The Effects of Trapidil on Fibroblastic Activity, Schwann Cell Proliferation, and Platelet-derived Growth Factor Receptor Levels in the Experimental Sciatic Nerve Injury

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Background: Trapidil, an antianginal drug with inhibitory effects on platelet-derived growth factor (PDGF), has been shown to reduce reactive fibrosis in models of central nervous system injury. Therefore, we evaluated trapidil's effects on fibroblast activity, Schwann cell proliferation, and PDGF receptor levels in rats after transection and anastomosis of the sciatic nerve. We also evaluated the effect of Schwann cell proliferation on regeneration of anastomosed sciatic nerves.

Methods: Wistar rats were randomly divided into 3 groups. Group 1 (N = 10) was used to determine normal peripheral nerve morphology (via light and electron microscopy) and PDGF receptor levels. Group 2 (N = 20) underwent sciatic nerve anastomosis after nerve transection to examine the influence of the surgical procedure on PDGF receptor levels and the microscopic findings. Group 3 (20 rats) received a single intraperitoneal dose of trapidil (40 mg/kg) immediately after the surgical procedure to investigate its effects on fibroblast activity, Schwann cell proliferation, and PDGF levels.

Results: PDGF-A and PDGF-B receptor levels were lower in group 3, the trapidil-treated group, than group 2 on all posttransection days examined. Ultrastructural analysis revealed that group 3 also had lower levels of fibroblast activity and Schwann cell proliferation than group 2. However, the peripheral nerve ultrastructure and degree of axonal regeneration were similar between groups 2 and 3 at 14 days posttransection.

Conclusions: Trapidil treatment significantly decreased reactive fibrosis and PDGF receptor expression after peripheral nerve injury, and thus, may be useful therapeutically. These results also suggest that Schwann cells alone are not effective in promoting neurite formation.

Key Words: growth factor, peripheral nervous system, PDGF, Schwann cell, trapidil

INTRODUCTION

Peripheral nervous system (PNS) injury induces a wide range of responses in neuronal and surrounding tissues. If the peripheral nerve is completely severed, a proximal stump neuroma develops. It is composed of whorls of disorganized and branching fine axons, mixed with a proliferation of connective tissue and Schwann cells.¹ Sensory and motor axons are capable of regenerating over long distances and reestablishing synaptic connections with their targets. They regenerate toward the distal stump, and this attraction depends on the presence of live Schwann cells in the distal nerve segment.²

The distal stump also undergoes marked changes after peripheral nerve transection. These changes, collectively known as Wallerian degeneration, include the recruitment of macrophages and inflammatory cells, the breakdown and clearance of myelin sheaths, and Schwann cell proliferation.³ This is accompanied by the up-regulation of growth-promoting substrates and a massive increase in the synthesis of neurotrophic factors. Schwann cells play a major role in neurite formation, providing a bioelectrical matrix for regenerating axons. Later, Schwann cells remyelinate the regenerated axons.

Regeneration should be clearly distinguished from regrowth. Regrowth is aimed solely at restoring the trophic link between the severed axon and other neurons, forming a neural network with no capacity to handle information. In contrast, regeneration is true functional recovery, allowing neural information to be handled as before the lesion. Although the factors that initiate nerve regeneration after axonal lesioning are not understood, some studies have shown Schwann cells are critical to promoting axonal regeneration.⁴⁻⁶ Therefore, 1 goal of the present study was to examine the effect of Schwann...
cell proliferation on regeneration of anastomosed sciatic nerves.

Connective tissue usually responds to nerve transection by proliferating in a haphazard and unstructured fashion. Mitogens for fibroblasts and Schwann cells include growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). The resultant reactive fibrosis (scar tissue) may represent a barrier to regenerating axonal sprouts. For example, Hermanson et al found that increased PDGF expression was a major cause of posttraumatic reactive gliosis after facial nerve axotomy in rats.

Trapidil is an antiangiogenic drug with diverse pharmacological properties, including a nitroglycerinlike vasodilating action, an inhibitory effect on platelet aggregation, and facilitatory actions on both prostacyclin synthesis and lipid metabolism. Several studies have shown that trapidil also has potent inhibitory effects on PDGF, leading some investigators to examine its effects after nerve injury. In experimental models of central nervous system (CNS) injury, trapidil treatment effectively suppressed or prevented the development of reactive fibrosis. Therefore, another objective of this study was to investigate the effects of trapidil on Schwann cell proliferation, fibroblast activity, and PDGF receptor levels after peripheral nerve injury.

**MATERIALS AND METHODS**

**Animals and Experimental Groups**

Male Wistar rats, weighing 280 to 330 g each, were used for this study. All surgical procedures were performed under an operating microscope with anesthesia induced by the intramuscular injection of ketamine hydrochloride (150 mg/kg).

The right sciatic nerve was exposed at the sciatic notch and transected with microscissors; the 2 nerve stumps were sutured end-to-end with 8.0 epineural suture.

The rats were randomly divided into 3 groups. Group 1 rats (N = 10) were used to determine normal PDGF receptor levels and light and electron microscopic features of normal peripheral nerve morphology; these animals did not undergo surgery or drug treatment. Group 2 rats (N = 20) underwent sciatic nerve saturation after nerve transection; these animals were used to determine the influence of this surgical procedure on PDGF receptor levels and peripheral nerve morphology (ie, light and electron microscopic findings). Group 3 rats (N = 20) received a single intraperitoneal dose of trapidil (40 mg/kg) (Rocornal, Rentschler-UCB, Kerpen, Germany) immediately after the surgical procedure; these animals were used to examine the effects of trapidil on fibroblast activity, Schwann cell proliferation, and PDGF receptors after peripheral nerve injury.

All experiments were conducted at the Experimental Research Center for Medical Sciences of Çukurova University, and the Medical Faculty’s Ethical Committee approved the study protocol.

**Sample Preparation**

The exposed sciatic nerve segments were immediately removed from the animals in all groups a certain interval after the surgical procedure. In groups 2 and 3, 5 rats each were killed 1, 3, 7, and 14 days after the surgical procedure.

For the light and electron microscope examinations, samples of sciatic nerve were obtained from the proximal, central, and distal portions of the suture line.

For PDGF immunohistochemistry and structural analysis, animals from each group were anesthetized and then immediately perfused. The initial perfusion was carried out with approximately 100 mL of 0.1-M phosphate-buffered saline (pH 7.3). Next, 150 mL of 4% paraformaldehyde was infused under constant pressure with an infusion pump. Ten minutes after the perfusion, each sciatic nerve was removed and tissues for the assay were obtained from the proximal, central, and distal portions of the suture line. The sample was divided transversely into 2 consecutive blocks, which were assigned sequential numbers from anterior to posterior. The first blocks were stored at −70°C and later used for PDGF immunohistochemical analysis; the second blocks were used for the structural analysis.

**Electron Microscopy**

Tissue specimens for electron microscopic examination were immediately placed in 5% glutaraldehyde buffered with Milloning’s phosphate buffer (pH 7.4) for 3 hours. Next, the samples were fixed in 1% osmium tetroxide for 2 hours, dehydrated with serial concentrations of ethanol, embedded in araldite, and processed for examination with a Zeiss EM 900 transmission electron microscope.

**PDGF Immunohistochemistry**

The frozen tissues were cut into 8-μm sections with a cryostat, fixed in cold acetone for 5 minutes, and then rinsed in Tris-buffered saline (TBS) × 3 for 5 minutes. Next, they were incubated in 3% H2O2 to block endogenous peroxidase activity and washed in TBS. The sections were then incubated with PDGF-A (Santa-Cruz, sc-128) or PDGF-B (Santa-Cruz, sc-7878) antibody for 60 minutes at room temperature. After incubation with the primary antibody, the sections were washed extensively with TBS. They were then incubated with a biotinylated second antibody for 10 minutes at room temperature and washed with TBS. Next, the sections were reacted with 3-amino 9-ethyl carbazole (AEC working color reagent) and counterstained with Mayer hematoxylin. Finally, they were processed with Zymed kit (Zymed Lab, cat. no.: 95-6143, San Francisco) and visualized using a streptavidin-biotin technique.

For negative controls, some of the sections were treated with a nonimmunogenic serum instead of the same concentration of primary antibody. Aminoethylcarbazole was used as the chromogenic substrate.

Immunohistochemical staining for PDGF-A and PDGF-B receptors was evaluated and scored on the basis...
of the percentage of stained cells observed with a light microscope; at least 100 cells were counted per field at a usual magnification of 40×. The cells were then categorized into the following groups:

- (none); no staining,
- + (sparse): <5% of the cells stained,
- ++ (moderate); 5% to 50% of the cells stained,
- +++ (severe); >50% of the cells stained.

**Statistical Analysis**

The Mann-Whitney U test was used for all statistical analyses. A P value < 0.05 was considered statistically significant.

**RESULTS**

**Immunohistochemical Examination**

Although a small amount of immunostaining was seen in perineurial cells, nerve tissue from group 1 rats was not immunoreactive for PDGF-A or PDGF-B (Fig. 1). In contrast, tissue from groups 2 and 3 rats showed widespread PDGF-A and PDGF-B immunoreactivity, involving structures such as the endoneurium, the cytoplasm of Schwann cells, mononuclear cells, macrophages, and the walls and tissue surrounding blood vessels.

Immunoreactivity for PDGF-A receptors was detected the first day after nerve transection in tissue from rats in group 2 and reached its maximum level by day 3 (Fig. 2A). The level of immunoreactivity had decreased by day 7 and seemed minimal by 14 days after transection. In contrast, immunoreactivity for PDGF-B receptors was not detected the first day after nerve transection, but was present by day 3. The level of immunoreactivity peaked on day 7 (Fig. 2B) and seemed minimal by 14 days after transection.

Tables 1 and 2 summarize semiqualitative data regarding the percentage of stained cells in groups 2 and 3. Compared with group 1, the number of PDGF-A–positive and PDGF-B–positive cells was markedly increased in group 2 (P < 0.05). Conversely, the number of PDGF-A–positive and PDGF-B–positive stained cells in group 3, the trapidil-treated group, was significantly lower than that in group 2 (P < 0.05). In group 3, the number of PDGF-A–positive cells did not significantly differ from the number of PDGF-B–positive cells (P > 0.05).

**Ultrastructural Findings**

**Group 1 (Controls)**

Electron microscopic examination of the sciatic nerve revealed normal myelinated and unmyelinated
nerve fibers. Schwann cells enclosed nerve fibers as neurilemma, and normal ultrastructural features such as Schwann cells, nerve fibers, and capillary vessels were visible (Fig. 4).

### Table 1. PDGF Immunoreactivity in Rat Nerve Tissue From the Experimental Group 2*

<table>
<thead>
<tr>
<th>Day</th>
<th>PDGF-A Staining Score†</th>
<th>PDGF-B Staining Score†</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+/–</td>
</tr>
<tr>
<td>3</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>–</td>
<td>+/–</td>
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</table>

*This group of rats (N = 20) underwent sciatic nerve transection and anastomosis alone.
†A semiquantitative scoring system (see Methods) was used to evaluate and compare levels of immunoreactivity for PDGF-A and PDGF-B receptors between groups.

PDGF indicates platelet-derived growth factor.

### Table 2. PDGF Immunoreactivity in Rat Nerve Tissue From the Experimental Group 3*

<table>
<thead>
<tr>
<th>Day</th>
<th>PDGF-A Staining Score†</th>
<th>PDGF-B Staining Score†</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3</td>
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<tr>
<td>7</td>
<td>+</td>
<td>+</td>
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<td>14</td>
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*A semiqualitative scoring system (see Methods) was used to evaluate and compare levels of immunoreactivity for PDGF-A and PDGF-B receptors between groups.
†This group of rats (N = 20) received a single dose of intraperitoneal trapidil (40 mg/kg) immediately after sciatic nerve transection and anastomosis.

PDGF indicates platelet-derived growth factor.

### Group 2 (Sciatic Nerve Transection + Suturation)

- **Group 2-A (Day 1):** Areas of edema and hemorrhage were visible between the nerve fibers. Axonal edema and separation and disruption of the myelin sheath lamellae were frequently noted. Although cytoplasmic vacuolization was visible in Schwann cells, the capillary vessels and connective tissue seemed similar to those in group 1.
- **Group 2-B (Day 3):** The myelinated nerve fibers showed moderate to severe degeneration of their myelin sheaths. Separation/disruption of the myelin lamellae and axonal changes were common, as were regions of fibrosis and edema. The endoplasmic reticulum cisternae of fibroblasts also seemed enlarged (Fig. 5A).
- **Group 2-C (Day 7):** Most of the myelinated nerve fibers revealed axonal and myelin sheath degeneration, although the degree was milder than that observed in groups 2-A and 2-B. The ultrastructure of Schwann cells and unmyelinated nerve fibers seemed normal, but diffuse fibrosis was noted.
- **Group 2-D (Day 14):** The electron microscopic appearance of the nerve fibers was similar to that in group 2-C. Myelinated nerve fibers revealed mild to severe myelin sheath degeneration, whereas unmyelinated nerve fibers, Schwann cells, fibroblasts, and capillary vessels seemed normal. Diffuse fibrosis was still noted among the nerve fibers (Fig. 5B).

### Group 3 (Trapidil-treated Animals)

- **Group 3-A (Day 1):** Most of the myelinated nerve fibers showed moderate to severe myelin sheath and axonal degeneration, and the unmyelinated nerve fibers revealed axonal changes. Furthermore, nuclear chromatin condensation and cytoplasmic vacuolization were visible in Schwann cells. The ultrastructure of Schwann cells and unmyelinated nerve fibers seemed normal, but diffuse fibrosis was noted.
- **Group 3-B (Day 3):** Although some of the myelinated nerve fibers revealed disruption of their myelin sheaths, axonal structures were generally normal. In addition, the degree of Schwann cell proliferation was milder than that observed in group 2-B. Enlargement of the endoplasmic reticulum cisternae and swelling of some mitochondria were visible in fibroblasts. The ultrastructure of the unmyelinated nerve fibers seemed normal.
- **Group 3-C (Day 7):** Most of the myelinated nerve fibers revealed normal myelin sheath and axonal structures,
although slight to moderate myelin sheath degeneration was seen in some. Schwann cells and fibroblasts had normal ultrastructures (Fig. 6B). Compared with group 2-C, fibrosis among nerve fibers was decreased.

**Group 3-D (Day 14):** The ultrastructures of both the myelinated and unmyelinated nerve fibers were generally normal. Furthermore, Schwann cells and fibroblasts seemed normal, with levels of proliferation that were similar to those observed in group 3-C. In most areas, the ultrastructure of nerve fibers seemed normal (Fig. 6C).

**Pathologic Evaluation of Neurite Formation**

Figure 6C shows the effect of trapidil treatment on neurite formation after experimental sciatic nerve injury. Although trapidil seemed to increase regeneration of anastomosed sciatic nerves, the degree of regeneration in group 3-D did not significantly differ from that in group 2-D.

**DISCUSSION**

In this study, we found increased levels of PDGF-A and PDGF-B immunoreactivity in transected and anastomosed sciatic nerve segments as early as 1 day after injury. We also found that experimental sciatic nerve transection and anastomosis in rats result in reactive fibrosis as early as 3 days after the trauma. The postinjury intraperitoneal administration of trapidil significantly decreased levels of PDGF immunoreactivity and reactive fibrosis. Thus, trapidil may be useful in the treatment of peripheral nerve injury.
As discussed earlier, peripheral nerve injury initiates a series of changes in which Schwann cells seem to play an important role. For example, the presence of live Schwann cells in the distal nerve segment attracts regenerating axons in the proximal stump.\textsuperscript{2} Schwann cells secrete a variety of factors that facilitate regeneration of the injured nerve fibers.\textsuperscript{19} Experimental evidence suggests that these signals, which are retrogradely transported from the site of injury to the nerve cell body, trigger responses including the redirection of RNA and protein synthesis required for regeneration.\textsuperscript{20} Schwann cell proliferation is also an important component of Wallerian degeneration, producing a bioelectrical matrix for the regenerating axons. However, proliferation

\textbf{FIGURE 6.} A, Group 3, day 1 after sciatic nerve transection+anastomosis and trapidil administration. Most of the myelinated nerve fibers exhibit severe myelin sheath (arrows) and axonal (a) degeneration. A Schwann cell (s) shows clumping of nuclear heterochromatin and changes involving cytoplasmic organelles (\( \times 10,100 \)). B, Group 3, day 7 after sciatic nerve transection+a-nastomosis and trapidil administration. Most of the myelinated nerve fibers reveal myelin sheath degeneration (arrows) and axonal (a) changes. Unmyelinated nerve fibers (+) and collagen fibers (c) are indicated (\( \times 6300 \)). C, Group 3, day 14 after sciatic nerve transection+anastomosis and trapidil administration. Most of the myelinated nerve fibers seem normal. Myelin sheath degeneration is less notable (arrows). A Schwann cell (s), fibroblast (f), collagen fibers (c), and axons (a) are indicated (\( \times 8100 \)).
may also result in reactive fibrosis, posing a barrier to regenerating axons.

Although the exact cause of Schwann cell proliferation during Wallerian degeneration is unknown, it may involve substances released during the breakdown of axons and/or myelin. Salzer et al reported that isolated neurites or neurite membranes were mitogenic for Schwann cells and that this stimulation was specific. In addition, Wallerian degeneration is associated with the up-regulation of growth-promoting substrates, and most growth factors seem to stimulate Schwann cell proliferation after peripheral nerve injury.

These growth factors, including the neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, are secreted by a variety of cell types. For example, Schwann cells and fibroblasts in injured nerve segments synthesize nerve growth factor, which, along with other neurotrophic factors, promotes the survival and repair of injured neurons. Although the neurotrophins do not seem to directly enhance peripheral nerve regeneration, they may play a role in remyelinating regenerated axons. Other growth factors include PDGF, ciliary neurotrophic factor, glial-derived neurotrophic factor, insulin-like growth factor-I (IGF-I), fibroblast growth factor, and EGF.

One growth factor with potentially important implications to the PNS is PDGF, a 30-kDa protein consisting of disulfide-bonded dimers of A and B chains. PDGF released from platelets plays a major role in cell biology, namely enhancing cell division and controlling maturation, especially that involving glial cells, fibroblasts, and Schwann cells. However, PDGF has also been linked to the development of reactive fibrosis after nerve injury in the CNS. PDGF binds to 2 types of PDGF receptors, α receptors and β receptors. A variety of cell types in the CNS and PNS express these receptors, including fibroblasts, Schwann cells, vascular smooth muscle cells, and glial cells. In addition, cultured Schwann cells have been shown to secrete PDGF. Thus, PDGF might play a role in the development of the PNS and in maintenance of peripheral neurons.

Studies of the mitogenic effects of PDGF have produced mixed results. For example, Westermark and Heldin reported that PDGF and EGF induce similar intracellular events that transmit a mitogenic signal to fibroblasts. However, Zhang et al reported that neither PDGF nor basic fibroblast growth factor was mitogenic for adult Schwann cells. Hermanson et al studied the expression of PDGF and its receptors in rat facial nerve after axotomy. They found strong immunoreactivity for PDGF in facial neurons and surrounding astrocytes 3 days postoperatively, which remained at high levels until day 26 in animals with a cut injury. Crush injury produced a similar pattern of immunoreactivity, although the level of immunoreactivity had decreased by postoperative day 19, when reinnervation occurred. On the basis of their findings, these investigators concluded that increased PDGF levels were a major cause of posttraumatic reactive gliosis after facial nerve injury. Other investigators have investigated the effect of PDGF administration after nerve injury. Oudega et al reported that the combination of IGF-I and PDGF enhanced myelination but diminished axonal regeneration in a rat model of spinal cord injury. Welch et al reported that the combined administration of PDGF and IGF-I did not enhance peripheral nerve regeneration of transected and anastomosed sciatic nerves in rats.

Several studies have shown that the antianginal drug trapidil has potent inhibitory effects on PDGF. Given the link between PDGF, Schwann cell proliferation, and reactive fibrosis, some investigators have examined the effects of administering trapidil after nerve injury. Takamiya et al reported that trapidil treatment dramatically suppressed the appearance of reactive astrocytes in areas of injury, suggesting that PDGF may play a role in posttraumatic gliosis after brain injury. Göçer et al reported that the immediate systemic administration of trapidil to rats after spinal cord trauma prevented fibrosis, increased Na⁺/K⁺/Mg²⁺ adenosine triphosphatase levels, and decreased lipid peroxidation.

Given these findings in models of CNS injury, we also decided to examine the effects of trapidil on axonal regeneration in an experimental model of peripheral nerve injury.

Our electron microscopic findings suggest that although trapidil treatment decreases cellular proliferation and fibrosis after peripheral nerve injury, it does not promote regeneration of anastomosed nerves. That is, the ultrastructural features and degree of axonal regeneration observed in the trapidil-treated rats 14 days after transection and anastomosis (group 3-D) were similar to those observed in the untreated rats (group 2-D) (P > 0.05). These findings may suggest that Schwann cells, by themselves, are not effective in supporting neurite formation. Trapidil did significantly reduce the amount of cellular damage and edema at the injury zone, possibly reflecting its ability to stabilize membranes by inhibiting thromboxane A₂ synthesis. The finding that levels of PDGF-A and PDGF-B receptors were similar in group 3 may reflect trapidil's nonspecific inhibitory effects on PDGF receptors.

CONCLUSIONS

The present findings support the notion that trapidil treatment decreases PDGF expression and reactive fibrosis after transection and anastomosis of the sciatic nerve. Thus, trapidil may be useful in the treatment of peripheral nerve injury. Further studies are needed to clarify the role of Schwann cells in neurite formation and to identify factors or drugs that promote axonal regeneration in this setting.

REFERENCES