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Fibrin Inhibits Peripheral Nerve Remyelination by Regulating Schwann Cell Differentiation

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Summary

Remyelination is a critical step for functional nerve regeneration. Here we show that fibrin deposition in the peripheral nervous system after injury is a key regulator of remyelination. After sciatic nerve crush, fibrin is deposited and its clearance correlates with remyelination. Fibrin induces phosphorylation of ERK1/2 and production of p75 NGF low-affinity receptor in Schwann cells and maintains them in a nonmyelinating state, suppresses fibronectin production, and prevents synthesis of myelin proteins. In mice depleted of fibrin(ogen), remyelination of myelinated axons is accelerated due to the faster transition of the Schwann cells to a myelinating state. Regulation of fibrin clearance and/or deposition could be a key regulatory mechanism for Schwann differentiation after nerve damage.

Introduction

A fundamental question in cellular neurobiology is the regulation of nerve regeneration after injury or disease. Functional nerve regeneration requires not only axonal sprouting and elongation, but also new myelin synthesis. Remyelination is necessary for the restoration of normal nerve conduction and for the protection of axons from new neurodegenerative immunologic attacks (Horner and Gage, 2000). Incomplete remyelination in the adult human CNS together with axonal damage contributes to clinical deficits in diseases such as multiple sclerosis (MS) (Bjartmar and Trapp, 2001).

In mammals, there are two types of myelinating cells: Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in the central nervous system (CNS). After a lesion within the PNS, Schwann cells respond to injury by proliferation and further differentiation to myelinating cells (Kioussi and Gruss, 1996). In the CNS, adult oligodendrocyte precursors are recruited to the sites of the lesion and exhibit a limited capacity for remyelination (Blakemore and Keirstead, 1999). Following demyelination, successful remyelination by both Schwann cells and oligodendrocyte precursors depends on a combination of signals that these cells receive from the inflammatory response and the demyelinated axons. These signals prompt them to first re-enter the cell cycle, and then differentiate into myelinating cells (Levine et al., 2001).

The molecular signals that drive the differentiation of Schwann cells toward myelin production are mainly derived from adherence to an axon destined for myelination and the basal lamina (Jessen and Mirsky, 1999b). Extracellular matrix (ECM) molecules of the basal lamina and their receptors are involved not only in the process of axonal elongation, via interactions with neurons/axons (Reichardt and Tomaselli, 1991), but also in remyelination, via interactions with glial cells (Bunge et al., 1986; Sobel, 1998). Laminin is a positive regulator of neurite growth and Schwann cell migration (Anton et al., 1994) and it also induces myelination in vitro (Eldridge et al., 1989). In addition, receptors of ECM molecules, such as β1 integrin, promote myelination by Schwann cells (Feltri et al., 2002; Fernandez-Valle et al., 1994). The sciatic nerve during regeneration upregulates laminin, fibronectin, and specific integrins (Lefcort et al., 1992) to resemble the constellation of ECM components and integrins that are present during development (Chernousov and Carey, 2000; Vogelezang et al., 1999). All of these results indicate the importance of the ECM for myelination.

The composition of the ECM can be altered after nerve injury by leakage of fibrinogen from the vasculature (Akassoglou et al., 2000). The tissue injury also causes a procoagulant state (Friedmann et al., 1999) resulting in conversion of fibrinogen to fibrin, which can be deposited in the matrix (Bugge et al., 1996). In nervous tissue, fibrin deposition has been reported in lesions of MS patients (Claudio et al., 1995), as well as in experimental models of CNS (Koh et al., 1993), and PNS demyelination after injury (Akassoglou et al., 2000) accompanied by leakage of the blood-nerve barrier. In these cases, therefore, fibrin becomes a new component of the ECM. However, a role for fibrin in nerve regeneration and new myelin formation has not been investigated.

In a previous study, we examined the degeneration phase of sciatic nerve injury (Akassoglou et al., 2000). Mice deficient in the fibrinolytic components tissue plasminogen activator (tPA) or plasminogen exhibit exacerbated damage after sciatic nerve crush. This effect is due to reduced fibrin clearance since fibrin depletion rescued the exacerbation observed in tPA-deficient mice. However, this work did not reveal any information on the regenerating phase of the injury or on the cellular and molecular mechanisms of fibrin action in the nervous tissue.

In our current work, we have focused on sciatic nerve remyelination and demonstrate a novel role for fibrin as a regulator of Schwann cell differentiation. Sciatic nerve crush in mice genetically (Suh et al., 1995) or pharmacologically (Akassoglou et al., 2000) depleted of fibrin(ogen) showed an acceleration in the formation of myelinated axons after injury. Schwann cells in fibrin(ogen)-depleted mice also had an earlier onset of myelin gene transcription, downregulation of p75 NGF low-affinity receptor (p75 NGFR), and elevated production of fibronectin, a beneficial ECM component for Schwann cell maturation. In vitro experiments demonstrated that fibrin induced ERK1/2 phosphorylation and downregulated myelin

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gene expression in Schwann cells and maintained them in a proliferating, nonmyelinating state.

These results identify the fibrin matrix as a critical regulator of remyelination of peripheral nerves, and provide a mechanism for how a blood-derived protein can affect the proliferation and differentiation of myelinating cells after peripheral injury. Persistent fibrin deposition, due to vascular leakage or inadequate clearance, might provide the basis for neuropathologies characterized by reduced myelin formation. If so, strategies aimed at removing the fibrin matrix could be beneficial in enhancing remyelination in neurodegenerative and demyelinating diseases.

Results

Fibrin Clearance Correlates with Nerve Repair

In our previous work, we demonstrated a correlation of fibrin deposition with sciatic nerve degeneration (Akassoglou et al., 2000). To examine how fibrin deposition is regulated during nerve regeneration, we examined the presence of fibrin at late time points after sciatic nerve injury. Uninjured sciatic nerve shows normal axonal morphology (Figure 1A) and no endoneurial staining for fibrin(ogen) (Figure 1B, arrows in figure indicate vascular staining). During degeneration after sciatic nerve crush, there is collapse of the myelin sheaths and axonal degeneration (Figure 1C). During the degeneration phase, fibrin(ogen) immunoreactivity increases in the endoneurium (Figure 1D). At late time points after injury, there are myelinated axons in the nerve with smaller diameter and thinner myelin sheath (Figure 1E), when compared to the axons of the adult uninjured nerve (Figure 1A). During the regeneration phase, fibrin(ogen) decreases to uninjured levels (Figure 1F), presumably due to the action of tPA, which is induced after crush (Akassoglou et al., 2000).

To further evaluate fibrin deposition at different time points after injury, we performed Western blot analysis using an antibody against γ-γ fibrin dimers, which are formed by the action of the coagulation system on the fibrinogen molecule (Chen and Doolittle, 1971; Lorand et al., 1969). The uninjured sciatic nerve did not show formation of γ-γ dimers, showing that there is no fibrin present constitutively (Figure 1G). Four days after injury, there was a dramatic increased formation of γ-γ dimers, indicating that the tissue during its degeneration phase has fibrin deposition (Figure 1G). Twenty-five days after crush injury, during the regeneration phase of the nerve, there were no γ-γ dimers present, demonstrating that the fibrin formed during the degeneration phase has been removed from the tissue (Figure 1G). Staining the membrane with Ponseau S before immunoblotting showed that equal amounts of total proteins were present in the blot (Figure 1H).

Fibrin(ogen)-Deficient Mice Show Increased Myelinated Axons after Sciatic Nerve Injury

To assess the involvement of fibrin in axonal remyelination in vivo, we examined the tissue morphology of the sciatic nerve 1 mm distal of the crush site 8 days after crush in wild-type mice and mice genetically or pharmacologically depleted of fibrinogen. In wild-type mice, axonal remyelination was not significant 8 days after crush, as evidenced by a small number of myelinated axons (Figure 2A), and there was substantial fibrin deposition evident (Figure 2D). However, at this time, there were numerous myelinating axons in *fib*$^{-/-}$ mice (Figure 2B), and as expected, no fibrin deposition (Figure 2E). To complement this result, we examined nerve remyelination in mice in which fibrinogen had been depleted pharmacologically. Administration of the Malayan pit viper (*Calloselasma rhodostoma*) venom protein, ancrod, leads to consumption of systemic fibrinogen and drastically reduces plasma fibrinogen levels (Bell et al., 1978). Therefore, under these conditions, fibrin deposition should be diminished. Ancrod was administered to wild-type mice for 3 days before crush and then throughout the experimental period, without any effects on survival. Consistent with experiments with genetic depletion of fibrinogen, ancrod treatment significantly enhanced the number of myelinating axons (Figure 2C), and prevented fibrin deposition (Figure 2F). Quantification of myelinating axons per 0.1 mm^2 showed: wild-type control, 5.9 $\pm$ 0.8; *fib*$^{-/-}$, 22.7 $\pm$ 4; wt + ancrod, 25.2 $\pm$ 5.8 (Figure 2G; difference, $p < 0.002$). Overall, these results demonstrate that fibrin deposition is an inhibitory factor for the early phase of sciatic nerve remyelination.

We additionally compared the status of regeneration in wild-type and *fib*$^{-/-}$ mice at late time points after sciatic nerve crush injury when there was clearance of fibrin in the wild-type mice. We observed that 35 days after crush, both wild-type and *fib*$^{-/-}$ mice showed the same extent of axonal regeneration and remyelination (data not shown). Overall these results demonstrate that fibrin deposition is an inhibitory factor for the onset and early time points of sciatic nerve remyelination and that its clearance is a physiological mechanism correlated with nerve repair.

Fibrin Deposition Does Not Affect Axonal Sprouting or Macrophage Infiltration

Functional nerve regeneration after injury is a multistep process that requires axonal regrowth of the damaged axons to their targets and remyelination. The analysis reported above relied on the detection of myelin to identify regenerating nerves. Thus, the delayed regeneration in the wild-type mice could be due to retardation of axon elongation or remyelination.

To identify which aspect of regeneration was affected by fibrin deposition, we first examined the progress of axonal growth after injury. GAP-43 is expressed by developing or regenerating axons, and can thus serve as a marker for axon sprouting and elongation (Benowitz and Routtenberg, 1997). Axonal expression of GAP-43 appears as early as 4 days after sciatic nerve crush injury (Woolf et al., 1990). Schwann cells can also express GAP-43 after injury, but that expression does not begin until 12 days after injury (Scherer et al., 1994). To examine whether fibrin depletion affected axonal regeneration, we stained longitudinal sections of sciatic nerve 8 days after crush injury. As expected, wild-type uninjured mice showed no staining for GAP-43 (Figure 3A). Eight days after crush injury, both wild-type (Figure 3B) and fibrin-depleted mice (Figure 3C) showed a similar pattern of axonal staining of GAP-43 and similar extent of

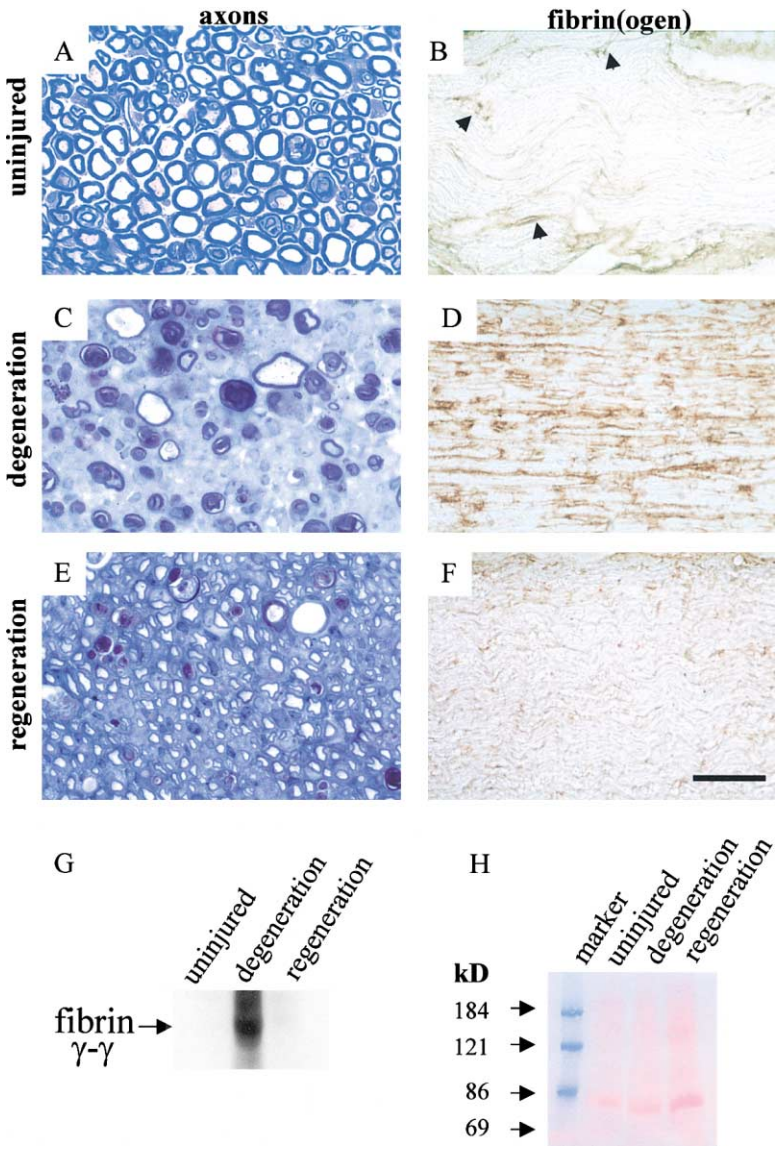

Figure 1. Fibrin Degradation Correlates with Remyelination after Injury

Immunocytochemistry for fibrinogen showed only vascular staining in the uninjured sciatic nerve (B, arrows). Tolouidine blue-stained semi-thin cross-sections of the sciatic nerve showed normal histology as demonstrated by numerous axons tightly packed and surrounded by myelin sheaths (A). Two days after crush, there was fibrin deposition in the nerve (D), which correlated with degeneration as detected by collapsed myelin sheaths and axonal loss (C). During the remyelination phase, 21 days after the crush injury, fibrin deposition was cleared (F) and its immunoreactivity dropped to uninjured wild-type levels (B). At this time, regenerating axons with small diameter and thin myelin sheath appeared (E). (G) Immunoblot for fibrin on sciatic nerve protein extracts showed absence of fibrin in the uninjured nerve, dramatic fibrin deposition during degeneration (4 days after crush injury), and clearance during regeneration (25 days after crush injury). (H) Ponseau staining demonstrated equal protein loading between samples. Bar: 150 μm (B, D, and F); 20 μm (A, C, and E).

axonal elongation. To further verify the axonal pattern of GAP-43 immunostaining, we stained longitudinal sections of wild-type and fibrin-depleted mice for GAP-43 and neurofilament, an axon-specific marker (Hsieh et al., 1994; Shaw et al., 1985). The morphology of the GAP-43-positive axons (Figure 3K) was similar to the morphology of the neurofilament-positive axons (Figure 3L), suggesting that axons are the major source of GAP-43 expression 8 days after crush injury.

To quantitate if there was a difference in axon sprouting and elongation, we also stained cross-sections of the sciatic nerve 1 mm distal to the crush site. Eight days after crush, sciatic nerves from control (Figure 3E) and fibrin-depleted wild-type mice (Figure 3F) showed a similar number of GAP-43-positive axons, suggesting that axonal sprouting is not affected by fibrin depletion. To examine whether the elongation rate differed between the two groups, GAP-43 staining was performed on cross-sections of the sciatic nerve 8 mm distal to the crush site 8 days after crush. No difference was observed in the number of the axons that had reached the distal tip of the nerve between wild-type (Figure 3H) and fibrin-depleted mice (Figure 3I). Quantitation of GAP-43-positive axons showed that there was no difference in the number of regenerating axons between wild-type and ancrod-treated mice 8 days after crush injury (Figure 3J). Similar results were obtained after examination of *fib*$^{-/-}$ mice (not shown). To examine whether there was a difference at earlier time points, we examined GAP-43 immunoreactivity 3 and 5 days after crush. In both earlier time points, there was no difference between wild-type mice and wild-type mice treated with ancrod in the number of GAP-43-positive axons (data not shown). Overall, these results indicated that the inhibitory effect of fibrin on regeneration after sciatic nerve injury was not on axonal sprouting and elongation.

We examined other possible mechanisms that fibrin might affect during peripheral nerve injury. Macrophages are known to accumulate in the sciatic nerve

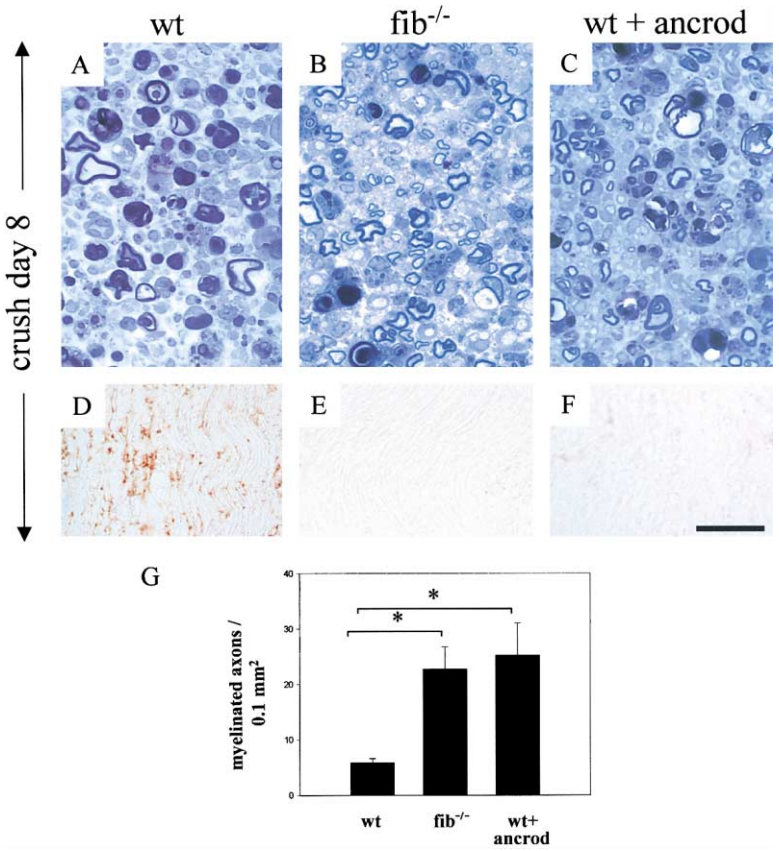

Figure 2. Fibrin Depletion Accelerates Appearance of Myelinated Axons after Injury

Eight days after crush injury, wild-type mice showed sciatic nerve degeneration characterized by axons with collapsed myelin sheaths and a few myelinated axons (A). At the same time point, fibrinogen-deficient mice (B) and wild-type mice treated with ancrod (C) showed numerous myelinated axons. Fibrin immunoreactivity was present in wild-type mice (D), with no staining in *fib*$^{-/-}$ (E) and little staining in ancrod-treated mice (F). (G) shows morphometric analysis of myelinated axons 8 days after sciatic nerve crush. Data are expressed as means ± SEM. Statistical comparisons between medians were made with the Student's t test. *fib*$^{-/-}$ mice ($n = 4$) and ancrod-treated wild-type mice ($n = 4$) showed significantly more myelinated axons compared to control mice ($n = 6$) ($p < 0.002$). Bar: 20 μm.

after crush (Stoll et al., 1989) and enhance axonal regeneration (Richardson and Lu, 1994). A possible mechanism therefore could be that fibrin inhibits macrophage infiltration in the tissue. In this hypothesis, the number of macrophages should be higher in *fib*$^{-/-}$ mice, when compared to wild-type mice. Immunostaining for Mac-1, a macrophage-specific marker (Ho and Springer, 1982) on longitudinal (Figure 4A) and cross-sections (Figure 4D) of wild-type nerve showed that there were no macrophages in the uninjured nerve. Four days after crush injury, macrophages were increased in both wild-type (Figure 4B) and *fib*$^{-/-}$ mice (Figure 4C) on longitudinal sciatic nerve sections. Cross-sections of wild-type and *fib*$^{-/-}$ mice 4 and 8 days after crush injury (Figures 4E, 4F, 4H, and 4I) showed infiltration of the injured nerve by macrophages. To verify whether there was a difference in numbers of macrophages, we quantitated the number of Mac-1-positive cells on cross-sections of sciatic nerve (Figure 4G). There was no statistically significant difference in the number of macrophages 4 or 8 days after crush injury in wild-type and *fib*$^{-/-}$ mice (Figure 4G). Similar data were obtained using F4/80 (not shown), as another macrophage marker (Austyn and Gordon, 1981), indicating that there is no significant difference in the number of macrophages in between the two genotypes after crush injury. As shown in Figure 4G, 4 days after injury, the number of macrophages increased in the nerve of both wild-type and *fib*$^{-/-}$ mice, as expected by results described in other studies (Perry et al., 1987; Stoll et al., 1989). The number of macrophages 8 days after injury remained constant (Siebert et al., 2000), probably due to macrophage removal by local apoptosis (Kuhlmann et al., 2001). Overall, these results suggest that fibrin is not the determining factor for macrophage infiltration in the sciatic nerve.

Fibrin Affects Schwann Cell Proliferation

The observation that fibrin-depleted mice have more newly myelinated axons after injury (Figure 2), but similar axonal elongation and sprouting (Figure 3), suggested that fibrin deposition might regulate nerve repair at the level of myelination. Remyelination is a crucial step for restoration of function since demyelinated axons may maintain both their afferent and efferent connections but have poor conduction due to loss of myelin (Horner and Gage, 2000). Therefore, we examined the effect of fibrin depletion on the myelinating cells of the sciatic nerve, the Schwann cells. After sciatic nerve injury, Schwann cells proliferate, establish contacts with the newly formed axons, stop proliferating, and differentiate into myelinating cells (Jessen and Mirsky, 1999a). To examine whether fibrin affects Schwann cell differentiation, we compared their proliferation in the presence or absence of fibrin 4 days after crush, the peak of Schwann cell proliferation after sciatic nerve injury (Bradley and Asbury, 1970). Sciatic nerves of wild-type and *fib*$^{-/-}$ mice were crushed and 4 days after injury, mice were injected intraperitoneally with BrdU, a thymidine analog that is incorporated in the DNA of cells that are in S phase of the cell cycle. Two hours after injection, mice were sacrificed and BrdU^{+} nuclei were detected with a biotinylated antibody against BrdU. Fibrin-

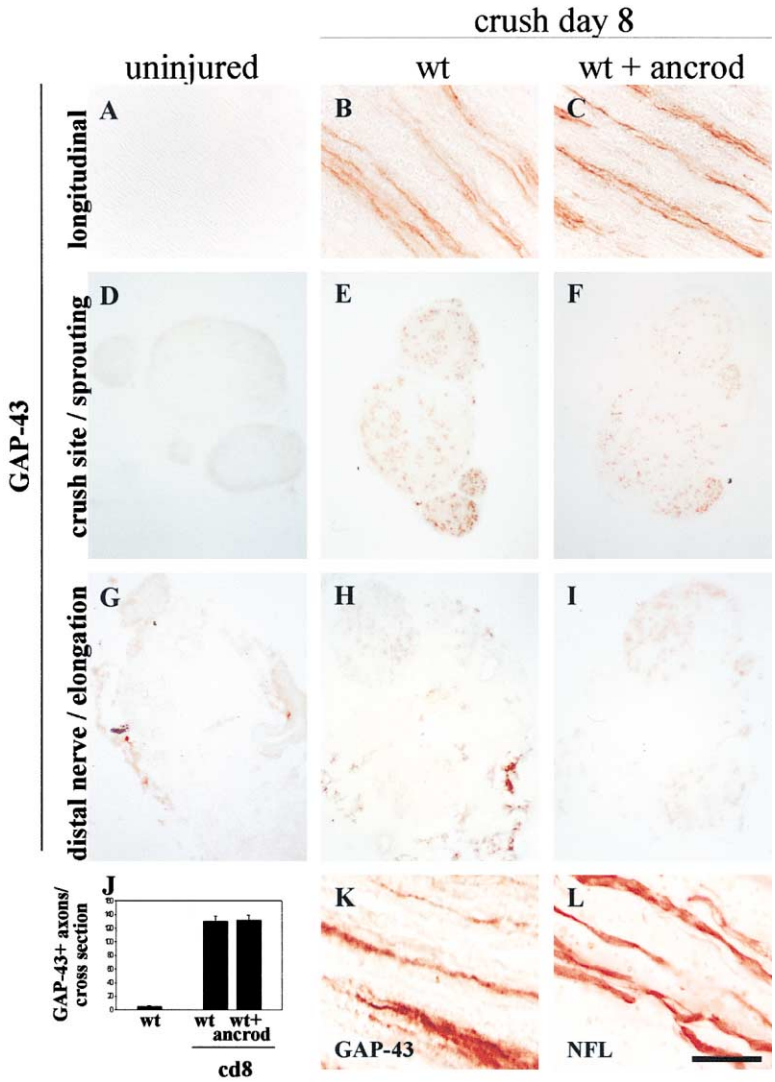

Figure 3. Depletion of Fibrin Does Not Affect Axonal Sprouting or Elongation

Immunostaining with an antibody against GAP-43 showed an axonal staining pattern in longitudinal sections of both wild-type (B) and fibrin-depleted (C) sciatic nerves 8 days after crush injury. Cross-sections of sciatic nerves showed the same extent of axonal sprouting close to the crush site in wild-type (E) and fibrin-depleted wild-type mice (F) and the same number of axons that have elongated toward the distal site of the crush (H and I). Uninjured sciatic nerve showed no staining for GAP-43 (A, D, and G). (J) Quantification of GAP-43-positive axons 8 days after crush. Data are expressed as means ± SEM. Wild-type mice treated with ancrod ($n = 3$) did not show any difference in the number of GAP-43-positive axons when compared to control mice ($n = 4$). High power of longitudinal sections of sciatic nerves show GAP-43-positive axons (K) with the same morphology as neurofilament-positive axons (L). Bar: 80 μm (A, B, and C); 350 μm (D to I); 25 μm (K and L).

depleted mice (Figure 5B) had a dramatic decrease in the number of proliferating cells compared to wild-type mice (Figure 5A). In vivo BrdU labeling of wild-type uninjured sciatic nerve showed no proliferating cells (Figure 5C). To determine if the $BrdU^+$ nuclei were from Schwann cells, we performed double staining using an antibody against S100, a general Schwann cell marker (Kioussi and Gruss, 1996). Almost all the cells with $BrdU^+$ nuclei were stained for S100 (Figure 5D). Quantification of the number of $BrdU^+$ nuclei showed that fibrin-depleted mice had 50% fewer $BrdU^+$ cells compared to wild-type mice (Figure 5E) and this difference was statistically significant ($p < 0.01$). Eight days after crush, when Schwann cell proliferation is still observed at the injured compared to the uninjured nerve (Bradley and Asbury, 1970), *fib*$^{-/-}$ mice also had fewer $BrdU^+$ cells compared to wild-type mice (data not shown). Overall, these results suggest that fibrin regulates Schwann cell proliferation.

Fibrin Induces ERK1/2 Phosphorylation

To examine if fibrin regulated a signaling pathway that affects proliferation, we examined the two forms of the extracellular signal-regulated kinase (ERK), p44 (ERK1) and p42 (ERK2), using an antibody specific for the phosphorylated form of ERK1/2. The ERK1/2 signaling pathway plays a critical role in the regulation of cell growth and differentiation (Kolch, 2000). ERK1/2 phosphorylation is induced by the Ras oncogene and blocks cell apoptosis. Since in the presence of fibrin, Schwann cells showed increased proliferation (Figures 5A and 5E), we examined if fibrin would affect ERK1/2 phosphorylation. We compared Schwann cells cultured on laminin, a major component of the ECM of the uninjured nerve, or on fibrin. Schwann cells cultured 3 days on fibrin showed a dramatic increase of phosphorylated ERK1/2 when compared to cells cultured on laminin (Figure 6A). The levels of total ERK1/2 were similar in both cells that grow on laminin and fibrin (Figure 6A), showing that the difference in phosphorylated ERK1/2 was due to regulation of ERK1/2 at the level of phosphorylation and not due to differences in total expression of ERK1/2.

We also examined the effect of fibrin on ERK1/2 phosphorylation at earlier time points in culture. We performed immunoblot analysis from Schwann cell extracts 1 day after plating on fibrin or on laminin. Quantitation

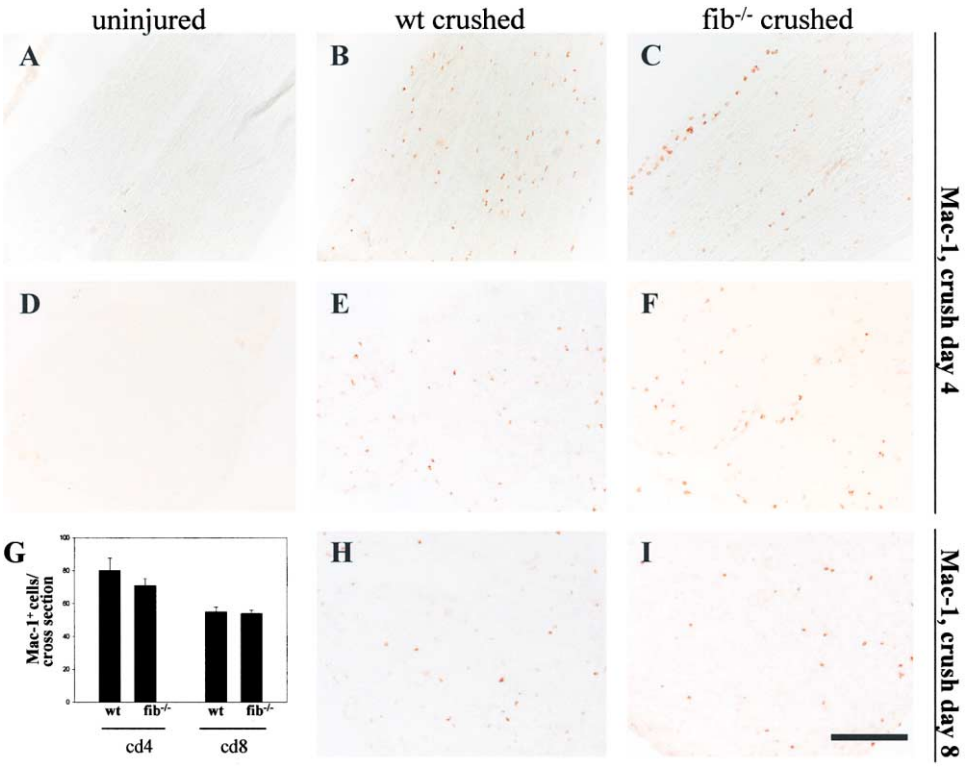

Figure 4. Fibrin Depletion Does Not Affect Macrophage Infiltration

Immunostaining for the macrophage marker Mac-1 on longitudinal sections showed macrophages in both wild-type (B) and *fib*$^{-/-}$ (C) mice 4 days after crush injury. Cross-sections of sciatic nerves stained with Mac-1 of wild-type mice 4 days (E) and 8 days after injury (H) showed no difference in macrophage infiltration when compared to *fib*$^{-/-}$ mice (F and I) at the same time points. Longitudinal (A) and cross-sections (D) of uninjured sciatic nerves did not show any staining for Mac-1. (G) Quantification of Mac-1-positive macrophages on cross-sections did not show any difference in the number of Mac-1-positive cells of *fib*$^{-/-}$ mice (n = 4) when compared to control mice (n = 4) 4 and 8 days after sciatic nerve crush injury. Data are expressed as means ± SEM. Bar: 100 μm.

showed a 19-fold increase of phosphorylated ERK1/2 of the cells grown on fibrin versus those on laminin for 1 day (Figure 6B). Longer culture times on fibrin for 3 days further increased the levels of ERK1/2 phosphorylation up to a 27-fold increase (Figure 6B). Schwann cells grown on laminin for 3 days also showed a 4-fold increase of ERK1/2 phosphorylation when compared to Schwann cells grown on laminin for 1 day (Figure 6B). These results show that fibrin is a potent inducer of ERK1/2 phosphorylation and that phosphorylation of ERK1/2 increases with cell culture time.

To examine whether inhibition of ERK1/2 phosphorylation affects Schwann cell proliferation on fibrin, we cultured primary Schwann cells in the presence of U0126, a specific inhibitor in the pathway that leads to ERK1/2 phosphorylation (Favata et al., 1998). We performed immunoblot analysis on extracts from Schwann cell cultured in the presence of the inhibitor and observed that U0126 completely inhibited ERK1/2 phosphorylation (Figure 6B). After 3 days in culture, the Schwann cells grew more vigorously on a fibrin matrix (Figure 6D), when compared to those cultured on laminin (Figure 6C). Addition of U0126, the inhibitor of ERK1/2 phosphorylation, had no effect on cells growing on laminin (Figure 6E). In contrast, addition of U0126 to Schwann cell cultures growing on fibrin dramatically reduced the number of cells (Figure 6F). Taken together, these results suggest that one mechanism by which fibrin maintains Schwann cells in a proliferating, nonmyelinating state is through induction of ERK1/2 phosphorylation.

Fibrin Regulates Schwann Cell Differentiation

The earlier withdrawal of the Schwann cells from a proliferating state in the absence of fibrin suggested their early differentiation to myelinating cells. Myelinating cells upregulate myelin genes and produce myelin proteins, such as P_0 protein and myelin basic protein, in contrast to the migrating-proliferating cells. Expression of these myelin genes can serve as a marker for the differentiated state. We investigated whether after injury, sciatic nerves from fibrin-depleted mice expressed myelin genes earlier than wild-type mice. In situ hybridization with a P_0 cRNA probe showed that 8 days after crush, Schwann cells from wild-type mice had not yet started P_0 mRNA expression (Figure 7A), whereas *fib*$^{-/-}$ mice had significant P_0 mRNA synthesis (Figure 7B). In addition, we examined P_0 mRNA expression in sciatic nerve extracts using semi-quantitative RT-PCR analysis. Using specific primers for P_0, we performed RT-PCR analysis on cDNA prepared from sciatic nerves of mice in postnatal day 6, adult wild-type, and wild-type and *fib*$^{-/-}$ 8 days after crush injury, using different cycles for amplification. RT-PCR for actin was performed on the

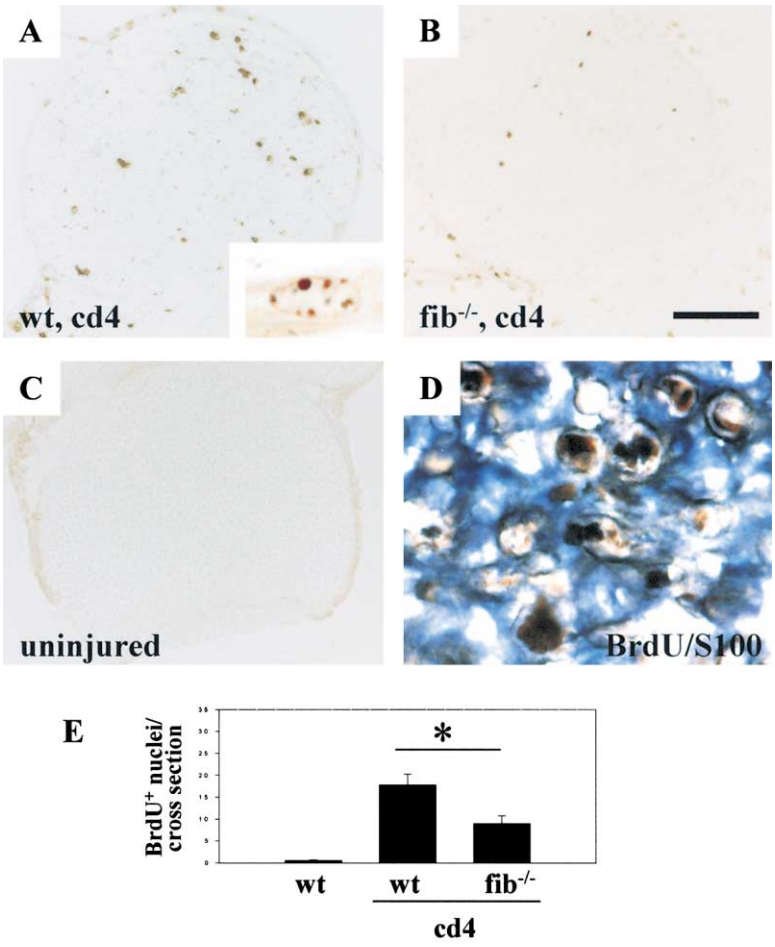

Figure 5. Fibrin Induces Schwann Cell Proliferation

Four days after sciatic nerve crush, *fib*$^{-/-}$ mice showed fewer BrdU-positive nuclei (B) than wild-type mice (A, inset shows a magnification of a BrdU^{+} nuclei). (C) Uninjured sciatic nerve did not show BrdU-positive nuclei. (D) In vivo BrdU labeling (brown) counterstained with anti-S100 (blue) showed that the BrdU^{+} cells are also positive for the S100 Schwann cel marker. (E) Quantification of BrdU^{+} nuclei 4 days after sciatic nerve crush. Data are expressed as means ± SEM. Statistical comparisons between medians were made with the Student's t test. *fib*$^{-/-}$ mice (n = 4) showed 50% less BrdU^{+} nuclei compared to control mice (n = 6) (*p < 0.01). Bar: 130 μm (A, B, and C); 25 μm (D).

same samples to determine cDNA integrity and amount between samples. As expected, sciatic nerve from P6 showed a high level of P_0 expression (Figures 7C and 7D) since at this state, the nerve actively myelinates (Jessen and Mirsky, 1999b). Consistent with our in situ hybridization data, after injury, sciatic nerve from *fib*$^{-/-}$ mice showed a 3-fold increase in P_0 mRNA, when compared to wild-type mice (Figures 7C and 7D). To examine whether fibrin could also have an effect in the expression of P_0 in vitro, we cultured Schwann cells in the presence of forskolin, a cAMP analog that is known to induce myelin gene expression (Lemke and Chao, 1988). We observed a 2-fold increase of P_0 mRNA expression of Schwann cells cultured on laminin versus those cultured on fibrin. These results suggest that fibrin both in vivo and in vitro is a downregulator of expression of P_0 myelin gene.

Since P_0 is a marker for differentiated myelinating Schwann cells, we also examined the expression of the p75 NGFR, a marker that identifies immature Schwann cells. It is well established that p75 NGFR is strongly activated by withdrawal of axons (Lemke and Chao, 1988) and its expression correlates with proliferating, nonmyelin-producing Schwann cells (Zorick and Lemke, 1996). Western blot analysis demonstrated expression of p75 NGFR in sciatic nerve extracts of postnatal day 6 (Figure 7E). Three or eight days after crush, expression of p75 NGFR is observed in sciatic nerve extracts of wild-type mice (Figure 7E). In contrast, *fib*$^{-/-}$ mice show a dramatic decrease in the production of p75 NGFR (Figure 7E). Overall, these results show that fibrin promotes the production of p75 NGFR after injury and provide further evidence that Schwann cells in the absence of fibrin are at a less immature state than in wild-type mice.

Our results that *fib*$^{-/-}$ mice show reduced cell proliferation raised the possibility that these mice might have a reduced number of Schwann cells compared to wild-type mice. To address this question, we counted total nuclei of wild-type and *fib*$^{-/-}$ sciatic nerves at different time points after the crush (Figure 7F). The total number of nuclei remained constant between the two genotypes. Our results suggest that wild-type and *fib*$^{-/-}$ mice have the same total number of cells, but the phenotype of these cells differs. In wild-type mice, Schwann cells are at a proliferating (Figure 5A), nonmyelinating state (Figure 7A), while in *fib*$^{-/-}$ mice, they are at a low-proliferating (Figure 5B), myelinating state (Figure 7B). Overall, these data demonstrate that depletion of fibrin accelerates Schwann cell differentiation to a myelinating, nonproliferating state.

Fibrin Inhibits Fibronectin Expression in the Sciatic Nerve

During nerve development and regeneration, the fibronectin level increases, which promotes Schwann cell

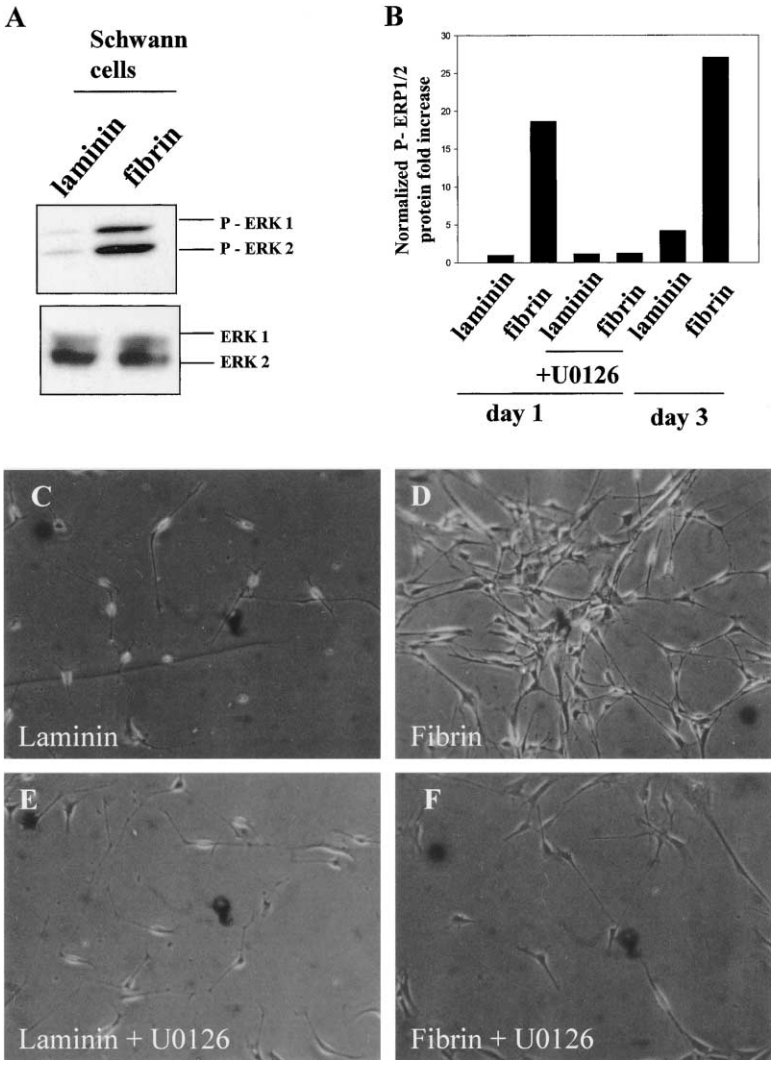

Figure 6. Fibrin-Induced Schwann Cell Proliferation Is Mediated through ERK1/2 Phosphorylation

(A) Sciatic nerve protein extracts from Schwann cells grown on laminin or on fibrin for 3 days were immunoblotted with an antibody that recognizes phosphorylated ERK1/2. Phosphorylated ERK1/2 was detected in Schwann cells grown on laminin, but a dramatic increase was observed in cells that grow on fibrin. Immunostatining for total ERK1/2 showed equal levels between samples. (B) Quantification of immunoblots of extracts of Schwann cells grown on fibrin for 1 day showed an 19-fold increase of ERK1/2 phosphorylation when compared to Schwann cells grown on laminin. Addition of U0126, a specific inhibitor of ERK1/2 phosphorylation, did not allow phosphorylation of ERK1/2 to occur. Schwann cells grown on laminin for 3 days showed a 4-fold increase compared to cells grown on laminin for 1 day, while Schwann cells grown on fibrin for 3 days showed a 27-fold increase of ERK1/2 phosphorylation. (C) Schwann cells grew on laminin after 3 days in culture and inhibition of phosphorylation of ERK1/2 by addition of U0126 does not inhibit their growth (E). Schwann cells grew vigorously on fibrin (D), while inhibition of ERK1/2 phosphorylation inhibited their growth on fibrin (F).

migration and correlates with their terminal differentiation to a nonproliferative, myelinating state (Chernousov and Carey, 2000). Since fibrin maintains Schwann cells in a proliferative state, it was possible that part of this effect might be mediated through inhibition of fibronectin expression. To address this question, wild-type and *fib*$^{-/-}$ sciatic nerves were stained for fibrin, laminin, and fibronectin 8 days after crush injury. Uninjured, wild-type mice did not show any staining for fibrin (Figure 8A; Akassoglou et al., 2000), while there was a significant deposit after crush (Figure 8B). *Fib*$^{-/-}$ mice after injury did not show any fibrin staining as expected (Figure 8C). Immunostaining for laminin showed that uninjured wild-type sciatic nerve had extracellular laminin (Figure 8D). After crush, both wild-type (Figure 8E) and *fib*$^{-/-}$ mice (Figure 8F) showed similar levels of laminin immunoreactivity, suggesting that fibrin deposition does not affect the synthesis and deposition of laminin at the sciatic nerve after injury. Immunostaining for fibronectin showed that the uninjured, wild-type nerve expressed very little fibronectin (Figure 8G). After injury, there was an increase in fibronectin in wild-type nerves (Figure 8H), and a much larger increase in *fib*$^{-/-}$ nerves (Figure 8I). Western blot analysis for fibronectin on sciatic nerve protein extracts also demonstrated increased production of fibronectin in wild-type mice treated with ancrod 8 days after crush (Figure 8J). These results suggest that fibrin deposition inhibits fibronectin production at the sciatic nerve after crush.

To further define fibronectin expression in myelinating and nonmyelinating nerve, we examined Western blots from extracts of sciatic nerves from P6 and adult mice with a fibronectin-specific antibody. Sciatic nerve protein extracts from mice at P6, when active myelination is robust (Jessen and Mirsky, 1999b), showed strong production of fibronectin, when compared to sciatic nerve from adult mice, when myelination is largely completed (Figure 8K). This result further establishes that an increase in fibronectin levels correlates with myelination. Fibronectin in the sciatic nerve is synthesized by Schwann cells (Vogelezang et al., 1999). To examine whether fibrin affects Schwann cell capacity to produce fibronectin, we cultured Schwann cells on laminin or on fibrin. After 4 days in culture, we prepared protein extracts and examined fibronectin production by immunoblot. While Schwann cells cultured on laminin pro-

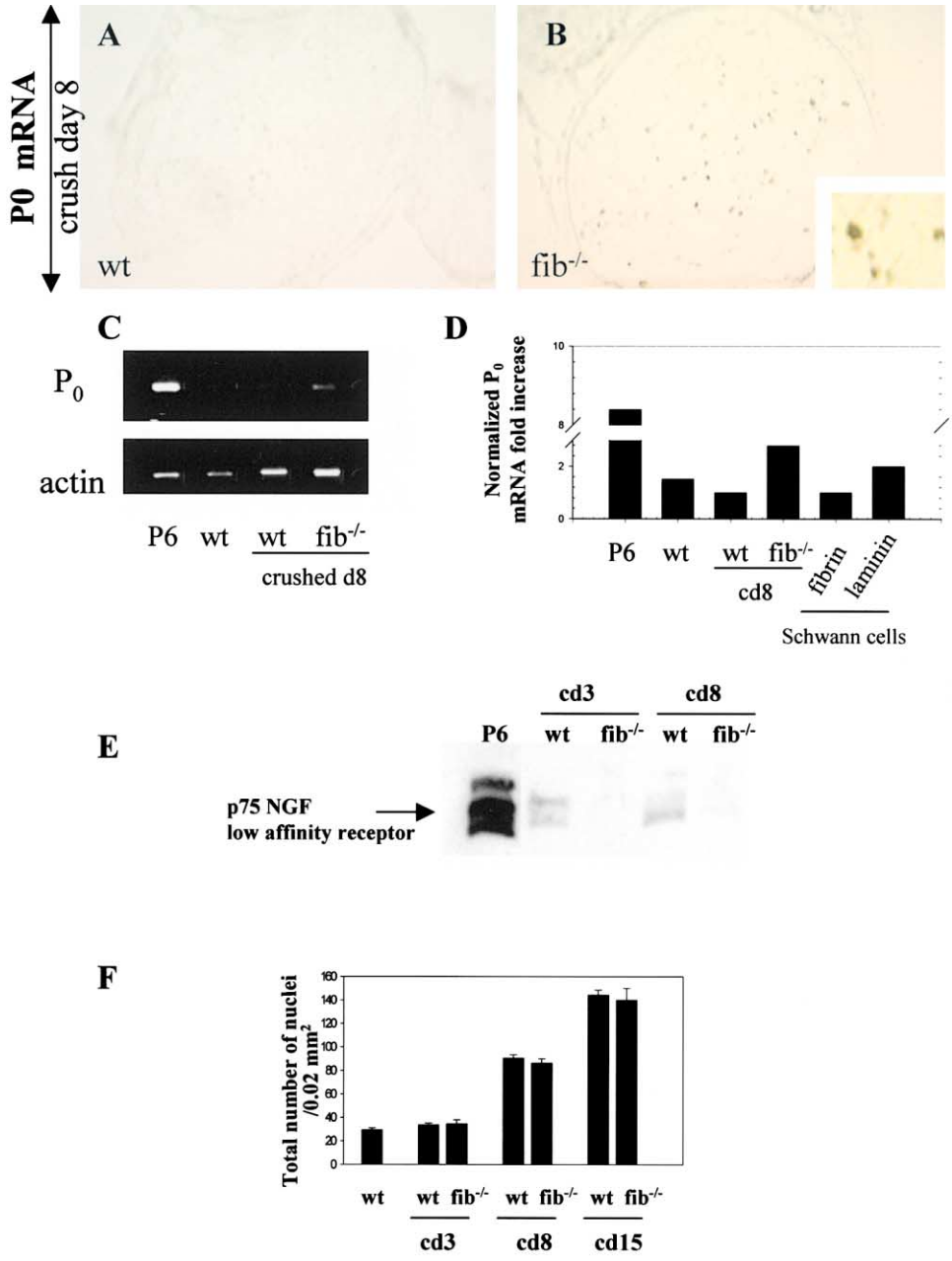

Figure 7. Fibrin Delays Myelin Gene Transcription and Regulates Schwann Cell Differentiation

In situ hybridization for P_0 mRNA 8 days after crush showed minimal expression in the wild-type sciatic nerve (A), while P_0 mRNA is detected in *fib*$^{-/-}$ mice (B, inset shows a high magnification of the P_0 positive cells). (C). Agarose gel of semi-quantitative RT PCR for P_0 and β-actin on sciatic nerve cDNA from P6, wild-type; wild-type and *fib*$^{-/-}$ 8 days after crush sciatic nerves. (D) Graphical representation of the quantified autoradiography of the RT-PCR normalized for actin showed a 3-fold increase in P_0 transcript in the *fib*$^{-/-}$ mice 8 days after crush when compared to wild-type mice. Quantitation of RT-PCR of forskolin-treated Schwann cells cultured on laminin showed a 2-fold increase of P_0 expression, compared to cells cultured on fibrin. (E) Western blot analysis for p75 NGFR showed decreased expression in *fib*$^{-/-}$ mice 3 and 8 days after crush. (F) Counting of total DAPI-stained cell nuclei per 0.02 mm^2 showed the same number of cells between wild-type and *fib*$^{-/-}$ mice at three different time points after the crush injury.

duce fibronectin, cells grown on fibrin produced undetectable levels of fibronectin (Figure 8K).

To determine if the inhibition of fibronectin expression by fibrin was at the mRNA or protein level, we performed in situ hybridization with a cRNA probe for fibronectin. Eight days after crush, wild-type mice treated with ancrod had a dramatic increase in the number of cells that synthesize the fibronectin transcript (Figure 8M), when compared to wild-type mice (Figure 8L). These data suggest that fibrin deposition after sciatic nerve crush injury inhibits expression of fibronectin mRNA and therefore fibronectin protein production by the Schwann cells.

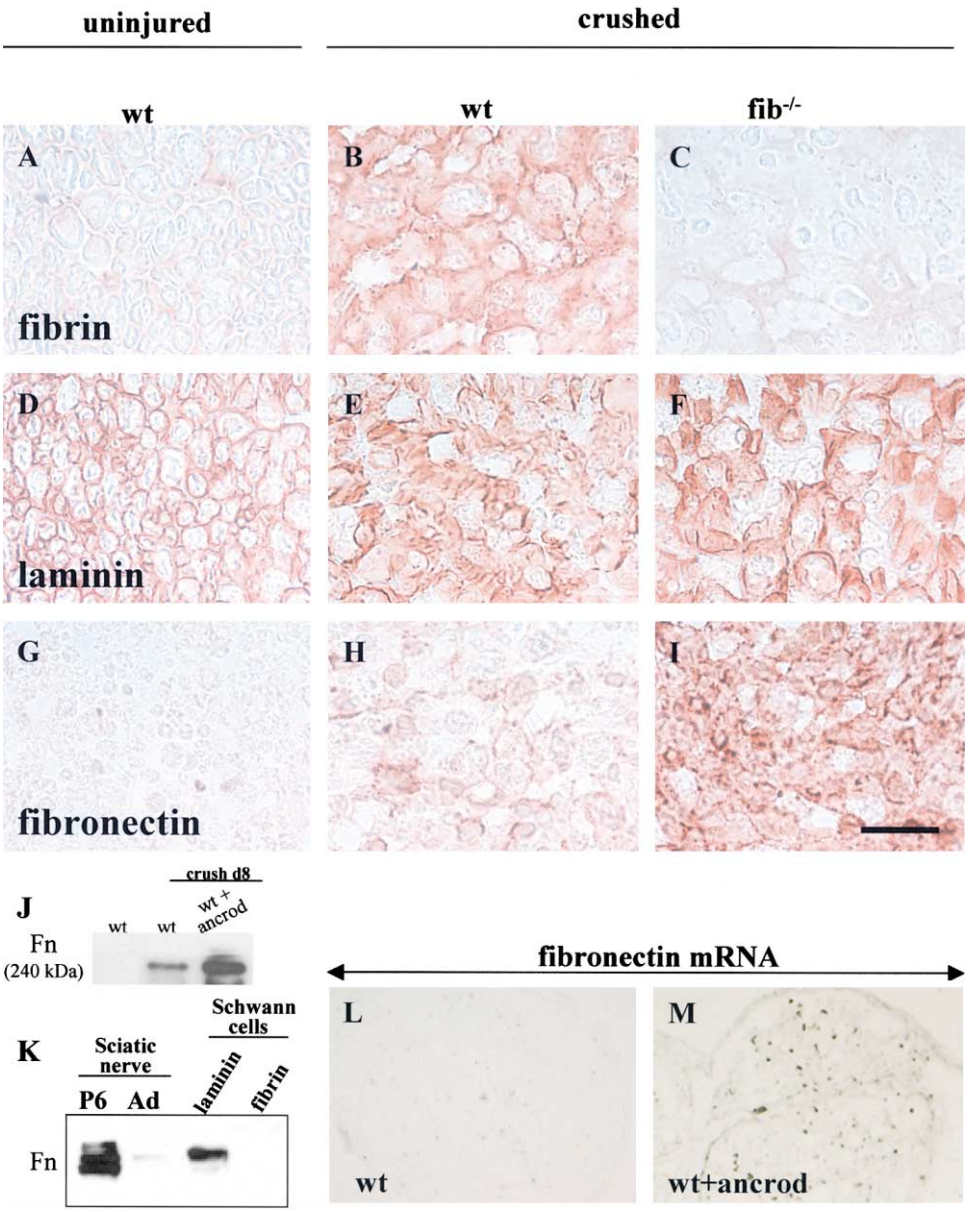

Figure 8. Fibrin Deposition Inhibits Fibronectin Production by Schwann Cells

Eight days after sciatic nerve crush immunostaining for fibrin showed increased deposition in wild-type sciatic nerve (B). Uninjured wild-type sciatic nerve (A) and crushed *fib*$^{-/-}$ nerve (C) did not show fibrin immunoreactivity. Immunostaining for laminin showed the same levels of laminin immunoreactivity in wild-type uninjured (D), wild-type crushed (E), and *fib*$^{-/-}$ crushed (F) sciatic nerves. Immunostaining for fibronectin showed no staining in uninjured sciatic nerve (G), minimal staining in wild-type crushed sciatic nerve (H), and a dramatic increase in staining in *fib*$^{-/-}$ crushed nerve (I). (J) Immunoblot for fibronectin on sciatic nerve protein extracts showed increased fibronectin production in fibrin-depleted mice 8 days after crush when compared to wild-type mice. (K) Immunoblot for fibronectin on sciatic nerve protein extracts showed increased fibronectin production at postnatal day 6, while the adult mouse had minimal levels of fibronectin. Protein extracts from Schwann cells cultured on laminin and on fibrin showed that there was fibronectin expression in the laminin cultures but not in fibrin cultures. In situ hybridization for fibronectin mRNA on crushed sciatic nerves showed many more fibronectin-producing cells in the fibrin-depleted mice (M), compared to wild-type mice (L). Bar: 30 μm (A to I); 150 μm (L and M).

Discussion

Fibrin and Peripheral Nerve Remyelination

Studies on the regulation of nerve regeneration have focused on molecules made by glial cells and neurons. Blood-derived fibrin, which is not present under physiological conditions as a component of the ECM, but which gains access to nervous tissue through disease or pathology, can interfere with repair progression by inhibiting the differentiation of Schwann cells to a myelinating state. Our results, and previous experiments, suggest the following model for the role of fibrin in remyelination after sciatic nerve injury (Figure 9). (1) Injury leads to a breakdown of the blood-nerve barrier, and fibrinogen can leak into the nerve where it is converted into fibrin by an activated coagulation pathway (Friedmann et al.,

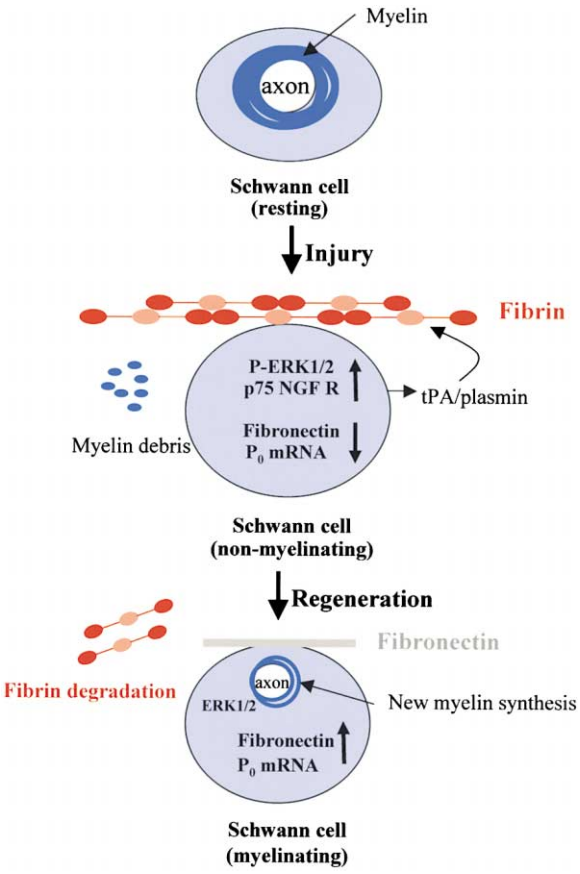

Figure 9. Proposed Mechanism for the Effects of Fibrin in Peripheral Nerve Remyelination

Myelinating Schwann cells in the adult normal sciatic nerve have established axonal contacts and produce myelin that enwraps the axons. After injury, axons degenerate, myelin gets degraded, and fibrin is deposited. Fibrin deposition regulates Schwann cell numbers by affecting two major cell survival pathways: phosphorylation of ERK1/2 and upregulation of p75 NGFR. A combined action of the proliferating ERK1/2 pathway and the p75 NGFR apoptotic pathway sustains the Schwann cells at a nonmyelinating state, as evidenced by the shut down of myelin genes transcription, such as P_0 and the downregulation of the fibronectin transcript. Downregulation of the P_0 or the fibronectin gene could either be due to the increased ERK1/2 phosphorylation as observed in transformed cells (Brenner et al., 2000), and/or by another mechanism. When fibrin is degraded during regeneration, ERK1/2 becomes nonphosphorylated, p75 NGFR is downregulated, fibronectin is produced and deposited in the nerve, and Schwann cells transcribe myelin genes and remyelination is initiated. Upregulation of P_0 associated with downregulation of p75 NGFR is a characteristic change associated with myelination (Morgan et al., 1991).

1999). (2) Coincident with fibrin deposition, tPA is induced in Schwann cells (Akassoglou et al., 2000; Clark et al., 1991). (3) Injury stimulates generation of proliferative Schwann cells, which do not produce myelin components (Kioussi and Gruss, 1996). This proliferation phase is necessary to allow Schwann cells to repopulate the regenerating axon as it elongates. (4) Schwann cells then establish axonal contacts and differentiate into a nonproliferative state, and begin the production of new myelin and ECM components. This transition is dramatically inhibited by fibrin. In the presence of fibrin, Schwann cells increase ERK1/2 phosphorylation, continue to proliferate, express p75 NGFR, and produce little fibronectin and P_0. This combination of effects retards the formation of new myelinated axons. In this scheme, the dissolution of fibrin by the tPA/plasminogen system allows the gradual return of the Schwann cell to its final, myelinating state. Fibrin clearance would allow remyelination to eventually occur in wild-type mice, which would explain why at later time points after injury, a difference is not observed between wild-type and *fib*$^{-/-}$ mice.

Although fibrin inhibits Schwann cell differentiation, overall it could play a beneficial role in nervous system repair. Interaction of Schwann cells with fibrin would signal that an injury had taken place, and that proliferation was required for restoration of myelinated nerves. As long as fibrin remained, indicating that the injury had not been healed, the cells would maintain their proliferative, undifferentiated state. The disappearance of fibrin would in turn signal that the composition of the ECM had normalized and that myelination could begin. Given the effect of fibrin on many other pathological situations (described below), Schwann cells may be an example of a general concept: that cells may be primed to detect fibrin so that they can gauge tissue damage, upregulate the production of proteins of the proteolytic pathway, and delay repair processes until the tissue has healed sufficiently to make it appropriate. In cases of chronic injury and fibrin deposition, or inadequate fibrin clearance, this beneficial mechanism could prove deleterious due to retardation of repair.

Schwann Cell Proliferation, Myelination, and Fibrin

The regulation of Schwann cell proliferation is critical for myelination. For example, mutations in the NF-1 tumor suppressor gene (Cichowski and Jacks, 2001); Krox-20, a transcription factor present only in myelinating Schwann cells (Zorick et al., 1999); or the PMP22 myelin gene (Robertson et al., 1997) trigger abnormal Schwann cell proliferation and hypomyelination. Alterations in growth factor pathways can also lead to abnormalities in proliferation and myelination. Neuregulin, a trophic factor that promotes Schwann cell proliferation during development (Dong et al., 1995), induces indefinite proliferation in Schwann cells cultured in the absence of hormones in low serum (Mathon et al., 2001), and inhibits myelination in neuron-Schwann cell co-cultures (Zanazzi et al., 2001). Taken together, these studies suggest a reverse correlation between Schwann cell proliferation and myelination. Our data demonstrate that in addition to oncogenes, transcription, and growth factors, the ECM protein fibrin can induce Schwann cell proliferation and therefore become a negative regulator of myelination.

The effects of fibrin on Schwann cell proliferation appear to be mediated through the ras signal transduction pathway via upregulation of ERK1/2 phosphorylation (p44/42 MAPK). In many cellular systems, activation of the MAPK pathway by trophic factors has been implicated in the regulation of gene transcription (Treisman, 1996) associated with proliferation and differentiation (Cowley et al., 1994). After deposition, fibrin degradation

by the tPA/plasmin system could trigger dephosphorylation of ERK1/2 and the differentiation of the Schwann cells to myelin-producing cells.

The effects of fibrin on Schwann cells may be regulated via integrin signaling since activation of the MAP kinase pathway is mediated by integrins (Aplin et al., 2001; Chen et al., 1994). Fibrin could either mediate ERK1/2 phosphorylation directly by binding to specific integrins or indirectly by upregulation of other factors that induce ERK1/2 phosphorylation and Schwann cell proliferation. One such factor is interleukin-1, since fibrin enhances transcription of interleukin-1 β in blood mononuclear cells (Perez et al., 1999; Perez and Roman, 1995). In the sciatic nerve, interleukin-1 β is secreted by Schwann cells (Rutkowski et al., 1999), fibroblasts, and macrophages and is one of the major cytokines that regulate the inflammatory response. In addition, interleukin-1 β induces ERK1/2 phosphorylation (Ng et al., 2001) and promotes Schwann cell proliferation (Lisak et al., 1994). A possible effect of fibrin on the cytokine profile of the sciatic nerve could be envisaged and this might be an additional mechanism that fibrin could utilize to affect ERK1/2-mediated Schwann cell proliferation.

While Schwann cell proliferation is less in the absence of fibrin, the total number of cells remains the same. This raises the possibility that fibrin not only regulates Schwann cell proliferation, but also affects Schwann cell survival mechanisms. For example, fibrin induces p75 NGFR expression, a receptor involved in Schwann cell apoptosis (Soilu-Hanninen et al., 1999; Syroid et al., 2000). Schwann cells proliferate less in *fib*$^{-/-}$ mice, but since the expression of p75 NGFR is also lower, they would be less prone to apoptosis and therefore their number would remain constant. A similar mechanism for the regulation of Schwann cell number has been proposed for *krox-20*$^{-/-}$ mice (Zorick et al., 1999). During early postnatal development, *krox-20*$^{-/-}$ mice exhibit both more proliferation and more apoptosis of Schwann cells, and the Schwann cell number remains constant between wild-type and *krox-20*$^{-/-}$ mice. Therefore, fibrin, by inducing ERK1/2 phosphorylation and p75 NGFR expression, could regulate both proliferation and apoptosis after sciatic nerve crush.

Since fibrin becomes a component of the ECM after injury, it could compete with other components of the ECM, such as fibronectin. Fibronectin signals primarily through heterodimeric integrin receptors binding to arg-gly-asp (RGD) and adjacent sequences in the central binding domain (Pytela et al., 1985). Schwann cell migration on fibronectin is blocked by RGD peptides, suggesting that migration is mediated by an RGD-dependent integrin (Milner et al., 1997). Since each fibrin molecule contains two RGD peptides (Thiagarajan et al., 1996), fibrin might act as an antagonist of fibronectin and possibly other members of the ECM that signal through RGD-dependent integrins.

Fibrin induction of ERK1/2 phosphorylation could be responsible for the downregulation of the fibronectin gene. In Ras-transformed cells, increase of ERK phosphorylation blocks induction of fibronectin (Brenner et al., 2000). In this view, as fibrin is cleared from the nerve by the PA/plasminogen system, the cells would increase production of fibronectin and differentiate. Therefore, fibrin might affect Schwann cell differentiation by preventing formation of the appropriate ECM that promotes myelination. The establishment of an appropriate ECM at later time points after crush when fibrin is cleared would facilitate remyelination in wild-type mice.

Our results demonstrate an inhibitory role for fibrin in remyelination in the PNS after traumatic injuries. Fibrinogen is not synthesized by nervous system cells, but it can enter in the nervous tissue when there is a leakage of the blood-nerve barrier (BNB). BNB disruption is considered a key mechanism of inflammatory demyelination in traumatic injuries and autoimmune peripheral nervous system diseases, such as Guillain-Barré syndrome (Hughes et al., 1998; Kieseier et al., 1999). Fibrin(ogen) therefore could be one of the pathogenic components of BNB disruption that could contribute to nerve damage. A deleterious role for fibrin deposition has been shown to contribute to degeneration in various pathologies in other tissues (Busso et al., 1998; Drew et al., 1998; Kitching et al., 1997; Tang and Eaton, 1993), including the central nervous system (Inoue et al., 1996; Paterson, 1976), and it can also delay wound healing (Bugge et al., 1996). Given the effects of fibrin in the PNS, fibrin might regulate ERK1/2 phosphorylation and cell survival in other pathologies associated with fibrin deposition.

Our observations that defibrinogenated tPA$^{-/-}$ mice show enhanced regeneration efficiency (our unpublished data) provide evidence that clearance of fibrin, even in the absence of innate proteolytic activity, enhances regeneration capacity. This result suggests that depletion of fibrinogen or prevention of fibrin deposition might improve regeneration in peripheral and possibly central nervous system injuries. The identification of fibrin deposition as a new inhibitory aspect of regeneration may yield additional strategies to promote repair in the nervous system.

Experimental Procedures

Animals and Sciatic Nerve Crush

Mice deficient for the fibrinogen *A*α chain gene (*fib*$^{-/-}$; Suh et al., 1995) were of mixed genetic background, and *fib*$^{+/+}$ littermates were employed as controls in all studies. For the pharmacological depletion of fibrinogen, we used C57Bl/6J mice. No differences were observed between C57Bl/6J mice and *fib*$^{+/+}$ mice in the crush injury model. All mice were 8–15 weeks old at the start of the experiment. The genotype of all mice was confirmed at the end of the experiments by PCR analysis of genomic DNA extracted from mice tails. Sciatic nerve crush and systemic defibrinogenation with ancrod delivery via subcutaneous implantation of mini-osmotic pumps were performed as described (Akassoglou et al., 2000).

Immunohistochemistry

Dissected nerves were embedded in Tissue-Tek OCT (Sakura, Torrance, CA), then immediately frozen on dry-ice and stored at −70°C until use. Sections were cut longitudinally on a motor-driven Leica cryostat with a retraction microtome and a steel knife at a cabinet temperature of −20°C. Immunohistochemical staining was performed on cryostat sections (Akassoglou et al., 1998). Primary antibodies were as follows: goat anti-human fibrin(ogen) (Chemicon, Temecula, CA) (1/500); rabbit anti-human fibronectin (Dako, Carpinteria, CA) (1/500); rabbit anti-mouse laminin (Sigma, St. Louis, MO) (1/1000); rabbit anti-GAP-43 (Chemicon) (1/5000); rat anti-mouse Mac-1 (Boehringer-Mannheim) (1/500); rabbit anti-neurofilament H (Chemicon) (1/500). Bound antibodies were visualized using the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (AEC) (Sigma) as a chromogen. Staining specificity for the fibrin(ogen)

antibody was confirmed using tissue from *fib*$^{-/-}$ mice. Staining specificity for the other antibodies was confirmed using rabbit IgG. Incubation without the first antibody served as a negative control.

In Situ Hybridization for P_0 and Fibronectin

In situ hybridization was performed as described in Akassoglou et al., 1998. For the P_0 probe, 5′-CACTATGCCAAGGGACAAC-3′ sense and 5′-CAGACATAGTGGGCAAGAC-3′ antisense oligonucleotides were used to PCR amplify 175 bp of exon 3 of the P_0 gene. For the fibronectin gene, 5′-AAACACTTGTCTTTCCACAG-3′ sense and 5′-TCTTATGGTTTGGTCTGG-3′ antisense oligonucleotides were used to PCR amplify 324 bp of the fibronectin gene. The PCR products were cloned using the TA PCR-cloning system (Invitrogen, Carlsbad, CA). Cloning was performed in both orientations and accordingly sense and antisense mRNA probes were produced using the T7 promoter of the PCR 2.1 vector (Invitrogen).

Semi-Quantitative RT-PCR

Total RNA was extracted from sciatic nerves and Schwann cell cultures using Trizol (Life Technologies, Rockville, MD). cDNA was generated using Superscript II reverse transcriptase (Life Technologies). Amplification of β-actin was performed using the 5′-GTC CTGTATGCCTCTGGTC-3′ sense and the 5′-TCGTACTCCTGCTTG CTGAT-3′ antisense oligonucleotides. For P_0, the primers described above were used. PCR reactions were performed in different cycles (15, 20, and 25). PCR products were run on agarose gels. Agarose gels were transferred to nylon membranes and were processed for hybridization. The ^{32}P DNA probes used for hybridization were prepared by amplification of genomic DNA with the oligonucleotides for P_0 and β-actin described above. Hybridization was performed in Rapid Hyb-Buffer (Amersham, UK). Autoradiographies were scanned, analyzed using Scion Corporate Image Analysis Software, and the P_0 levels were normalized against actin expression.

Quantification of Myelinated Axons and Total Nuclei

Eight days after injury, the crushed sciatic nerve was removed and approximately 2.5 mm above and below the lesion was isolated and prepared for semi-thin sectioning (Akassoglou et al., 2000). The noninjured sciatic nerve (contralateral) served as a control. A morphometric grid (100 mm^2) was adapted to the microscope and a minimum of three grids per sample of myelinated axons was counted. For counting the total number of cells, cross-sections were stained for DAPI (Vector Laboratories) and were observed under a Zeiss Axiophot microscope. A minimum of four areas per cross-section was counted.

Cell Culture

Schwann cells were isolated as described (Milner et al., 1997). Briefly, sciatic nerves from neonatal P0–P2 mice were minced and incubated in 0.1% trypsin (Sigma) and 0.03% collagenase (Sigma) at 37°C, 5% CO_2 for 30 min. The enzymatic reaction was blocked by trituration in blocking solution (3 mg/ml BSA, Sigma; 10 μg/ml DNase, Sigma; 0.5 mg/ml trypsin inhibitor, Sigma; all in Hanks' buffered solution, Life Technologies, Rockville, MD). After centrifugation, the cells were resuspended in DMEM containing 10% FCS and penicillin and streptomycin (Life Technologies), and grown at 37°C, 5% CO_2. The cells were left on bacteriological plastic dish plates for 3 hr to allow fibroblast adhesion, following which nonadherent cells were shaken off and plated onto poly-D-lysine-coated dishes in DMEM (Life Technologies) containing 10% FCS. After 1 day, the cells were trypsinized briefly to remove Schwann cells while leaving contaminating fibroblasts on the dish. The cells were used within 3 to 5 days. For the induction of expression of myelin genes, cultures were treated for with forskolin as described (Lemke and Chao, 1988). For the inhibition of ERK1/2 phosphorylation, U0126 (Cell Signaling Technology, Beverly, MA) was used at 1.5 μM in the tissue culture medium and was replaced every 24 hr. Control cultures received equal amounts of DMSO (Sigma).

Immunoblots

Coating with fibrin was prepared as described (Lansink et al., 1998). Briefly, 2 mg of murine fibrinogen (Sigma), 0.1 U/ml thrombin, 2.5 U factor XIII were added to 1 ml of DMEM and 300 μl added per 1 cm^2 of plate. Plates were placed in the incubator for 2 hr and the formation of gel was detected macroscopically. Schwann cells were cultured as described above. After 3 days in culture they were plated on 6-well plates that were covered either with laminin or fibrin. Cells were cultured for 1 or 3 days and protein extracts were prepared with tissue homogenization in SDS lysis buffer. Protein concentration was determined using the Bradford protein assay and equal amounts of protein were loaded on an SDS-PAGE gel. Electrophoresis was performed according to standard procedures and the proteins were transferred to a nylon membrane which was incubated with an antibody against fibronectin (DAKO, 1/5,000), rabbit anti-mouse fibrin(ogen) (kind gift of Jay Degen, Children's Hospital, Cincinnati, OH), phosphorylated ERK1/2 (Cell Signaling Technology, 1/1,000), or rabbit anti-p75 NGFR (Chemicon, 1/1,000) followed by incubation with anti-rabbit HRP (Cell Signaling Technology, 1/10,000). All membranes were stained with Ponsau to assess equal loading and transfer for all samples. Detection of HRP was performed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Autoradiographies were scanned, analyzed using Scion Corporate Image Analysis Software, and normalized levels were used for the calculation of the differences between different extracts.

In Vivo and In Vitro BrdU Labeling

BrdU (Roche, Indianapolis, IN) was dissolved in water and injected intraperitoneally at a concentration of 100 mg/kg. Mice were sacrificed 2 hr after injection. Sciatic nerves were isolated and cryostat blocks were prepared. $BrdU^+$ nuclei were detected using the BrdU labeling kit (Oncogene Research Products, San Diego, CA). BrdU/S100 double staining was performed as described (Gaiano et al., 2000) with the following alterations. Briefly, PFA-fixed cryostat sections of BrdU-injected mice were incubated with an antibody against S100 (1:500, Sigma). Immunoreactivity was detected using the alkaline phosphatase ABC kit (Vector) and developed with the Blue Alkaline phosphatase substrate (Vector). Sections were then processed with the BrdU labeling kit, with the modification that instead of acid denaturation, sections were treated with 5 μg/ml DNase I (Sigma) in TBS with 10 nM $MgCl_2$ and 10 nM $MnCl_2$ for 30 min at 37°C. BrdU-positive nuclei were detected with DAB (Oncogene Research Products).

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