (Lin et al., 1993). GDNF belongs to a family of growth factors that bind glycosyl-phosphatidylinositol-linked family receptor (GFR) α1–4 (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Trupp et al., 1998). GFRα1–4 preferentially binds GDNF, neuturin, artemin, and persephin, respectively, whereas the transmembrane protooncogene, c-Ret, mediates the transmembrane signaling by these factors (Baloh et al., 1997; Eketjall et al., 1999; Jing et al., 1996). Recently, GDNF has been shown to signal in a Ret-independent manner via a Src family tyrosine kinase Fyn (Paratcha et al., 2003). Since GFRαs do not have an intracellular domain, coupling of GFRα1 with Src family kinases would require a coreceptor, recently identified as the 140 kDa isoform of neural cell adhesion molecule (NCAM) (Iwase et al., 2005; Paratcha et al., 2003).

The Schwann cell (SC) is one of the most widely studied cell types for repair of the injured central (CNS) and peripheral nervous system (PNS). In the PNS, SCs de-differentiate, proliferate, migrate, express growth promoting factors, and form myelin on regenerating axons after injury (Mirsky and Jessen, 1999; Oudega and Xu, 2006; Weinstein, 1999). One critical feature of SCs is their ability to produce an array of trophic factors (Acheson et al., 1991; Heumann et al., 1987; Neuberger and De Vries, 1993) such as GDNF (Springer et al., 1994) that support the growth of regenerating axons after acute (Chen et al., 2001; Munson and McMahon, 1997) or delayed peripheral nerve injuries (Boyd and Gordon, 2003). In fact, both GDNF and GFRα1 transcripts are up-regulated in distal segments of lesioned sciatic nerves, suggesting a growth-promoting role of GNDF following injury (Trupp et al., 1997). However, SCs express only one of the two coreceptors, i.e., GFRα1 but not Ret, that are required for GDNF signaling (Paratcha et al., 2003; Trupp et al., 1999). Previously, we demonstrated that transplantation of SC-seeded guidance channels into completely transected or hemisected spinal cords promoted substantial axonal regeneration in the CNS following injury (Xu et al., 1997; Xu et al., 1995b; Xu et al., 1999). To further increase their growth supportive ability, we coadministered GDNF with SCs and found that GDNF promoted marked axonal regeneration and remyelination in this model (Iannotti et al., 2003). Among the regenerated axons, many of them originated from dorsal root ganglion neurons (DRGN). However, the cellular target through which GDNF enhances SC-mediated axonal regeneration and remyelination remains unclear. In this study, we sought to determine whether GDNF-mediated axonal regeneration and remyelination was a direct effect of GDNF on neurons or an indirect effect on grafted SCs. To delineate these possibilities, we employed both *in vivo* SC transplantation and *in vitro* SC and DRGN single or coculture models. We found that GDNF primarily act on neurons and secondarily on SCs to effect axonal regeneration and myelin formation.

MATERIALS AND METHODS

Schwann Cell Culture

The procedures for harvesting purified populations of rat Schwann cells (SCs) were described in previous publications (Morrissey et al., 1991; Xu et al., 1995b; Xu et al., 1999). Briefly, SCs were harvested from the sciatic nerves of adult female Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN) under aseptic conditions and purified and expanded in culture. The purity of the SCs was ascertained by previously described methods (Xu et al., 1997; Xu et al., 1995b). Purified SCs (purity >98%) at the third or forth passage were collected for either *in vitro* experiments or seeding into miniguidance channels for transplantation.

DRG-Dissociated Neuronal Culture, Explant Culture, and Neuron/Schwann Cell Coculture

Dissociated dorsal root ganglion (DRG) cultures were established from DRGs obtained from the embryonic (E) day 15 SD rats. DRG neurons (DRGN) were dissociated and seeded onto Aclar coverslips coated with rat tail tendon collagen (4 mg/mL) as described previously (Kleitman et al., 1998; Plant et al., 2002). The cultures were maintained in Neurobasal medium with B27 Supplement (Invitrogen, Grand Island, NY, hereafter designated as NB medium) with added nerve growth factor (NGF, 100 ng/mL, Roche, Indianapolis, IN). Nonneuronal cells were eliminated by applying two cycles (once a week) of fluorodeoxyuridine (10 μM) and uridine (10 μM, both from Sigma, St Louis, MO). Three weeks after the initial seeding of dissociated DRG cells, ~5×10^4 purified SCs were seeded onto these purified DRGN cultures that contains elongated, meshwork-like neurites. Two weeks later, the cultures were divided into serum-free and serum-containing (10% fetal bovine serum, FBS, Invitrogen) groups. The serum-free group ($n = 2$) was further receiving either (1) NB medium only (control), (2) NB medium plus NGF (100 ng/mL, Roche), or (3) NB medium plus recombinant human GDNF (rhGDNF, 10 ng/mL, Amgen, Thousand Oaks, CA) ($n = 4$/subgroup). Myelination was initiated by the addition of ascorbic acid (50 μg/mL, Sigma) which lasted for 2 weeks. The serum-containing group ($n = 32$) was receiving either (1) NB medium plus ascorbic acid or (2) NB medium plus ascorbic acid plus rhGDNF (10 ng/mL) ($n = 16$/sub-group). Cultures were fixed at 4, 8, 12, and 16 days after the addition of ascorbic acid for immunostaining of myelin basic protein (MBP) to investigate the myelin sheath formation ($n = 4$/time point). After immunofluorescence staining, photomicrographs were taken from five randomly selected areas in each culture. The area occupied by MBP-labeled myelin segments was calculated from these micrographs using ImageJ software (NIH). The ratio of the MBP-labeled area to that of entire captured image was defined as the MBP area ratio.

For DRG explant cultures, DRGs from neonatal (postnatal day 1) rats were dissected and digested with 0.25% Trypsin/EDTA (Invitrogen) for 5 min, then placed on Aclar coverslips coated with rat tail collagen, and maintained in NB medium or in NB medium plus rhGDNF (10 ng/mL) ($n = 4$/group) for 3 days *in vitro* before being fixed and immunostained, as described below. After immunostaining, axonal length from the edge of DRG explants to the tip of axons and the area occupied by axons in both groups were measured using a Neurolucida System (MicroBrightField, Williston, VT) and statistically compared.

Seeding Schwann Cells Into Mini-Guidance Channels

Semi-permeable 60:40 poly-acrylonitrile/poly-vinyl-chloride (PAN/PVC) copolymer guidance channels with an outer diameter of 1.25 mm (Provided by Dr. Xuejun Wen, Clemson University, Charleston, SC) were cleaned and sterilized according to the established methods (Bamber et al., 2001; Iannotti et al., 2003). SCs were suspended in a 60:40 (v:v) of DMEM and Matrigel (MG, Collaborative Research, Bedford, MA) at a final density of 120×10^6 cells/mL. In channels containing GDNF, the amount of medium was replaced with an equal volume of concentrated GDNF to achieve a final concentration of GDNF at 1 μg/μL. Channels seeded with SCs but no GDNF served as a control. After seeding, the channel was closed at both ends with PAN/PVC glue and kept in DMEM for 2–3 h at 37°C to allow polymerization of the MG.

Spinal Cord Hemisection and Transplantation of SC-Seeded Guidance Channels

Adult female SD rats (180–200 grams, Harlan) were randomly divided into two groups in which the grafted channel contained either a mixture of SC-vehicle (DMEM) or SC-GDNF ($n = 12$/group). In both groups, rats were sacrificed at 2, 4, and 6 weeks postimplantation ($n = 4$/subgroup). The procedures for spinal cord hemisection and mini-guidance channel implantation, as well as for preoperative and postoperative animal care, were described in

detail in previous publications (Bamber et al., 2001; Iannotti et al., 2003; Xu et al., 1999). Briefly, after the removal of a 2.8 mm piece of the hemi-cord at T10 on the right side, a mini-guidance channel of 3 mm long was implanted into the hemisection gap. The dura and wound were closed in layers. All animal handling, surgical procedures, and postoperative care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committees of University of Louisville.

Tissue Preparation for Light and Electron Microscopy

The preparation for light and electron microscopy were described previously (Xu et al., 1999). Briefly, at 2, 4, and 6 weeks after transplantation, rats were given an overdose of sodium pentobarbital and were transcardially perfused and fixed with 4% paraformaldehyde. A 1 mm transverse section was removed from the midpoint of the guidance channel and transferred to 2.5% glutaraldehyde and 5% sucrose in 0.1 M cacodylate buffer (pH 7.4) overnight followed by 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr. Tissues were then dehydrated and embedded with EM-812 (Electron Microscopy Sciences, Hatfield, PA). Transverse 1 μm-thick semi-thin plastic sections were stained in 1% toluidine blue and 1% sodium borate. The number of myelinated axons, blood vessels, and the mean tissue cable cross-sectional areas for each group were quantified as described previously (Xu et al., 1995b). For electron microscopy (EM), ultrathin sections (70–90 nm) were collected and subsequently counterstained with 4% uranyl acetate in 50% ethanol and Reynolds' lead citrate and examined using a Philip CM10 electron microscope. The number of myelinated axons, the ratio of myelinated to unmyelinated axon numbers, the axon diameter (measured to the inner border of myelin), and the *g*-ratio, defined as the ratio of the axon diameter to the total diameter of the myelinated fiber (Friede and Bischhausen, 1982), were quantified from randomly captured images (four fields per tissue cable) within the guidance channel and statistically compared between groups using one-way ANOVA followed by Tukey's post hoc test (Graphpad Prism 4.0, GraphPad Software, San Diego, CA).

DRGN-SC Cocultures for Sudan Black Staining and Electron Microscopy

To quantitate the amount of myelin in the DRGN-SC cultures, purified DRGN-SC cultures were prepared as described above and maintained in NB medium. Ten days after plating the SCs onto the purified DRGN networks, myelination was initiated by the addition of ascorbic and at the same time either vehicle or GDNF was added to the medium (n = 4/group). Cultures were fixed 8 days later and stained for Sudan black according to a previously reported method (Eldridge et al., 1987). Briefly, cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed with 0.1% osmium tetroxide for 1 h at room temperature, dehydrated to 70% ethanol, stained with 0.5% Sudan black (Sigma) in 70% ethanol for 1 h, rehydrated, and mounted in glycerin jelly. Myelinated axons were counted using a square grid eyepiece during three scans across the coverslip. The number of Sudan black-stained myelin sheaths crossing the scan line was counted. For ultrastructural identification of myelin, DRGN-SC cocultures were transferred to 2% glutaraldehyde and 3% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) for 30 min and followed by 2% osmium tetroxide in 0.1 M PB for 1 h. Cultures were then dehydrated in graded ethanol and embedded with EM-812. Areas of myelination were randomly selected, blocked, and mounted on a capsule. Ultrathin sections (70–90 nm) were made in an orientation that axons were cut transversely. All counterstaining and examination followed those described previously.

Schwann Cell Proliferation Assays

Effects of GDNF on the proliferation of SCs was investigated in two conditions: (1) purified SC culture and (2) a SC-DRGN coculture in which purified SCs were seeded onto a

meshwork of SC-free DRG axons (n = 8/ group). All cultures were fed with NB medium containing either GDNF (10 ng/mL, Amgen) or medium alone for 3 days (n = 4/subgroup). 5-Bromodeoxyuridine (BrdU, 10 mM, Sigma) was applied at 24 h prior to the fixation of cultures and the BrdU immunostaining followed that described below. To determine the SC's proliferation level, a BrdU labeling index was calculated as the number of BrdU labeled nuclei divided by the number of Hoechest labeled nuclei in each randomly selected area.

ELISA and Immunoblotting Analyses

To test the effect of GDNF on the production of other neurotrophic factors and cell adhesion molecules by SCs, purified SCs at passage 3 or 4 were fed with culture media (DMEM with 10% FBS, Invitrogen) containing GDNF (10 ng/mL) or vehicle for 3 days (n = 12/group). Then the culture supernatants were collected for measuring the production of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) by ELISA analysis (n = 4/subgroup). Meanwhile, cells isolated from the same culture were used to measure protein levels of cell adhesion molecules, particularly the 140 kDa neural cell adhesion molecule (NCAM) and L1 using Western blotting (n = 6/ subgroup). Neurotrophins in 100 μL SC-conditioned culture supernatants were measured using NGF-ELISA, BDNF-ELISA, and NT-3-ELISA kits (Promega, Madison, WI) following manufacture's protocol. For immunoblots, SC monolayers were solublized by the addition of 200 μL of 1% SDS in Tris-EDTA buffer with proteinase inhibitors (10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM PMSF) and sonicated. After centrifuging at 14,000 rpm, supernatant was assayed for protein concentration using a BCA kit (Pierce, Rockford, IL), diluted with 3X Laemmli's buffer, and 50 mg of protein loaded for each sample. After running the sample in 7.5% SDS-poly-acrylamide gel, proteins were transferred to nitrocellulose membranes. Membranes were blocked using Odyssey (LI-COR, Biosciences, Lincoln, NE) blocking buffer. Proteins of interest were identified using mouse antihuman L1 (monoclonal 74-5H7; gift from Dr. V. Lemmon, Case Western Reserve University) and mouse antirat NCAM (1:1000; Chemicon, Temecula, CA). After a 2 h incubation in primary antibody, the membranes were washed five times for 10 min in Tris-buffered saline with 0.1% Tween 20 and incubated in goat antimouse IgG (1:7500; Invitrogen) conjugated with Alexa-Flour 680 for 1 h. Membranes were washed as above and images were captured using an Odyssey infrared imaging system (LI-COR, Biosciences) at 700 nM.

Immunocytochemistry

DRG cultures were permeabilized and blocked with 0.3% Triton X-100/10% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) in 0.01 M PBS (pH 7.4) for 30 min and primary antibodies were then applied to the cultures overnight at 4°C. The following day, cultures were incubated with either fluorescein-conjugated goat anti-rabbit (1:100, Cappel/ Organon Teknika Corp., Aurora, OH) or rhodamine-conjugated goat anti-mouse (1:100, Cappel/Organon Teknika Corp.) antibodies, and Hoechest 33342 (10 μg/mL, Sigma), a fluorescent nuclear dye. The following primary antibodies were used: monoclonal mouse anti-BrdU antibody (1:100, Sigma) for identifying proliferating SCs; polyclonal rabbit anti-S100 protein antibody (S-100, 1:100; Dako Corporation, Carpinteria, CA) for identifying SCs; monoclonal mouse or polyclonal rabbit anti-neurofilament (1:100, Sigma); and monoclonal mouse anti-myelin basic protein (MBP; 1:100, Chemicon) were used for identifying axons and myelin sheathes, respectively. Primary antibody omission and mouse and rabbit isotype controls (Zymed Lab, San Francisco, CA) were used to confirm the specificity of the antibodies.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA was used for statistical comparison of the means. Significant results were followed by Tukey's post hoc testing (GraphPad Prism 4.0). A P value of <0.05 was considered statistically significant.

RESULTS

GDNF Accelerated the Time Course and Increased the Amount of Axonal Regeneration and Remyelination *In Vivo*

Previously, we demonstrated that GDNF enhanced axonal regeneration and myelin formation within SC-seeded guidance channels implanted into hemisected spinal cords (Iannotti et al., 2003); however, the time dependency of these effects was not determined. We thus examined GDNF-mediated axonal regeneration and myelination at three time points, i.e., 2, 4, and 6 weeks posttransplantation in the same model. Application of GDNF to channels caused a considerable increase in the size of regenerative tissue cables, the number of myelinated axons, and the number of blood vessels when compared with the vehicle-treated group (see Fig. 1). At all three time points examined, the size of the regenerative tissue cable was significantly greater in the GDNF treatment group than the vehicle-treated group (Fig. 1E–G,I, $*P < 0.05$, $**P < 0.01$). At the 6th week posttransplantation, the size of the GDNF-containing tissue cable reached 0.75 mm^2, 2.6-fold greater than that of the vehicle-treated tissue cable (0.28 mm^2). Consistent with the increase in regenerative tissue cable size, the number of myelinated axons increased significantly in GDNF-treated group compared with the non–GDNF-treated group at all three time points examined (Fig. 1J, $*P < 0.05$, $**P < 0.01$). An earlier initiation of myelination was found only in the GDNF-treated group at 2 weeks posttransplantation, relative to controls. Few, if any, myelinated axons were found in the vehicle-treated group at this time point. At the 6th week posttransplantation, the number of myelinated axons reached 5,475 ± 1,307 in the GDNF-treated group, a 3.5-fold increase from the vehicle-treated control (1,546 ± 327). The difference in the amount and density of myelination of regenerated axons between GDNF-treated and vehicle-treated groups can be notably appreciated at high magnifications (Fig. 1D,H). Thus, our data provide strong evidence showing that GDNF both accelerated the time course for the onset of myelination and increased the numbers of regenerated axons undergoing myelination in this SC bridge transplantation model. In addition, GDNF treatment also significantly increased the number of blood vessels that grew into the graft at 4 and 6 weeks posttransplantation (Fig. 1K, $**P < 0.01$). At the 6th week posttransplantation, there was a 3.8-fold increase in the number of blood vessels at the graft-midpoint in the GDNF-treated group (221.8 ± 13.1) compared with the vehicle-treated group (58.5 ± 8.9; $P < 0.01$).

To further determine the effect of GDNF on axonal regeneration and subsequent myelination within the SC-graft, quantitative measures of axon and myelin perimeters were performed at the EM level. Consistent with the light microscopic observation, early initiation of myelination on regenerated axons at 2 weeks posttransplantation was clearly seen in tissue cables treated with GDNF (Fig. 2D) compared with the lack of myelination in the vehicle control (Fig. 2A). In addition, the number of myelinated axons per field was markedly increased in tissue cables treated with GDNF compared with the vehicle control at all three times examined (Fig. 2G, $*P < 0.05$, $**P < 0.01$). Furthermore, significantly higher ratio of myelinated (MA) to unmyelinated (UMA) axons was found in grafts treated with GDNF than those treated with vehicle (Fig. 2H, $*P < 0.05$, $**P < 0.01$). According to the ratio of MA and UMA, the total number of axons, myelinated and unmyelinated, was estimated. At the 6th week posttransplantation, the estimated total number of regenerated axons per tissue cable was 15,613 in the GDNF-treated group as compared with 7,272 in the

vehicle-treated group. Thus, GDNF enhanced both the number of regenerated axons and the percentage of myelinated axons in this model. When axonal diameter was compared (Fig. 2I), a clear shift from small axon caliber in vehicle-treated group (peaked at 1.5–1.8 μm in diameter) to larger caliber in GDNF-treated group (peaked at 2.1–2.4 μm in diameter) was found. Increased axon caliber has been implicated as a determining factor for inducing myelination in rodents (Voyvodic, 1989). Although myelin thickness increased over time (data not shown), there was no statistically significant difference in the *g*-ratio (an indicator of the proportionality between axon diameter and myelin thickness) between groups treated with and without GDNF (Fig. 2J). This finding supports the interpretation that the effect of GDNF is mainly on neurons.

GDNF Enhanced Neurite Outgrowth from DRG Explants

Since GDNF significantly promoted axonal regeneration and remyelination *in vivo*, we examined whether GDNF had a direct effect on axons. We assessed this possibility by using a neurite outgrowth assay from DRG explants *in vitro*. In this experiment, DRGs obtained from neonatal rats were used since the expression of GFRα1 and Ret in DRG neurons is well established (Molliver et al., 1997). Two parameters were measured: the area occupied by axons derived from a single DRG and the average length of the five longest axons. In cultures treated with GDNF (10 ng/mL), the average area occupied by DRG axons was 2.9 mm^2, ~4 times higher than that treated with the vehicle (0.77 mm^2 , Fig. 3C, $^{**}P < 0.01$). Similarly, the average length of five longest axons in the GDNF-treated cultures was significantly longer than that in the vehicle-treated group (Fig. 3D, $^{**}P < 0.01$). These results, along with the *in vivo* transplantation data, collectively support the interpretation that GDNF has a direct effect on axonal process growth.

GDNF Promoted Proliferation of SCs Only in the Presence of Axons

We also tested the possibility that GDNF might modify axonal regeneration indirectly, by modifying the functions of grafted SCs. In this regard, two possibilities exist. First, GDNF could promote SC proliferation which enhances axonal regeneration by providing increased substrate for growth. Second, GDNF could induce or enhance the production of axon-growth promoting trophic factors by SCs. To address the first possibility, SC proliferation was assessed in two separate paradigms. First, GDNF (10 ng/mL) or vehicle was added to neuron-free cultures of purified population of SCs for 3 days. Our results showed that there was no significant difference in the percentage of SCs labeled with BrdU between the two groups (Fig. 4A,C,E; $P > 0.05$), indicating that GDNF has no direct effect on the proliferation of SCs. Second, we seeded purified SCs onto dissociated and purified DRGNs after an extensive neurite network had been formed. Addition of GDNF for 3 days did not induce significant proliferation of SCs compared with the control (Fig. 4B,D,F; $P > 0.05$). However, if the GDNF treatment was extended to 4 weeks, the number of SCs increased significantly after GDNF treatment when compared with the nontreated control (Fig. 7K, $^{**}P < 0.01$). Because it is well known that axons are mitogenic for SCs and we have shown above that GDNF stimulates the growth of axons, the increased proliferation of SCs in the presence of GDNF could be due to the increased axon growth and not a direct effect of GDNF on the SCs. However, these experiments do not rule out the possibility that long-term treatment with GDNF might also somehow increase the responsitivity of SCs to axonal mitogenesis.

GDNF had no Effect on the Secondary Production of Neurotrophins by SCs

A second hypothesis to explain the effect of GDNF on increased axonal growth into SC grafts is that GDNF promotes the production of other neurotrophic factors by SCs. Since SCs can produce a number of neurotrophic factors such as nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), and neurotrophin-3 (NT-3) (Acheson et al.,

1991; Meier et al., 1999), we examined the GDNF effect on the production of these factors. We observed that treatment of SC cultures with GDNF (10 ng/mL) for 3 days had no statistically significant effect on the production of these factors, compared with the vehicle-treated controls (Fig. 5A–C; $P > 0.05$). The reduced NT-3 production in the GDNF-treated SC cultures appears to be significant, but statistical analysis showed no significant difference between the two groups possibly because of large standard deviation.

GDNF Enhanced SC Production of NCAM but not L1

SCs and neurons express the adhesion molecules L1 and NCAM. Both molecules are involved in neuron-SC recognition and neurite outgrowth on SCs. We therefore examined the effect of GDNF on the production of L1 and NCAM in purified SC cultures using Western blot analysis. The density of specific bands was determined by densitometry and expressed as a ratio of β-actin. After a 3 day treatment of GDNF (10 ng/mL), NCAM production increased about two-fold compared with the vehicle control (Fig. 6A,B; $*P < 0.05$). In contrast, no statistically significant change was found in the SC production of L1 between GDNF-treated and non–GDNF-treated groups (Fig. 6A,C). It has been shown that GDNF stimulates axonal growth from hippocampal and cortical neurons via an NCAM-mediated mechanism (Paratcha et al., 2003). Thus, increased NCAM expression in the SCs in response to GDNF treatment could increase their capacity to interact with axons and thereby promote neurite outgrowth.

GDNF Enhanced Myelination by SCs *In Vitro*

To better identify the cellular targets by which GDNF enhances myelination within SC grafts *in vivo*, we studied the effect of GDNF on SC myelination in DRGN-SC cocultures. To determine whether such myelin formation was GDNF-specific, the effect of a second trophic factor, NGF, was studied. After seeding purified SCs onto DRGNs, cultures were maintain in serum-free media containing GDNF, NGF, or vehicle for 2 weeks followed by the addition of ascorbic acid for myelin initiation for another 2 weeks. We found that in all 3 groups, axon fascicles were formed; however, larger axon fascicles were found only in cultures treated with GDNF (Fig. 7B) compared with the vehicle-treated (Fig. 7A) or NGF-treated (Fig. 7C) groups, confirming that GDNF has a direct effect on axonal growth described above. Strikingly, GDNF-treatment promoted significantly more myelination than the vehicle-treated and NGF-treated groups, as evidenced by both the MBP immunostaining (Fig. 7D– F) and EM (Fig. 7G–I). Statistical analysis revealed that the MBP-labeled area in the GDNF-treated group is significantly higher than that in the NGF-treated or vehicle-treated group (Fig. 7J, $**P < 0.01$). Interestingly, both GDNF and NGF administration increased significantly the number of SCs as compared with the vehicle control (Fig. 7K, $**P < 0.01$) although more SCs were found in the GDNF-treated group than the NGF group (Fig. 7K, $*P < 0.05$). Given that GDNF does not have a direct effect on isolated SC proliferation, as shown above, the increased SC number seen here must result from the presence of axons with sustained exposure to GDNF. Whether proliferation of SCs was a result of direct contact with axons or indirectly through factors released from axons remains to be investigated.

To further confirm the GDNF effect on SC myelination, GDNF treatment of the cocultures was delayed until the addition of ascorbate. This approach allows a more precise observation of effects of GDNF on the process of myelination itself. This greater precision results from the fact that myelination is triggered by ascorbic acid addition and myelin sheath formation can be observed as soon as 5 days later. At the time of ascorbic acid/GDNF addition, the cultures have already been fully populated with SCs and neurites. Eight days after adding ascorbic acid, few myelin sheaths, stained with Sudan black, were seen in control cultures

(Fig. 7L). In contrast, addition of GDNF resulted in a significant increase in the number of myelin sheaths in these cultures (Fig. 7M,N; $^{**}P < 0.01$).

GDNF Accelerated the Time of Onset of Myelination by SCs *In Vitro*

To investigate if GDNF treatment affected the time course of myelination in the cocultures, we examined the expression of MBP, a marker for myelin, in DRGN-SC cocultures at 4, 8, 12, and 16 days after the addition of ascorbic acid. In the vehicle-treated group, no MBP labeling was found at 4 and 8 days after the addition of ascorbic acid (Fig. 8A,B) until at day 12 and increased at day 16 postascorbic acid addition (Fig. 8C,D). In contrast, MBP labeling was found as early as day 4 postascorbic acid addition after GDNF-treatment (Fig. 8E) and significantly increased at and beyond day 8 compared with the vehicle-treated controls (Fig. 8E–I, $^{**}P < 0.01$). Thus, GDNF not only enhanced the amount of SC myelination on DRG axons, but also accelerated the time course of such myelination, further confirming our *in vivo* results described above.

DISCUSSION

The goal of this study was to determine whether the action of GDNF on axonal regeneration and SC myelination was because of an effect on neurons, on SCs or on both. Our results demonstrate significant effects of GDNF on neurite outgrowth *in vitro* and accelerated time course and increased amount of axonal regeneration and myelination *in vivo*, suggesting a direct effect of GDNF on neurons. In addition, GDNF treatment of SCs in isolation from axons caused a significant increase in the expression of the cell adhesion molecule NCAM, demonstrating that GDNF might also affect SC function. These results suggest that GDNF influences both axonal properties and SC function, leading to improved regeneration and myelination.

GDNF Induced the Recruitment of More Axons to Regenerate or Sprout

In this study, we observed that GDNF significantly increased the number of myelinated axons as well as the total number of axons that regenerated into the SC-seeded guidance channel, confirming our previous finding (Iannotti et al., 2003). The GDNF-mediated effect on axonal regeneration was further confirmed *in vitro* since GDNF increased both the area and length of growing neurites from cultured DRGs. Our previous work also showed that axons from DRGs regenerated into SC-seeded guidance channels implanted into transected spinal cord (Xu et al., 1997). Previous study showed that GDNF up-regulated rapidly in denervated SCs after sciatic nerve injury (Hoke et al., 2002) and is retrogradely transported by motoneurons (Yan et al., 1995). And a long-term continuous exogenous GDNF treatment significantly increased the number of motoneurons which regenerate their axons, accompanying with an increase in axon sprouting within the distal nerve stump (Boyd and Gordon, 2003). Therefore, it is possible that, following SCI and GDNF administration, GDNF may be retrogradely transported along damaged axons to the responsive neurons to exert a direct neurotrophic effect. This in turn rescues injured neurons or increases the number of neurons that regenerate or sprout their axons. Up-regulation of GFRα1 and c-Ret gene expression following SCI (Widenfalk et al., 2001) may further increase the responsiveness of injured neurons and axons to GDNF for neuronal survival and axonal regeneration.

Importantly, GDNF markedly increased the number of axons regenerating into SC grafts. For example, at the 6th week posttransplantation, an average of 5,475 myelinated axons and 15,613 total axons were observed in SC grafts in GDNF-treated animals. This was 3.5 and 2.1 times higher, respectively, than those observed when GDNF was not used. Since the amount of axonal regeneration could be positively correlated to the amount of functional

recovery (Takami et al., 2002), the ability of GDNF to recruit significantly more axons to regenerate is particularly encouraging. The increased number of regenerated axons in the SC grafts after the GDNF treatment could be a result of increased number of neurons that regenerate their axons or branching of existing axons; such possibilities remain to be further elucidated.

GDNF Increased the Caliber of Regenerated Axons, a Mechanism for Increased Myelination

In addition to recruiting more axons through regeneration and/or sprouting, GDNF administration also increased the caliber of regenerated axons. At the EM level, GDNF was shown to induce a shift from smaller axon diameter to larger ones, as compared with the vehicle-treated group. Previous studies demonstrated that increased axon diameter is a determining factor for the initiation of myelination (Voyvodic, 1989). Increased numbers of myelinated axons in GDNF-treated animals may be a result of caliber increase of existing regenerated axons or the recruitment of additional large caliber axons. But the current experiments did not allow differentiation between these two possibilities.

Although how GDNF increases the diameter of regenerated axons remains unclear, several factors may affect the size of axon caliber. For example, axon caliber can be affected by neurofilament (NF) gene expression (Hoffman et al., 1987) and the phosphorylation state of the long COOH-terminal tails of the medium and heavy subunits of NF (Elder et al., 1998) in axons. Also, myelination plays a critical role in determining the axon caliber (Windebank et al., 1985). It has been shown that myelinating SCs exert a significant influence on axon caliber by modulating NF phosphorylation and NF packing density in the axons of peripheral nerves (Nixon et al., 1994; Yin et al., 1998). However, axonal ensheathment by SCs does not guarantee the increase in axonal caliber of unmyelinated fibers, suggesting that a molecule enriched in myelinating but not nonmyelinating SCs regulates axonal caliber. Myelin-associated glyco-protein (MAG) has been suggested to be one such molecule (Yin et al., 1998). Whether GDNF plays a role on neurofilament reorganization or SC expression of MAG remains to be determined.

GDNF did not Promote the Secondary Production of Neurotrophins

One important feature of SCs as a promising cell type for transplantation is their ability to produce a variety of trophic factors that are growth-promotive [for review, see (Guenard et al., 1993; Oudega and Xu, 2006)]. Among these factors, members of the neurotrophin family play particular roles. It was demonstrated that SCs expressed NGF (Heumann et al., 1987), BDNF (Acheson et al., 1991), and NT-3 (Offenhauser et al., 1995). We previously demonstrated that BDNF and NT-3 enhanced regeneration of propriospinal and supraspinal axons into SC-seeded guidance channels (Xu et al., 1995a). When administered distally, they promoted reentry of axons from the SC graft into the distal host spinal cord (Bamber et al., 2001). Although the neurotrophins are growth-promotive, our results suggest that their effects were independent of the action of GDNF since GDNF had no effect on the secondary production of neurotrophins by SCs.

GDNF Enhanced Myelination of Regenerated Axons: Possible Mechanisms

A recent study has demonstrated that regenerated axons have electrophysiological abnormalities reminiscent of chronic demyelination for at least 6 months post-injury and that potential restoration of myelination could enhance functional recovery (Tan et al., 2007). A striking finding in the present study is that GDNF consistently and significantly enhanced SC myelination. Our data showing that GDNF did not affect the *g*-ratio of the myelinated fibers strongly suggest that this effect of GDNF is exerted on the neuron and not on the SC. This interpretation is consistent with the observation that GDNF increased axonal caliber, a

known determinant of myelin thickness (Aguayo et al., 1976; Weinberg and Spencer, 1976). Recently, it has been reported that the axonal determinant for myelin thickness may be neuregulin 1, Type 3, a surface molecule on axons that activates the erbB2/erbB3 receptor dimer on SCs (Michailov et al., 2004; Taveggia and Salzer, 2007). It is possible that GDNF upregulates the expression of neuregulin on the axon.

GDNF Enhanced Expression of NCAM: A Possible Mechanism for GDNF Signaling in SCs

SCs and axons express adhesion molecules L1 and NCAM, both involved in neuron-SC recognition and neurite outgrowth on SCs (Bixby et al., 1988). *In vitro*, L1 and NCAM were detected at the premyelinating stage and their expression decreased when myelin formed (Wood et al., 1990). In MAG-deficient mice, overexpression of NCAM at sites normally expressing MAG was found, suggesting an important role of NCAM in myelin formation (Montag et al., 1994). In our study, a two-fold increase in 140 kDa NCAM but not L1 in SCs was found after GDNF treatment. This indicates that GDNF regulates the production of NCAM, but not L1. The increase in NCAM production by SCs may play a dual role in GDNF-mediated action. First, NCAM may exert a direct effect on SC migration, axon-SC recognition, SC-mediated neurite outgrowth, and myelination. For example, NCAM-Fc and F3-Fc chimeras enhanced migration of cultured SCs (Thomaidou et al., 2001). Secondly, increased NCAM may serve as a coreceptor for GDNF signaling in SCs in a Ret-independent pathway. It has been shown that SCs express only GFRα1 but not Ret that is required for GDNF signaling (Paratcha et al., 2003; Trupp et al., 1999). Given that both GDNF and GFRα1 transcripts are up-regulated by SCs in distal segments of lesioned PNS (Trupp et al., 1997), it is conceivable that the action of GDNF on axonal regeneration and myelination is mediated, at least in part, through an autocrine mechanism involving GFRα1 and NCAM.

In summary, we provide evidence that GDNF can enhance axonal regeneration and myelination in both the *in vivo* and *in vitro* experimental settings. The data reported here supports the idea that the effect of GDNF is mediated primarily through a direct effect on neurons but the possibility of effects of GDNF on SCs is not ruled out. Since SCs have also been shown to promote axonal regeneration, combination of GDNF and SC transplantation may represent an effective strategy for promoting axonal regeneration in the injured CNS, including the spinal cord.

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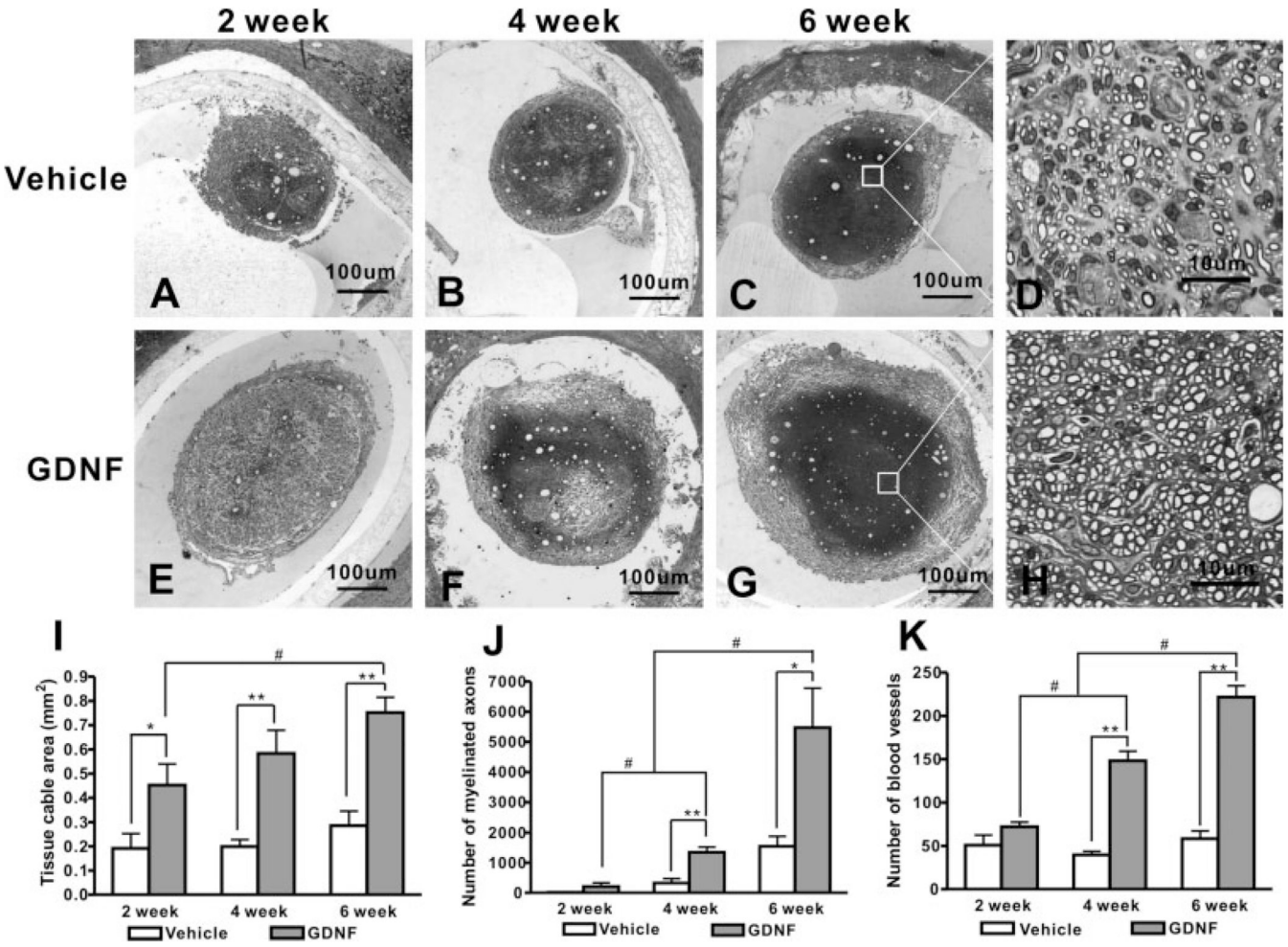

Fig. 1.

GDNF increase the graft diameter and the number of myelinated axons and blood vessels within SC grafts at different times after transplantation. (**A–C**) Transverse sections of SC grafts within mini-channels in the control (vehicle) group at 2, 4, and 6 weeks after transplantation. (**D**) Magnified area from C. (**E–G**) Transverse sections of SC grafts within the mini-channels in the GDNF group at 2, 4, and 6 weeks after transplantation. (**H**) Magnified area from G. (**I**) The cross-sectional area of SC grafts within channels containing GDNF are bigger than those in channels without GDNF at all posttransplantation time points (**P*< 0.05, ***P*< 0.01). A significant increase in graft size was observed in the GDNF group between 2 and 6 weeks after transplantation (#*P* < 0.01). (**J**) The number of myelinated axon increased over time both in channels with or without GDNF (*P* < 0.01). In addition, myelinated axon numbers were significantly increased in GDNF groups compared with vehicle groups at 4 and 6 weeks after transplantation (**P* < 0.05, ***P*< 0.01). (**K**) The number of blood vessels in regenerated cables significantly increased over time in GDNF groups after transplantation (#*P* < 0.01) and is much higher in the GDNF group than the vehicle group at 4 and 6 weeks after transplantation (***P* < 0.01). The number of blood vessels among vehicle groups did not differ significantly. Scale bar: A–C and E–G, 100 μm; D and H, 10 μm. Graph bars: mean ± SD.

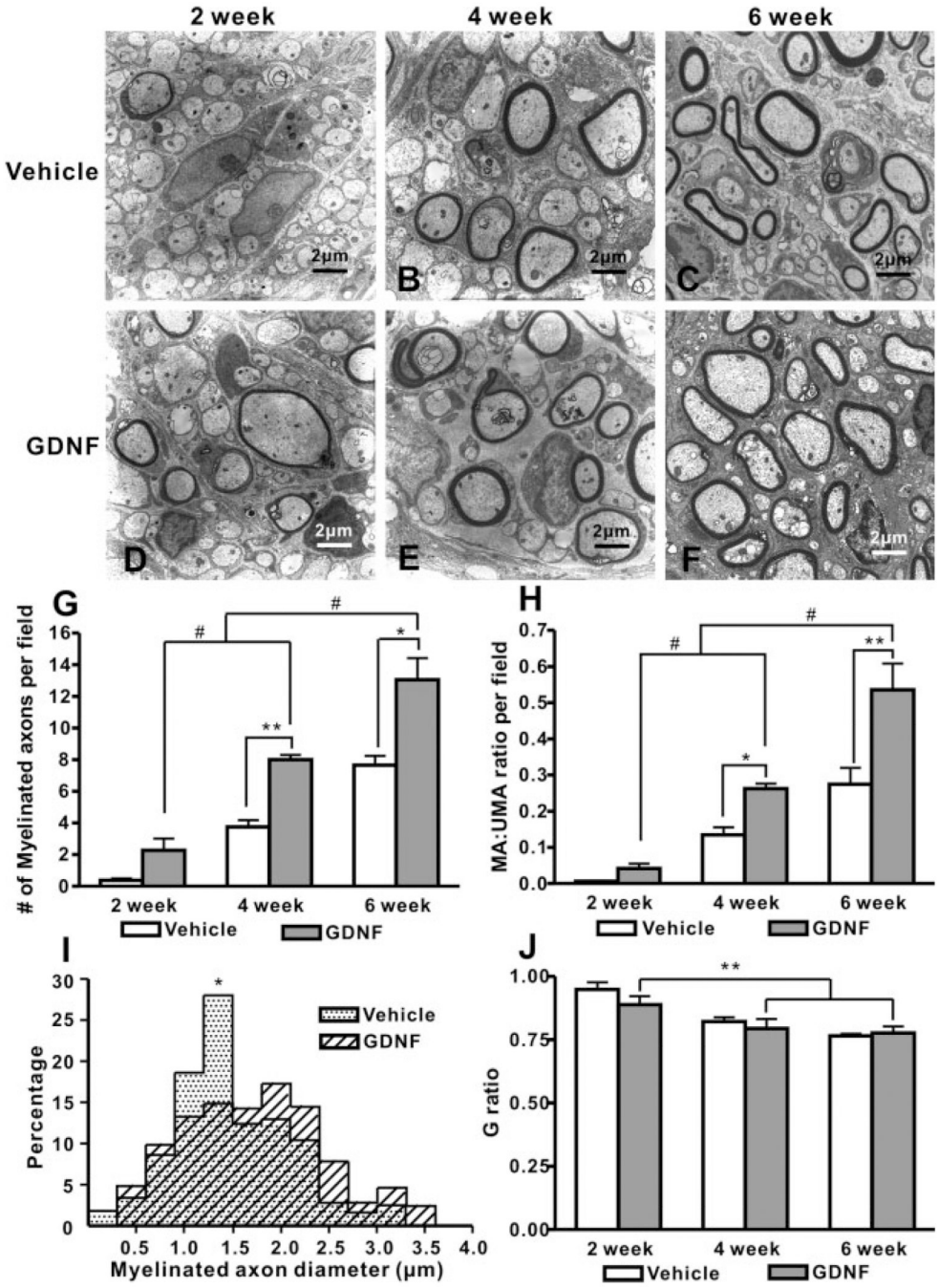

Fig. 2.

Electron microscopic analysis of changes in myelinated axons in vehicle-treated and GDNF-treated SC grafts. (**A–C**) Representative micrographs of vehicle-treated grafts at 2, 4, and 6 weeks after transplantation. (**D–F**) Representative micrographs of GDNF-treated grafts at 2, 4, and 6 weeks after transplantation. (**G**) The number of myelinated axon per microscopic field increased over time in both the vehicle-treated and GDNF treated groups (#$P < 0.01$). However, SC grafts treated with GDNF have more myelinated axons than those without GDNF at 4 and 6 weeks (*$P < 0.05$, **$P < 0.01$). (**H**) In both vehicle-treated and GDNF-treated grafts, the ratio of myelinated axons (MA) to unmyelinated axons (UMA) also significantly increased over time (#$P < 0.01$); however, the MA/UMA ratio showed a

significantly greater increase in the GDNF-treated grafts by 4 and 6 weeks, compared with vehicle-treated grafts (*$P < 0.05$, **$P < 0.01$). (I) Distribution of myelinated axon diameter of vehicle (dotted) and GDNF (slash) groups 6 weeks after transplantation. GDNF group shows increased axon diameter compared with the vehicle group. (**J**) The *g*-ratios were calculated from micrographs at 2, 4, and 6 weeks after transplantation. These ratios increase over time but no differences were found between the two groups at each time point. Scale bar: A–F, 2 μm. Graph bars: mean ± SD.

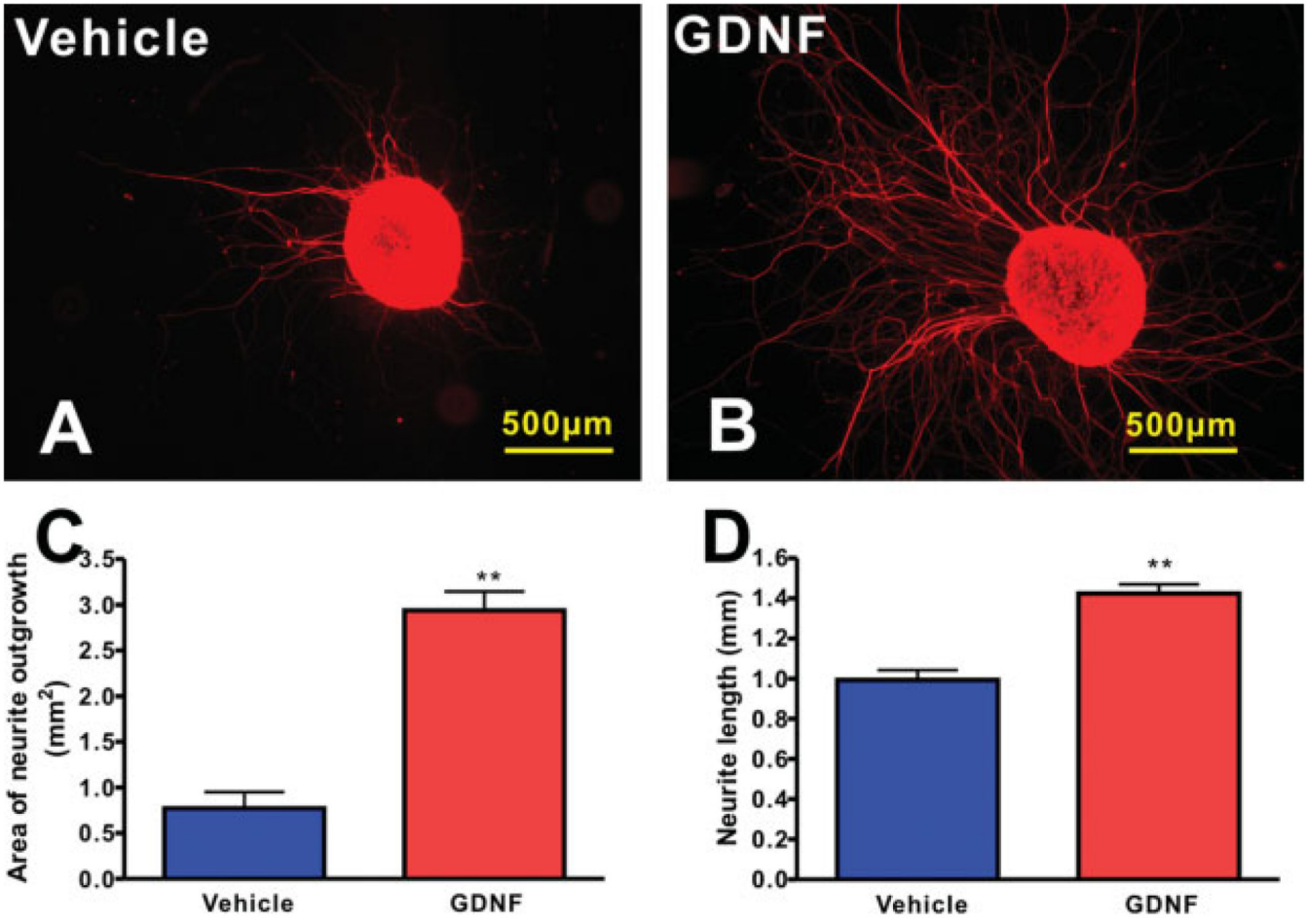

Fig. 3.

Effect of GDNF on neurite outgrowth. (**A** and **B**) Micrographs show the extent of axonal outgrowth from DRG explants at 3 days of culture in the vehicle-treated (A) or GDNF-treated group (B). (**C** and **D**) Comparison of the total area covered by neurites (C) or average length of the five longest axons (D) in vehicle-treated or GDNF-treated DRG explant cultures (**$P < 0.01$). Scale bar: A–B, 500 μm. Graph bars: mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

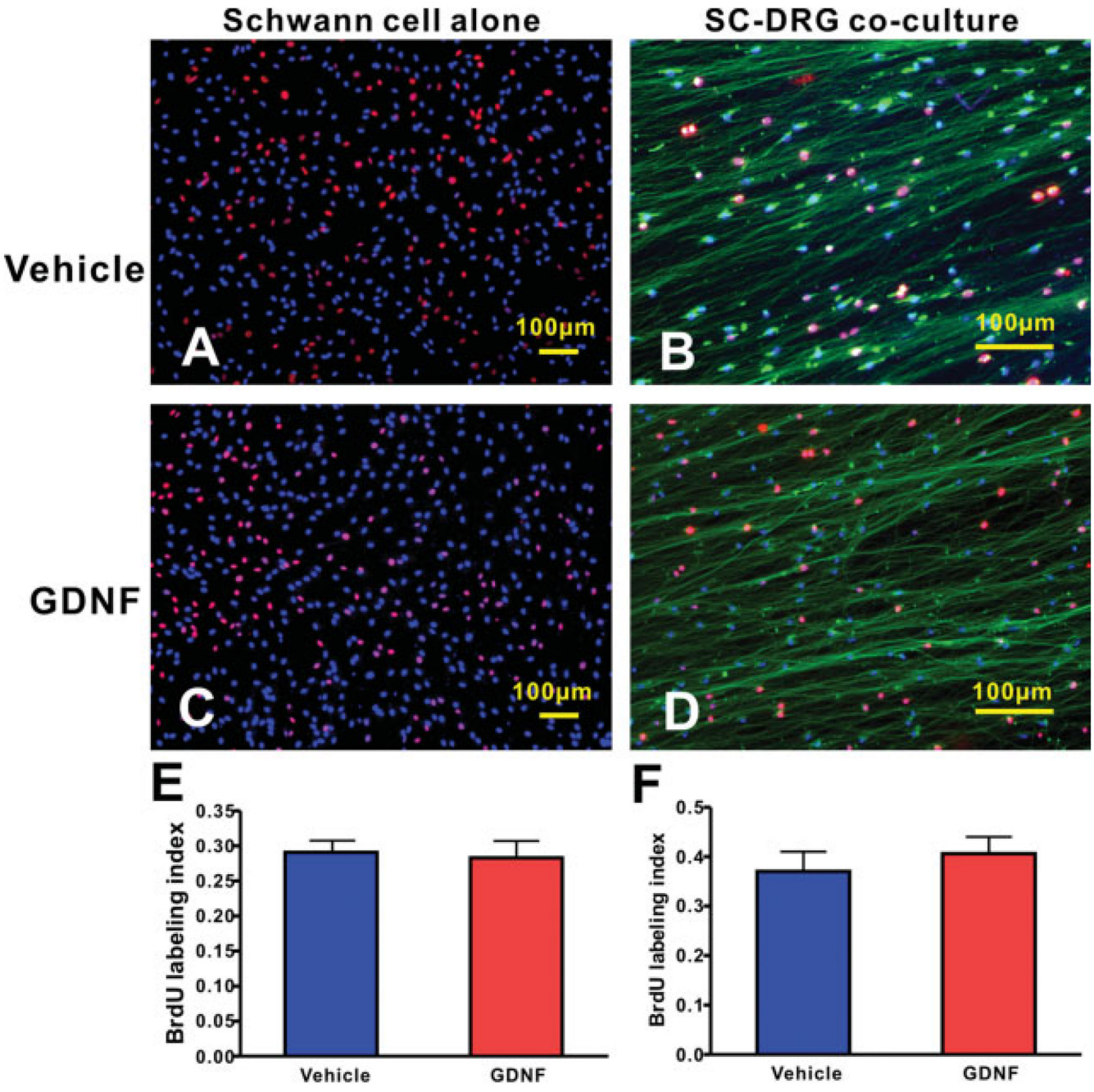

Fig. 4.

Effects of GDNF on Schwann cell proliferation in the absence and presence of axons. (**A, C**, and **E**) Isolated SC cultures were treated with vehicle or GDNF for 3 days and labeled with BrdU (red) for the final 24 h of the treatment period. No difference was found in the number of BrdU labeled cells between the two groups. (**B, D**, and **F**) When SCs were seeded onto purified DRGN cultures, received GDNF treatment for 3 days, and BrdU labeling for the final 24 h, GDNF treatment did not increase the BrdU labeling index compared with the vehicle control. Scale bar: **A–D**, 100 μm. Graph bars: mean ± SD.

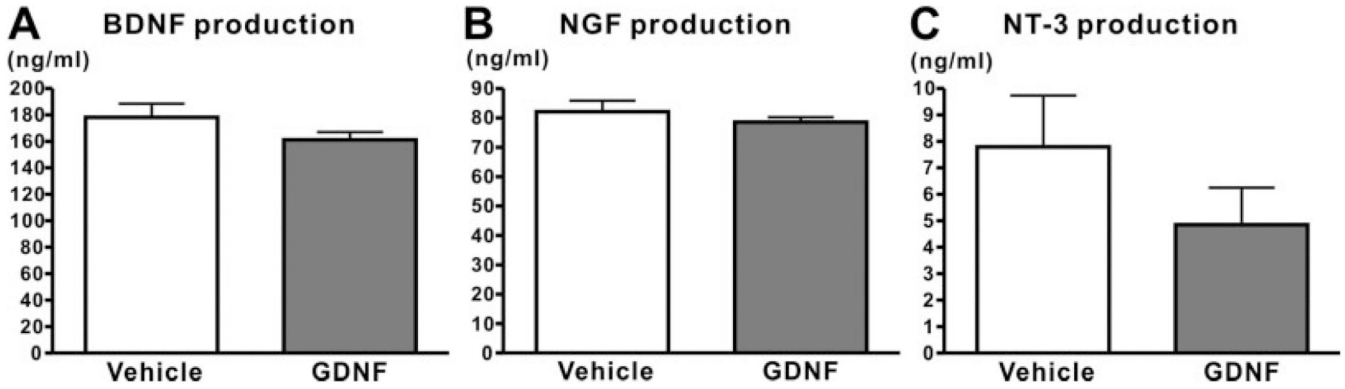

Fig. 5.

Effect of GDNF on production of neurotrophins by SCs. (**A**–**C**) Cultures of isolated SCs were treated with vehicle or GDNF as described in the text. Conditioned media were analyzed for neurotrophins by ELISA assay. No statistically significant differences in concentrations of BDNF (**A**), NGF (**B**), and NT-3 (**C**) in Schwann cell media were found between the vehicle-treated and GDNF-treated groups. Graph bars: mean ± SD.

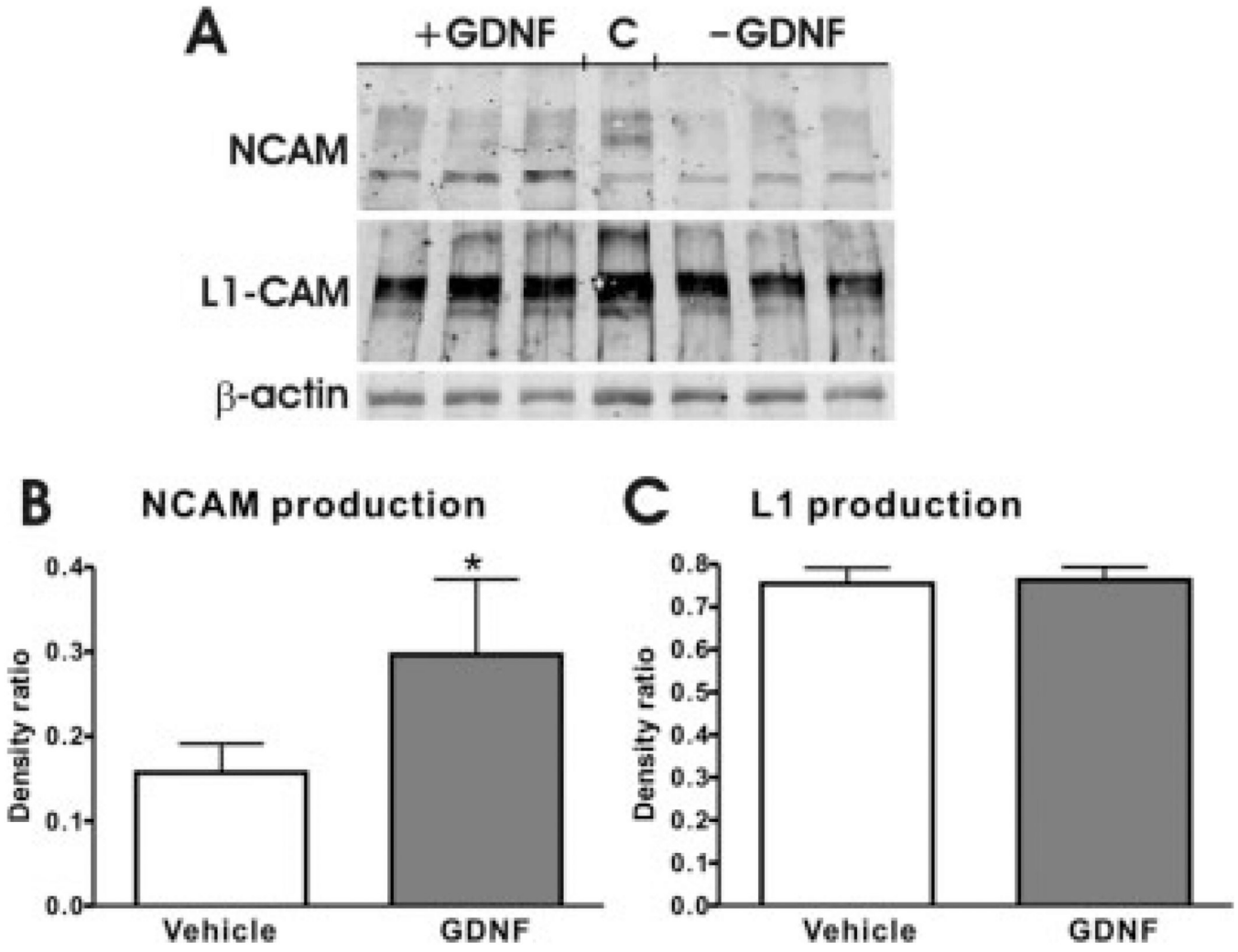

Fig. 6.

Effect of GDNF on the expression of cell adhesion molecules by isolated SCs. (**A**) The effect of GDNF on NCAM and L1expression was analyzed by Western blotting. The density of specific bands was determined by densitometry, a ratio of β-actin. (**B**) After 3 days of GDNF treatment, the expression of NCAM was significantly increased (*$P < 0.05$) in the GDNF-treated group compared with the vehicle-treated group. (**C**) In contrast, no significant difference in L1 expression was found between the two groups. Graph bars: mean ± SD.

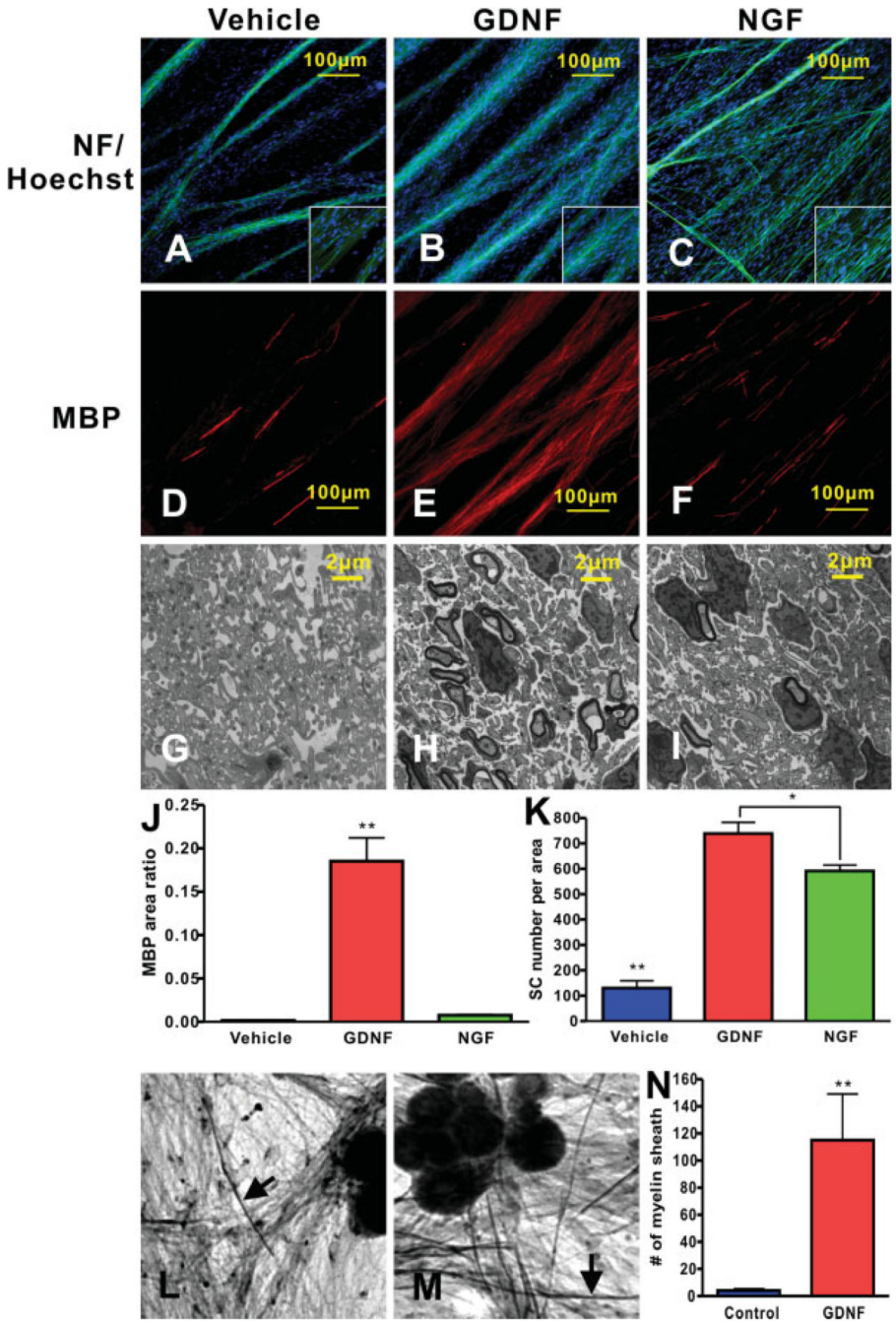

Fig. 7.

GDNF promotes myelin formation *in vitro*. (**A–C**) In both vehicle-treated and NGF-treated DRGN-SC coculture, some axon fascicles were formed (A, C), while in GDNF-treated culture, larger axon fascicles were formed (**B**). Inserts in A–C show association of SCs with axon fascicles in the three treatment conditions. (**D**–**F**) 2 weeks after the initiation of myelin formation by addition of ascorbic acid to the culture medium, substantially more myelin sheathes were formed in the GDNF-treated group (**E**) than in either the vehicle-treated (D) or NGF-treated (F) group. (**G–I**) EM images of myelin formation in the vehicle, GDNF-treated and NGF-treated cultures. The greatest amount of myelin was found only in the GDNF-treated cultures (**H** and **J**). Myelin formation was represented by the ratio of the area

taken by MBP-labeled myelin sheaths to total culture area sampled. GDNF significantly enhanced Schwann cell mediated myelin formation (**$P < 0.01$). (**K**) Both GDNF and NGF increased the number of Schwann cells during the process of myelin formation (**$P < 0.01$) and GDNF has stronger effect on Schwann cell proliferation than NGF (*$P < 0.05$). (**L** and **M**) Comparison of myelin formation in cultures treated with either vehicle (L) or GDNF (M) during the myelination period only. Myelin was visualized by Sudan black staining; myelin sheathes are indicated by arrows. (**N**) Quantitative comparison of the number of sheathes in vehicle-treated and GDNF-treated cultures showing a significant increase of myelin sheathes in the GDNF-treated than the vehicle-treated cultures. Scale bar: A–F, 100 μm; G–I, 2 μm. Graph bars: mean ± SD.

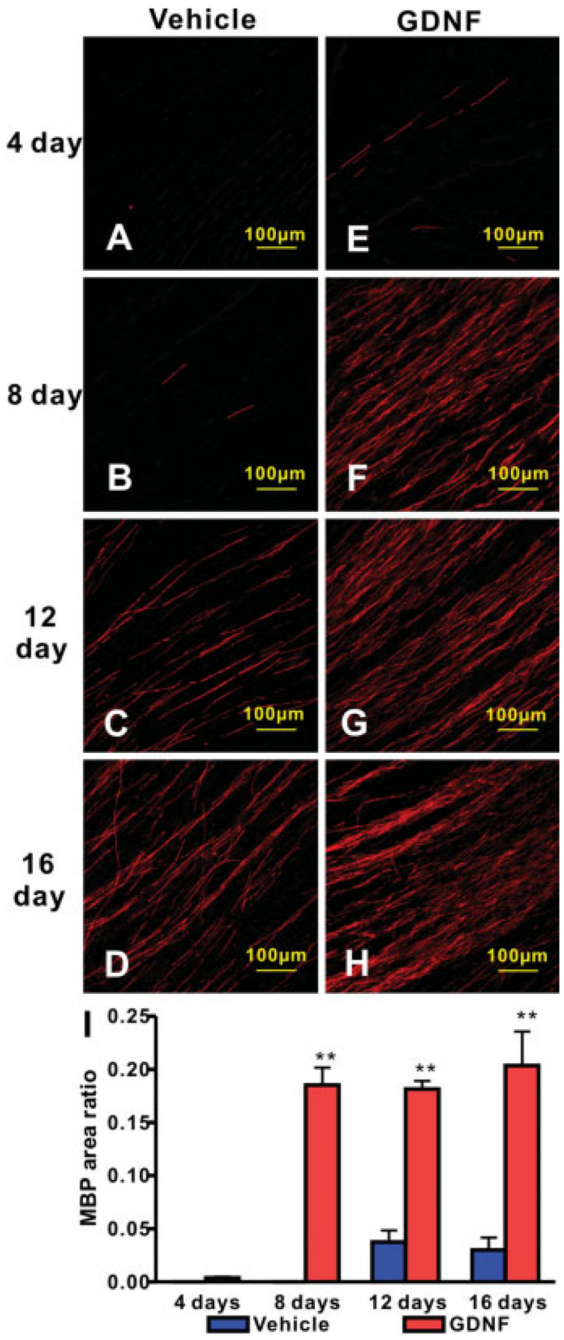

Fig. 8.

Effect of GDNF on the rate of myelin formation *in vitro*. DRGN-SC cocultures were stained for MBP at various times after the addition of ascorbic acid as indicated. (**A–D**) In the vehicle-treated cultures, only a few myelin sheaths were detected at 4 and 8 days after adding ascorbic acid (A and **B**), while more myelin formed thereafter (**C** and **D**). (**E–H**) In the GDNF-treated group, a few myelin sheaths were found as early as at 4 days after adding ascorbic acid (E) and, by 8 days, a large number of myelin sheaths were observed (F). Interestingly, the number of myelin sheaths did not appear to significantly increase after 8 days (**G** and **H**). (**I**) Quantitative measurement of MBP + myelin profiles in randomly selected culture areas at various times after ascorbic acid addition in vehicle-treated and

GDNF-treated cultures. The MBP-positive area was significantly increased in the GDNF-treated groups at and after 8 days of adding ascorbic acid, compared with the vehicle-treated group (**$P < 0.01$). Scale bar: A–H, 100 μm. Graph bars: mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]