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The effect of the platelet derived wound healing formula and the nerve growth factor on the experimentally injured spinal cord

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The main purpose of this study is to investigate the effect of platelet derived wound healing formula (PDWHF) and nerve growth factor (NGF) in the treatment of experimental spinal cord injury. PDWHF is a conglomerate of growth factors which include platelet derived growth factor (PDGF), platelet derived angiogenesis factor (PDAF), transforming growth factor-beta (TGFβ) and platelet factor IV (PF4). Complete spinal cord transection was performed at T12 in rats and the treatment of the spinal cord injury was achieved by filling the dead space with type 1 collagen gel impregnated with PDWHF, or with 2.5S-NGF. Controls were treated with only type 1 collagen gel. Animals were sacrificed at 1, 2 or 3 months. Histopathologically, tissue autolysis and cavity formation by phagocytosis expanded 1–3 mm into the cord stumps and the volume of cavitation was less in the two treated groups. In the NGF group, a greater number of surviving nerve cells were observed in this region. Most of the control animals formed only thin, short axonal bundles, however, increased axonal regrowth was noted in animals treated with trophic factors, especially in the NGF group. The NGF group formed thick axonal bundles and abundant neuroma. Increased angiogenesis was observed in the collagen gel matrix and the injured spinal cord parenchyma, in the PDWHF group. Recent studies have shown that mammalian adult CNS possesses the ability for structural and/or functional plasticity following injury under appropriate circumstances. In this *in vivo* study, exogenous NGF appeared to induce axomal outgrowth and nerve cell survival. PDWHF produced notable angiogenesis which seemed to improve the extracellular microenvironment. This may be important for the delivery of exogenous trophic factors, nutrients and for the changes of extracellular matrices to support nerve cells and axons.

Keywords: platelet derived wound healing formula (PDWHF); nerve growth factor (NGF); spinal cord injury; axonal regeneration; angiogenesis

Introduction

The purpose of this study is to investigate the possibility of improving the injured spinal cord microenvironment by rescuing the adult spinal cord from secondary neuronal death and by inducing axonal regrowth and revascularization.

In the adult central nervous system (CNS), neuronal cell death or axonal degeneration may occur as a consequence of traumatic injuries. It is tempting to speculate that adult CNS neurons, like those in early development, continue to depend on neurotrophic support for their maintenance, functional competence and repair capabilities.[1] Axotomy of CNS neurons will deprive them of all or part of their target derived and glia derived supply of neurotrophic factors and thus account for their retrograde degeneration or their inability to regenerate.[2] There have also been reports that the intrinsic blood supply and blood flow in the spinal cord at the site of an injury decreased, and the injured spinal cord is more sensitive to ischemia than the peripheral nervous system.[3–10]

To prevent secondary spinal cord degeneration and to induce neural tissue repair, two growth factors were applied in this study. NGF was administered to evaluate whether exogenous neurotrophic factor played a direct role in nerve cell survival or axonal regrowth in the adult spinal cord lesion. PDWHF was administered to evaluate whether improvement in the perineural microenvironment by local neovascularization induced survival and repair of neural tissue. In addition, type 1 collagen gel was evaluated as a matrix for axonal regeneration and as a vehicle for trophic factors.

Materials and methods

Thirty-six adult female Wistar rats (200–250 g) were used. The rats were anesthetized with intraperitoneal pentobarbital (Nembutal) 40 mg/kg. Surgical sites

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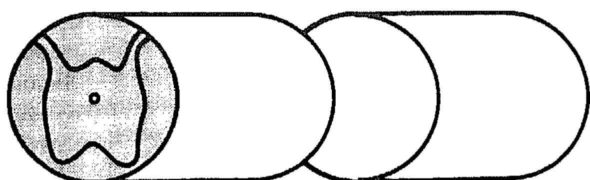
were then prepared and draped for sterile surgery. Laminectomy was performed at T12 and the spinal cord was cut transversely. Immediately after the transection, the spinal cord stumps separated from each other through a distance of 2 to 3 mm. Treatment of the spinal cord injury was achieved by filling this dead space with 0.2 ml of Vitrogen 100 (purified bovine dermal type I collagen, Collagen Corporation, Palo Alto, California) as a matrix for axonal growth and vehicle for trophic factors (Figure 1). This collagen is 95 to 98% type I collagen and was used as a sterile liquid solution at a temperature of 4–6°C. This liquid collagen later turned to a state of gelatin (fibrillogenesis) by warming to the rat body temperature of 37°C in 10 to 20 min and linked the spinal cord stumps with collagen matrix. This collagen solution was impregnated with PDWHF or NGF prior to gelatin. PDWHF is a conglomerate of growth factors which include platelet derived growth factor (PDGF), platelet derived angiogenesis factor (PDAF), transforming growth factor-beta (TGFβ) and platelet derived factor IV (PF4). PDWHF was produced from mouse platelets which had been obtained by platelet pheresis and supplied by Curative Technologies, Inc. (Setauket, NY). Details regarding the production of PDWHF were reported in previous papers.[11,12] The 2.5S-NGF used was supplied by Department of Pharmacology, Washington University (St. Louis, MO).[13–19] In the PDWHF group a solution of 100 μg PDWHF and 0.2 ml type I collagen was administered locally in 12 rats. In the NGF group a solution of 100 μg 2.5S-NGF and 0.2 ml type I collagen gel was administered locally in 12 rats. In the control group, 12 rats were treated with only type I collagen. Animals received appropriate care postoperatively and were sacrificed at 1 month (mo), 2 mo and 3 mo. There were four rats in each treatment group. Daily evaluation of neurological and locomotive function as well as manual emptying of the paralyzed urinary bladder were done twice every day. On the day of sacrifice, the animals were anesthetized with intraperitoneal pentobarbital and perfusion fixation with 10% parafolmaldehyde was performed. The injured spinal cord segments with adjacent normal spinal levels were excised and were fixed in buffered 10% formaldehyde. Longitudinal sections of the spinal cord were made at a thickness of 60 μm and stained with routine H&E, Biel's silver, Laidlaw's silver, LFB-PAS and Masson's trichrome stain. The following numerical figures were obtained by observing whole area of the midline section in the sagittal plane. To evaluate axonal regeneration, a nine-grade (0–8 points) classification by length of axonal growth into the scar tissue, width of axonal bundle, and neuroma formation, was used (Table 1). The size of cavity formation by autolysis was determined by measuring the largest diameter of the largest cavity in the spinal cord stump. To evaluate neovascularization, the number of vessels per 0.25 mm^2 was counted with a computer assisted device. Significance was determined by the student t-test. Animal surgery, postoperative management, and histological evaluation were performed by the first and fourth authors, and others contributed as academic advisors.

SPINAL CORD TRANSECTION



COLLAGEN GEL MATRIX

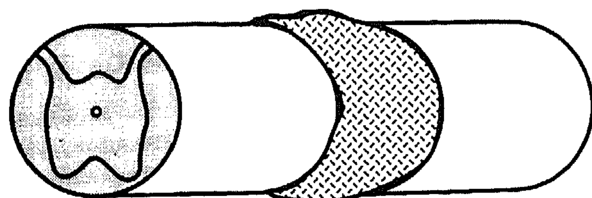


Figure 1 Treatment of transected spinal cord stumps. The dead space caused by spinal cord transection was filled with type I collagen solution which became gelatinated and linked the spinal cord stumps. This collagen solution was impregnated with PDWHF or NGF prior to gelatin

Results

All animals showed complete paralysis of the lower extremities throughout 3 months and developed a kyphotic spinal deformity with the apex at T12. Neither voluntary movement of lower extremities nor locomotive function recovered within 3 months of the observation period in this spinal cord transection study.

Table 1 Classification of axonal growth

Grade	Description
0:	no axonal growth into scar tissue
1:	<1 mm axonal growth into scar tissue
2:	<1 mm axonal growth of a thick bundle into scar tissue
3:	1–2 mm axonal growth into scar tissue
4:	1–2 mm axonal growth of a thick bundle into scar tissue
5:	1–2 mm axonal growth of a thick bundle into scar tissue with neuroma formation
6:	>2 mm axonal growth into scar tissue
7:	>2 mm axonal growth of a thick bundle into scar tissue
8:	>2 mm axonal growth of a thick bundle into scar tissue with neuroma formation

Changes in the collagen gel matrix between the spinal cord stumps

In the control group, the space which had been produced by spinal cord transection and had been filled with collagen gel matrix, was infiltrated by macrophages and fibroblasts at 1 month and was gradually replaced by fibrous connective tissue which synthesized collagen fibers. Cysts of cavities were observed in the spinal cord stumps which represented phagocytosis by macrophages (Figure 2A). At 3 months, the number of lymphocytes and macrophages decreased. The production of collagen fibers in the fibroblast network increased and collagen fiber bundles became thicker with the passage of time (Figure 2B). In the PDWHF group, macrophages also migrated into the spinal cord cut ends. New vessel formation was active in the collagen gel matrix and the spinal cord parenchyma. This was more common in the PDWHF group than in the control group (Figure 2C). In the NGF group, the number of macrophages which infiltrated into the spinal cord cut ends was fewer than in the PDWHF group. Migration of fibroblasts was also less in the NGF group than in the PDWHF group (Figure 2D).

Axonal regrowth and nerve cell survival in the spinal cord stumps

In the control group, within the injured spinal cord parenchyma, autolysis by phagocytic reactions were active in the acute phase and numerous dead nerve cells were found (Figure 3A). At 3 months, tissue autolysis and cavity formation by phagocytosis in the injured spinal cord stump expanded 1–3 mm (2.1 ± 1.3 mm) into the spinal cord parenchyma. By 3 months, cavities had become surrounded by dense collagen fibers as the scar matured (Figure 3B). In eight of 12 control animals, thin, short axonal bunches growing from the nerve roots were observed (Figure 3C). In seven of 12 control animals, sparse axonal regrowth from the spinal cord parenchyma was observed. The substrate of the injured spinal cord was insufficient to support axons and nerve cells (Figure 3D). No obvious neuromas were observed in the control group but were seen in the PDWHF and the NGF groups. No axonal regrowth occurred in the remaining four control animals.

In the PDWHF group, numerous macrophages were present around the surface of injured spinal cord stumps at 1 month. Astroglia also proliferated in this region (Figure 4A). Spinal cord autolysis and subsequent cavity formation was less in the PDWHF group (1.3 ± 0.6 mm) compared to the control group. This was statistically significant ($P<0.05$). Apparent axonal growth was seen from the proximal motor nerve roots as well as the distal sensory nerve roots which were derived mostly from the dorsal root ganglion cells (Figure 4B). Axonal regeneration in the spinal cord parenchyma was also observed but not as abundantly as in the nerve roots. The number of

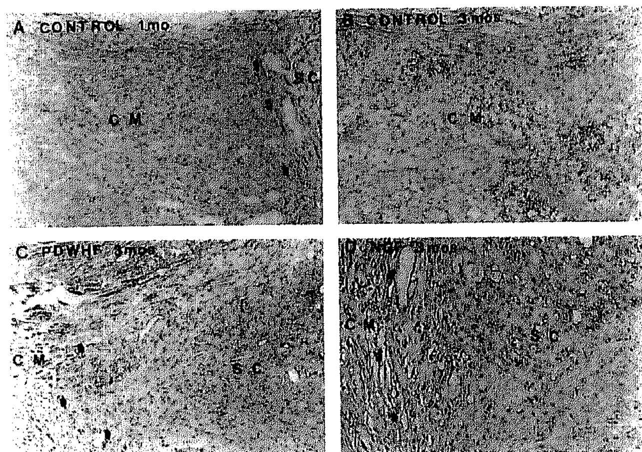


Figure 2 Changes in the collagen gel matrix between the spinal cord stumps - Masson's trichrome stain. (A) Control group, 1 mo, × 10, (B) Control group, 3 mos, × 10, (C) PDWHF group, 3 mos, × 10, (D) NGF group, 3 mos, × 20. (A) In the control group, a number of macrophages migrated into the collagen matrix (CM) and cysts were produced in the injured spinal cord stumps (SC) (→) by phagocytic activities in the acute phase. (B) The collagen matrix was later replaced by dense fibrous scar tissue. (C) In the PDWHF group macrophage invasion was observed mainly on the surface of injured spinal cord (→). (D) In the NGF group, migration of macrophages on the edge of the injured spnial cord (→) was less than the PDWHF group. Phagocytosis was less dominant and cavity formation was relatively smaller than the control group

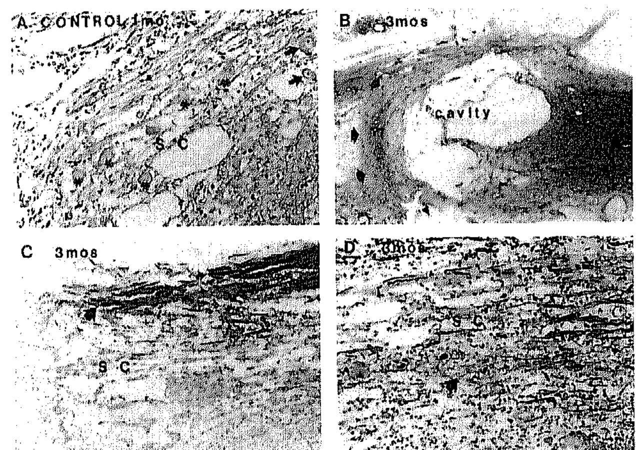


Figure 3 Changes of neural elements in the injured spinal cord (control group). (A) Masson's trichrome stain, 1 mo, × 20, (B) Biel's silver stain, 3 mos, × 4, (C) Biel's silver stain, 3 mos, × 10, (D) Biel's silver stain, 3 mos, × 20. (A) In the control group, phagocytic activity was evidenced by numerous cysts or cavities in the injured spinal cord (SC). Numerous dead nerve cells were found (*) and only few live nerve cells were seen (→) in this area of autolysis. (B) The end of injured spinal cord stump was seen surrounded by a thick glial scar (→) and a large autolytic cavity. No axonal growth was observed from the spinal cord or nerve roots in this case. (C) Only a short segment of axonal regrowth from the nerve root (→) was seen. (D) Scant axonal regrowth within substance of the spinal cord (→) could be seen

nerve cells surviving in the injured gray matter in the PDWHF group was much greater than the control group (Figure 4C). Growing axonal bundles could be seen forming neuromas in the fibrous scar tissue. These growing axons were forced to change their direction of growth by the dense and thick barrier of collagen fibers which were produced by migrating fibroblasts and glia. This prevented effective growth of the axons to their targets (Figure 4D).

In the NGF group, a number of living nerve cells as well as oligodendrocytes and astrocytes in the gray matter were observed even in the 3 month animals. These cells survived in greater numbers even though small amount of autolysis was observed. The amount of cavity formation was less (1.4±0.7 mm) compared to the control group, although there was no statistical significance (Figure 5A). Axonal growth was notable, and the most active axonal growth was observed from the nerve roots (Figure 5B). Axonal growth was also oberved from the spinal cord tissue, but not as abundantly as from the nerve roots (Figure 5C). Rostrally, axonal regrowth was from the descending motor neurons or the ventral root fibers; caudally, from the ascending sensory neurones or the dorsal root fibers. They migrated into the fibrous scar tissue, however, the growth of these regenerated axons was blocked by dense fibrous scar and resulted in neuroma formation (Figure 5D). In the NGF group thick axonal bundles were observed in 11 and neuromas in seven of 12 animals. Statistically, both the NGF and PDWHF groups produced more apparent axonal growth than the control group. Axonal growth in the NGF group became constantly longer and larger during the time course than in the PDWHF group, although there was statistically no significant difference between the NGF group and the PDWHF group (Table 2).

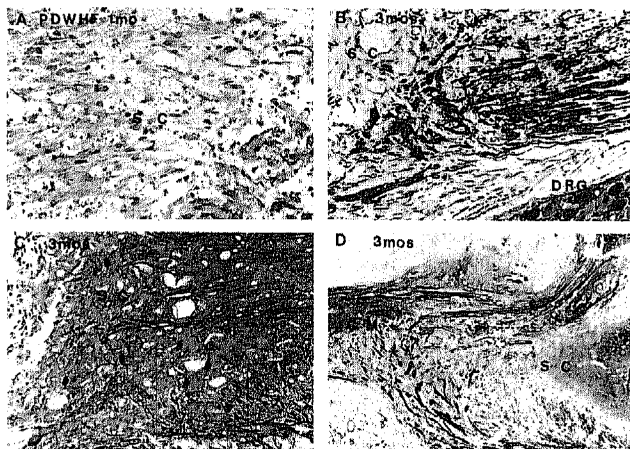


Figure 4 Changes of neural elements in the injured spinal cord (PDWHF group). (A) Masson's trichrome stain, 1 mo, ×20, (B) Biel's silver stain, 3 mos, ×20, (C) Biel's silver stain, 3 mos, ×20, (D) Biel's silver stain, 3 mos, ×4. (A) Numerous macrophages migrated into the injured spinal cord in the acute phase. (B) Axonal growth (→) occurred from the dorsal nerve roots and grew into the spinal cord. Numerous live dorsal root ganglion cells (DRG) were observed. (C) Apparent axonal growth from the spinal cord parenchyma was also observed. The microenvironment surrounding the axons and nerve cells appeared healthy. Numerous live nerve cells were seen (→). (D) Axonal growth was supported by surrounding substrates until it reaches the fibrous scar tissue in which the direction of axonal growth changed and neuroma (*) was found within the dense collagenous fibrous barrier

Neovascularization in the injured spinal cord

New vessel formation was observed both in the collagen gel matrix and the injured spinal cord parenchyma. In the control group, vessels were observed predominantly in the collagenous matrix but not in the spinal cord parenchyma (Figure 6A). In the PDWHF group, dominant new vessel formation was observed in the collagen matrix (Figure 6B) as well as in the injured spinal cord parenchyma (Figure 6C). Several large vessels also migrated from the nerve roots, pia matter or epidural adipose tissue. In the

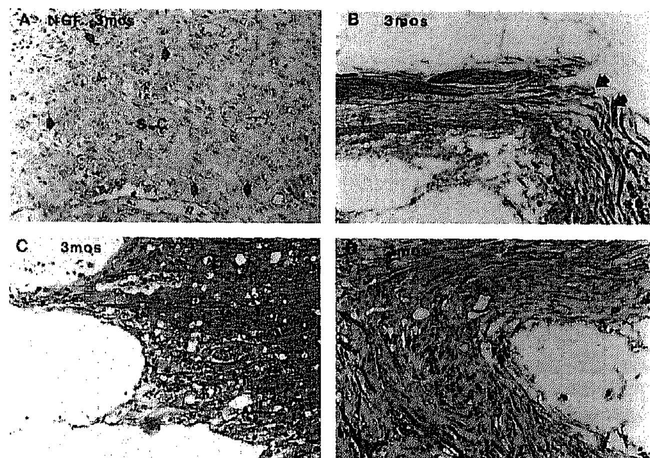


Figure 5 Changes of neural elements in the injured spinal cord (NGF group). (A) Masson's trichrome stain, 3 mos, ×20, (B) Biel's silver stain, 3 mos, ×10. (C) Biel's silver stain, 3 mos, ×20, (D) Biel's silver stain, 3 mos, ×10. (A) In the NGF group, migration of macrophages was much less than in the PDWHF group and numerous live nerve cells (→) were seen. (B) Axonal growth occurred from the nerve root (→) and formed a neuroma (*). (C) Axonal growth was also seen in the spinal cord parenchyma. (D) Growing axons were being blocked by a dense fibrous barrier with the formation of a whirling neuroma (*)

Table 2 Activity in axonal regeneration

	Control	*PDWHF*	*NGF*
1 month	1.7±1.4 (pts)	2.1±0.6 (pts)	2.8±2.1 (pts)
2 months	2.2±1.5	3.1±1.4	3.4±1.6
3 months	2.7±2.7	3.8±1.5	4.1±1.7
mean	2.1±1.8	3.1±1.4*	3.4±1.8*

*$P<0.05$ against the control group

NGF group, findings similar to those in the PDWHF group with neovascularization were noted in both the collagen matrix area and the degenerating spinal cord stumps (Figure 6D). However, the angiogenic reactions were greater in the PDWHF group. Stastistically, there was a significant increase in number of vessels in both of the PDWHF and NGF groups compared to the control group. The number of vessels in the PDWHF group compared to the NGF group was also significantly increased (Table 3).

Discussion

Possibility of regeneration of the injured spinal cord
Immediately after severe CNS trauma, neuronal death occurs at the site of injury accompanied by local breakdown of the blood-CNS barrier.[20-22] Accumulations of blood-derived macrophages are observed acutely after the initial injury. These macrophages proceed to engulf degenerating myelin and other cell debris. Astrocytes also appear to participate in this process.[23] Due to such microenvironmental changes which surround neural elements, neurons not directly affected by injury begin to die, and gradually enlarging cysts develop and coalesce to form cavities within the CNS parenchyma.[24] They probably arise from multiple causes such as ischemia, absence of nutrients or neurotrophic factors, and metabolic imbalances followed by autolytic processes.[25,26] This wave of secondary neuronal death after the primary injury is probably responsible for a larger proportion of neuronal death than the initial injury itself. While phagocytosis is going on, astrocytes adjacent to the lesion proliferate and their enlarged fibrous processes isolate the surfaces of the injury from the surrounding tissue.[27,28] At about the same time, fibroblasts from the connective tissue invade the injury site overlaying the CNS surfaces with a dense layer of collagen.[29,30] The so-called glial 'scar' is thus formed.[26]

Historically, is has been assumed that the spinal cord is unable to regenerate a pathway through a lesion in adult mammals, even if the axons are provided with a favorable glial environment for growth.[31,34] Current opinion is in many instances the opposite.[35-39] The adult CNS in mammalian species possesses the capability of axonal regrowth following injury under appropriate circumstances both in ventral motoneurons and dorsal sensory neurons, if provided appropriate extracellular microenvironment.[40-42] Thus, an important area for research concerns the prevention of degeneration of nerve cells in the period immediately following injury to the spinal cord followed by induction of axonal regrowth[38] and synaptogenesis.[43] Both soluble and membrane-associated growth factors may be necessary for neural regeneration.[44] In the injured spinal cord, there are deprival of target derived and glia derived supply of neurotrophic factors. There are also problems of local microvasculature in the injured spinal cord which is important for delivery of exogenous trophic factors or nutrients[26,45,46] and problem of local mircoenvironment such as matrices which play an important supportive role in regenerating axons and synaptogenesis. Thus, recovery of the appropriate microenvironment such as extracellular matrices and microvasculature surrounding nerve cells and axons are important in maintaining function and repairing process of the spinal cord.

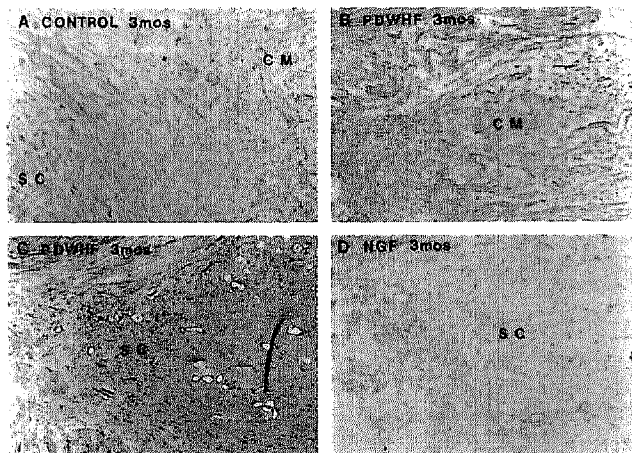


Figure 6 Neovascularization in the injured spinal cord tissues - Laidlaw's silver stain. (A) Control group, 3 mos, ×10, (B) PDWHF group, 3 mos, ×10, (C) PDWHF group, 3 mos, ×10, (D) NGF group, 3 mos, ×10. (A) In the control group, new vessel formation occurred mainly in the collagenous matrix (CM) but very little within the substance of the spinal cord (SC). (B) In the PDWHF group, neovascularization in the collagenous matrix (CM) was greater. (C) In the PDWHF group, prolific neovascularization in the injured spinal cord parenchyma (SC) was also observed. (D) In the NGF group, neovascularization in the spinal cord parenchyma (SC) was observed but less than in the PDWHF group

Table 3 New vessel formation

	Control	*PDWHF*	*NGF*
1 month	6.0±5.6 (/0.25mm^2)	10.5±8.2 (/0.25mm^2)	8.9±5.6 (/0.25mm^2)
2 months	5.8±5.0	13.2±9.6	7.5±5.0
3 months	3.7±4.0	16.7±10.0	9.0±5.3
mean	5.9±4.9	13.1±9.5$^{+,++}$	8.0±5.3^{+}

$^{+}P<0.001$ against the control group, $^{++}P<0.01$ against the NGF group

NGF effects on CNS
In peripheral sympathetic neurons, one well-known metabolic requirement of neurons is the need for their appropriate target-derived trophic factor.[47,48] The loss of this trophic factor, NGF, results in chromatolytic reactions and in many cases cell deaths.[49] Perez-Polo *et al*[50] considered that effects of NGF on oxidant-antioxidant balance might be relevant to the regula-

tion of axonal sprouting, growth, and synaptogenesis at critical periods during development, following injury and throughout aging. Exogenous NGF has been reported to support the survival and general growth capabilities of axons in peripheral sympathetic and dorsal root sensory neurons in the development or after injury.[51-53] Recent investigations have shown that NGF is produced in the CNS areas surrounding the lesion[14,35,54-57] and central neurons have NGF receptors.[58-61] Thus, NGF supposed to be necessary for survival or regeneration even in the CNS, however, this seems not to have been proven in *in vivo* spinal cord lesions. In this *in vivo* study with spinal cord injured animals, exogenous trophic factors supported cell survival and induced axonal outgrowth in the gray matter of the spinal cord parenchyma as well as from the nerve roots.

Importance of microvasculature in the injured spinal cord
Spinal cord regeneration is thought to be heavily influenced by locally acting trophic factors. There are reports that after spinal cord lesion, the blood-CNS barrier is broken down.[22] There have also been reports that the intrinsic blood supply and blood flow in the spinal cord at the site of an injury are decreased.[3-10] This creates difficulties in the delivery of these crucial trophic factors. PDWHF is a conglomerate of growth factors. They are PDGF, PDAF, TGFβ, and PF4. These factors could induce directly or indirectly angiogenesis.[62,63] They are commonly a chemoattractant for monocytes which may induce macrophage derived angiogenesis.[43,64-67] TGFβ[66] and PDAF[68,69] are also chemoattractant for capillary endothelial cells. In addition, PDGF stimulates proliferation and differentiation of oligodendrocytes which are supportive to neural tissue.[70] Our previous reports showed evidence for an angiogenic effect of PDWHF in incomplete spinal cord lesions using a double-blind method.[11] In this study, we used the complete spinal transection model fililng the dead space with a collagen matrix impregnated with PDWHF, and investigated whether improvement of extracellular microenvironment by angiogenesis induces survival and repair of neural tissue. Abundant angiogenesis was observed in the injured spinal cord parenchyma, and PDWHF appeared to be useful in delivery of trophic factors and to improve the extracellular microenvironment which is supportive in nerve cell survival and axonal regeneration.

Importance of extracellular matrices in the injured spinal cord
Natural extracellular matrices act as tissue repair promoters by providing a spatial framework for cell migration, cell interactions and scar remodeling. Collagen, a major element of extracellular matrices, can be used as a hydrogel in cell culture to feature properties of extracellular matrices and to support neuronal differentiation and axonal growth.[71] In this study the type 1 collagen matrix completely filled the injured site providing a template structure for tissue repair that leads to the formation of a well-vascularized collagen rich neotissue different from the normally occurring dense glio-connective scar. This secondary matrix favored tissue ingrowth and axonal regeneration. However, this collagenous matrix generally became denatured within 2–3 months, and could not prevent the implantation site from penetration by fibroblasts which formed dense fibrous connective barrier. Further studies regarding more supportive extracellular matrix will be required.

Classical observations suggest that following damage to the CNS, astrocytes produce a glial scar and thus the barrier at the edge of the spinal cord is formed. The reasons for the arrest of axonal growth and subsequent degeneration are not understood, but one of the many hypotheses maintains that the glia-collagen scar, formed in CNS wounds, represents a 'barrier' to axonal rerouting of the regenerating axon to its target.[22,30,33,72-74] However, several recent studies showed astrocytes were associated with the restoration of neural structures,[60,75,76] in particular, astrocytes have been implicated in the regulation of axonal growth both during development[76,77] and during regeneration of the central nervous tissue.[78-81] It has also been demonstrated that *in vitro* astrocytes can produce NGF[60,82-85] and *in vivo*.[86] Astrocytes are also believed to produce laminin.[87] Migration of macrophages and fibroblasts may not always be harmful to neural cell survival.[35] Macrophages secrete some trophic factors such as PDGF, PDAF, PF4[63,88] and fibroblasts secrete fibronectin.[65-67] The synthesis of NGF by macrophages[89] and fibroblasts[83,90-93] has also been suggested *in vitro*. The neuronal response to a lesion is an apparently more complex event,[35] and less is known about the molecular signals that act on glia and macrophages as part of the inflammation, gliosis, and scarring associated with neuronal injury.[49] The challenge is whether these properties of the glial scar can be modified to make them more conductive to axonal outgrowth. A successful result would have to be coupled with further studies on fibrous scar tissue and appropritae substrates for axonal growth within the CNS.

Conclusions

Regeneration is difficult in the mature CNS in mammals, due not to any intrinsic incapability of the nerve cell, but rather to the complex organization of the CNS microenvironment, in which the lesioned central neurons have to regenerate.[35] This study regarding the effects of NGF and PDWHF on degenerative and regenerative features of injured spinal cord may serve to highlight the multifaceted action of trophic agents and their interplay. NGF and PDWHF seem to play a role in the healing processes of

NGF group, findings similar to those in the PDWHF group with neovascularization were noted in both the collagen matrix area and the degenerating spinal cord stumps (Figure 6D). However, the angiogenic reactions were greater in the PDWHF group. Stastistically, there was a significant increase in number of vessels in both of the PDWHF and NGF groups compared to the control group. The number of vessels in the PDWHF group compared to the NGF group was also significantly increased (Table 3).

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Historically, is has been assumed that the spinal cord is unable to regenerate a pathway through a lesion in adult mammals, even if the axons are provided with a favorable glial environment for growth.[31,34] Current opinion is in many instances the opposite.[35-39] The adult CNS in mammalian species possesses the capability of axonal regrowth following injury under appropriate circumstances both in ventral motoneurons and dorsal sensory neurons, if provided appropriate extracellular microenvironment.[40-42] Thus, an important area for research concerns the prevention of degeneration of nerve cells in the period immediately following injury to the spinal cord followed by induction of axonal regrowth[38] and synaptogenesis.[43] Both soluble and membrane-associated growth factors may be necessary for neural regeneration.[44] In the injured spinal cord, there are deprival of target derived and glia derived supply of neurotrophic factors. There are also problems of local microvasculature in the injured spinal cord which is important for delivery of exogenous trophic factors or nutrients[26,45,46] and problem of local mircoenvironment such as matrices which play an important supportive role in regenerating axons and synaptogenesis. Thus, recovery of the appropriate microenvironment such as extracellular matrices and microvasculature surrounding nerve cells and axons are important in maintaining function and repairing process of the spinal cord.

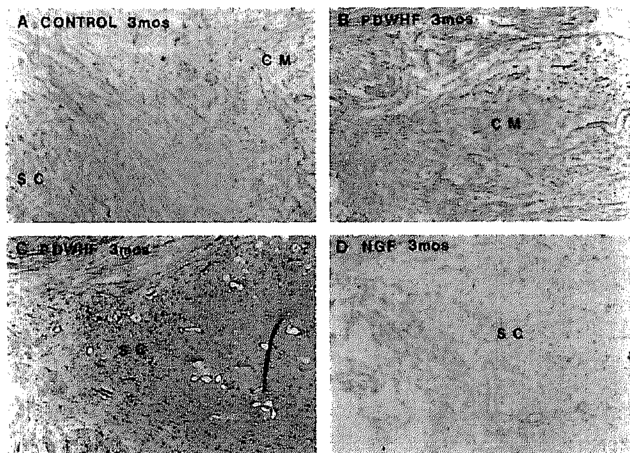

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Table 3 New vessel formation

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2 months	5.8 ± 5.0	13.2 ± 9.6	7.5 ± 5.0
3 months	3.7 ± 4.0	16.7 ± 10.0	9.0 ± 5.3
mean	5.9 ± 4.9	$13.1 \pm 9.5^{+,++}$	$8.0 \pm 5.3^{+}$

$^{+}P<0.001$ against the control group, $^{++}P<0.01$ against the NGF group

NGF effects on CNS
In peripheral sympathetic neurons, one well-known metabolic requirement of neurons is the need for their appropriate target-derived trophic factor.[47,48] The loss of this trophic factor, NGF, results in chromatolytic reactions and in many cases cell deaths.[49] Perez-Polo *et al*[50] considered that effects of NGF on oxidant-antioxidant balance might be relevant to the regula-

tion of axonal sprouting, growth, and synaptogenesis at critical periods during development, following injury and throughout aging. Exogenous NGF has been reported to support the survival and general growth capabilities of axons in peripheral sympathetic and dorsal root sensory neurons in the development or after injury.[51–53] Recent investigations have shown that NGF is produced in the CNS areas surrounding the lesion[14,35,54–57] and central neurons have NGF receptors.[58–61] Thus, NGF supposed to be necessary for survival or regeneration even in the CNS, however, this seems not to have been proven in *in vivo* spinal cord lesions. In this *in vivo* study with spinal cord injured animals, exogenous trophic factors supported cell survival and induced axonal outgrowth in the gray matter of the spinal cord parenchyma as well as from the nerve roots.

Importance of microvasculature in the injured spinal cord
Spinal cord regeneration is thought to be heavily influenced by locally acting trophic factors. There are reports that after spinal cord lesion, the blood-CNS barrier is broken down.[22] There have also been reports that the intrinsic blood supply and blood flow in the spinal cord at the site of an injury are decreased.[3–10] This creates difficulties in the delivery of these crucial trophic factors. PDWHF is a conglomerate of growth factors. They are PDGF, PDAF, TGFβ, and PF4. These factors could induce directly or indirectly angiogenesis.[62,63] They are commonly a chemoattractant for monocytes which may induce macrophage derived angiogenesis.[43,64–67] TGFβ[66] and PDAF[68,69] are also chemoattractant for capillary endothelial cells. In addition, PDGF stimulates proliferation and differentiation of oligodendrocytes which are supportive to neural tissue.[70] Our previous reports showed evidence for an angiogenic effect of PDWHF in incomplete spinal cord lesions using a double-blind method.[11] In this study, we used the complete spinal transection model fililng the dead space with a collagen matrix impregnated with PDWHF, and investigated whether improvement of extracellular microenvironment by angiogenesis induces survival and repair of neural tissue. Abundant angiogenesis was observed in the injured spinal cord parenchyma, and PDWHF appeared to be useful in delivery of trophic factors and to improve the extracellular microenvironment which is supportive in nerve cell survival and axonal regeneration.

Importance of extracellular matrices in the injured spinal cord
Natural extracellular matrices act as tissue repair promoters by providing a spatial framework for cell migration, cell interactions and scar remodeling. Collagen, a major element of extracellular matrices, can be used as a hydrogel in cell culture to feature properties of extracellular matrices and to support neuronal differentiation and axonal growth.[71] In this study the type 1 collagen matrix completely filled the injured site providing a template structure for tissue repair that leads to the formation of a well-vascularized collagen rich neotissue different from the normally occurring dense glio-connective scar. This secondary matrix favored tissue ingrowth and axonal regeneration. However, this collagenous matrix generally became denatured within 2–3 months, and could not prevent the implantation site from penetration by fibroblasts which formed dense fibrous connective barrier. Further studies regarding more supportive extracellular matrix will be required.

Classical observations suggest that following damage to the CNS, astrocytes produce a glial scar and thus the barrier at the edge of the spinal cord is formed. The reasons for the arrest of axonal growth and subsequent degeneration are not understood, but one of the many hypotheses maintains that the glia-collagen scar, formed in CNS wounds, represents a 'barrier' to axonal rerouting of the regenerating axon to its target.[22,30,33,72–74] However, several recent studies showed astrocytes were associated with the restoration of neural structures,[60,75,76] in particular, astrocytes have been implicated in the regulation of axonal growth both during development[76,77] and during regeneration of the central nervous tissue.[78–81] It has also been demonstrated that *in vitro* astrocytes can produce NGF[60,82–85] and *in vivo*.[86] Astrocytes are also believed to produce laminin.[87] Migration of macrophages and fibroblasts may not always be harmful to neural cell survival.[35] Macrophages secrete some trophic factors such as PDGF, PDAF, PF4[63,88] and fibroblasts secrete fibronectin.[65–67] The synthesis of NGF by macrophages[89] and fibroblasts[83,90–93] has also been suggested *in vitro*. The neuronal response to a lesion is an apparently more complex event,[35] and less is known about the molecular signals that act on glia and macrophages as part of the inflammation, gliosis, and scarring associated with neuronal injury.[49] The challenge is whether these properties of the glial scar can be modified to make them more conductive to axonal outgrowth. A successful result would have to be coupled with further studies on fibrous scar tissue and appropritae substrates for axonal growth within the CNS.

Conclusions

Regeneration is difficult in the mature CNS in mammals, due not to any intrinsic incapability of the nerve cell, but rather to the complex organization of the CNS microenvironment, in which the lesioned central neurons have to regenerate.[35] This study regarding the effects of NGF and PDWHF on degenerative and regenerative features of injured spinal cord may serve to highlight the multifaceted action of trophic agents and their interplay. NGF and PDWHF seem to play a role in the healing processes of

spinal cord injury and may have important interactions with other growth factors. These effects are important to help the spinal cord to escape from the diaschisis and to orient the metabolism of the neurons and glial cells towards the reparative responses.

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