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Laboratory study

Immunohistochemical profile of transforming growth factor-β1 and basic fibroblast growth factor in sciatic nerve anastomosis following pinealectomy and exogenous melatonin administration in rats

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Abstract

Collagen scar formation at the cut end of a peripheral nerve, an important problem in clinical practice for neurosurgeons, obstructs sprouting of axons into appropriate distal fascicles, and thereby limits the regeneration process. Researchers have attempted to control collagen accumulation and neuroma formation with various physical and chemical methods, but with limited functional success. Recently, it has been demonstrated that transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF) play an important role in collagen production by fibroblasts and in Schwann cell activity. In our study, rats were divided into a control group, a melatonin-treated group, a surgical pinealectomy group, and a group treated with melatonin following pinealectomy. They then underwent a surgical sciatic nerve transection and primary suture anastomosis. At 2 months after anastomosis, the animals were sacrificed and unilateral sciatic nerve specimens, including the anastomotic region, were removed and processed for immunohistochemical study from two animals in each group. For each antibody, immunoreactivity was assessed using a semiquantitative scoring system. Strong TGF-β1 and/or bFGF expression was observed in the epineurium of animals that underwent pinealectomy, but no or weak staining was observed in animals in the control and melatonin treatment groups. Based on these data, we suggest that both TGF-β1 and bFGF have important roles in control of collagen accumulation and neuroma formation at the anastomotic site, and that the pineal neurohormone melatonin has a beneficial effect on nerve regeneration.

Keywords: Basic fibroblast growth factor; Collagen; Pinealectomy; Melatonin; Regeneration; Transforming growth factor-β1; Sciatic nerve

1. Introduction

Numerous studies have shown prominent collagen accumulation in the nerve stump after complete sciatic nerve transection.[1–4] Furthermore, it has been suggested that collagen scar formation at the cut end of the nerve obstructs sprouting of axons into appropriate distal fascicles, and thereby limits nerve regeneration.[1,2,5–7] However, research attempting to inhibit collagen accumulation in neuroma formation has been of limited functional success.[1,2,5–8] Importantly, it has been reported that melatonin decreases wound collagen content, and removing the pineal gland increases collagen content, which can be reversed by melatonin adminstration following pinealectomy.[9–13]

Transforming growth factor (TGF)-β, from a family of regulatory polypeptides, is involved in many vital processes including inflammation and wound healing.[14] TGF-β mRNA and immunoreactivity have been found in normal peripheral nerve.[14] Pathophysiologically, it has been dem-

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onstrated that repair of a peripheral nerve lesion includes a series of well-defined step-wise cellular actions possibly controlled by TGF-β.[15] Interestingly, it has been postulated that TGF-β1 can particularly modify epineurial scarring.[15] Basic fibroblast growth factor (bFGF), a representative member of the heparin-binding growth factors, promotes proliferation of fibroblasts and developing neural cells.[16–18] Some authors report that it stimulates angiogenesis and proliferation of Schwann cells, as well as mesenchymal cells including endothelial cells, fibroblasts and vascular smooth muscle cells.[16,19,20] Furthermore, bFGF also induces injury-associated angiogenesis and granulation tissue formation, and thus accelerates wound closure in healing-impaired animal models.[21]

Recently, it has been reported that TGF-β and bFGF play an important role in collagen production by fibroblasts and Schwann cells.[8,14,22–33] The data suggest that these cytokines may be involved in neuroma formation after traumatic nerve section. Thus, it is possible that both TGF-β and bFGF have growth promoting effects for axonal sprouting during peripheral nerve regeneration. We have recently shown that exogenous application of melatonin significantly inhibits accumulation of both Type I collagen and Type III collagen, as well as macroscopic neuroma formation, at the suture repair site in the rats.[34] This paper describes the immunohistochemical profile of TGF-β1 and bFGF in the anastomotic region of the sciatic nerve following pinealectomy and exogenous melatonin administration, using a semiquantitative analysis.

2. Material and methods

2.1. Materials

Trypsin, glycerol vinyl alcohol aqueous-GVA, PAP pen, serum blocking solution, and glass slides were obtained from Zymed Laboratories (Invitrogen Corporation, Carlsbad, CA). Melatonin and 4% phosphate-buffered paraformaldehyde were products of Sigma Chemical Co (St. Louis, MO). TGF-β1 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). bFGF was a product of Oncogene Research Products (Cambridge, MA).

2.2. Animals, experimental design and surgical procedures

As described previously, the animals were used after approval of the experimental protocol by the local ethics committee.[34] Adult Wistar rats, weighing 210 g (±10 g), were housed with access to food and water *ad libitum*. All the animals were kept in 12 hour each light-dark conditions. All surgical procedures were performed under general anaesthesia by intraperitoneal injection of a mixture of ketamine hydrochloride (60 mg/kg) and dihydrothiazine (8 mg/kg). The animals were randomly divided into eight groups (two rats each) as follows: control group comprising intact animals; vehicle group comprising animals receiving drug vehicle only; melatonin group comprising animals receiving melatonin 30 μg/100 g body weight; sham operation group; surgical pinealectomy group; pinealectomy plus vehicle group; sham operation plus vehicle group; and pinealectomy plus melatonin group comprising animals receiving melatonin 30 μg/100 g body weight after pinealectomy.

The animals underwent pinealectomy or a sham operation under deep anaesthesia 3 weeks prior to sciatic neurotomy and primary suture anastomosis. Pinealectomy was performed with a modification of the method described previously by Bliss and Bates.[35] Briefly, the scalp was opened with a longitudinal midline incision and an area of skull removed between the sagittal and lambdoid sutures, holding the head steady. The dura mater was then opened and the pineal gland removed using iridectomy forceps held at an angle of 30° from the lambdoid suture and dissected downward at an angle of 15° from the horizontal towards the midline at the junction of the sagittal and lambdoid sutures. Meticulous care was taken to avoid injury to the venous sinuses adjacent to the pineal gland. Bleeding was controlled with cotton wool pledgets. At the end of the procedure, the skin was approximated with 5/0 nylon sutures.

In each rat, both sciatic nerves were exposed via a posterior thigh approach and isolated with a plastic sheet. Then, each nerve was sharply transected using a steel scalpel, and the nerve ends were approximated with four to six 10/0 nylon sutures placed around the nerve in the epineurium using standard microsurgical methods. Finally, the wound was closed with 4/0 nylon sutures and the rats were replaced unrestricted in their cages. All animals were checked for complications in the early postoperative period and then during weekly control examinations for neurological complications.

After sciatic nerve section, animals were subcutaneously injected as follows: either 2% (v/v) ethyl alcohol in physiological saline, 0.1 ml/100 g body weight as the vehicle control or melatonin 30 μg/100 g body weight dissolved in vehicle. All the injections were given once daily between 16.00 and 17.00 hours for 2 months.

2.3. Immunohistochemistry

Two months after sciatic nerve section and suture repair, the animals in all groups were sacrificed and unilateral sciatic nerve specimens including the anastomotic region from two animals from each group were carefully removed. The excised segments were gently stretched on pieces of index card and were processed for immunohistochemical analysis. The nerve segments were immediately fixed in 4% phosphate-buffered paraformaldehyde pH 7.4 for 24 hours at 4 °C and washed in phosphate buffered-saline (PBS). Following fixation the tissue was dehydrated through graded ethanol (80%, 95%, and 100% sequentially), cleared in xylene, embedded in paraffin, and 5 μm coronal sections were cut on microtome. Sections were floated in a sterile bath, were picked up onto poly-L-lysine

coated glass slides and were dried at room temperature. Selected slides were incubated at 55 °C overnight, deparaffinised in xylene (2 × 30 min) followed by a decreasing gradient wash in ethanol (90%, 80%, 70%, and 60%, sequentially, ×2 min each). They were then washed with distilled water, drawing around the tissue with a PAP pen for retention of reagents on glass slides, washed in PBS and trypsin was applied for enzyme digestion to tissue sections for approximately 15–30 min. They were then washed in PBS (3 × 5 min) at room temperature in a humidified chamber. Endogeneous peroxidase activity was quenched with 3% hydrogen peroxide for 5 minutes and washed with PBS. Thereafter, the sections were blocked with serum blocking solution. Primary antibodies specific for TGF-β1 and bFGF were applied to the sections and washed in PBS. Each antibody was dissolved in PBS to optimal dilution. This step was followed by the addition of a biotinylated secondary antibody for 30 min, wash, and staining for 30 min with a streptavidin-peroxidase enzyme conjugate, followed by thorough rinsing in PBS. Colour was developed with 3-amino-9-ethylcarbazole as chromogen for five minutes and counterstained with hematoxylin and mounted with glycerol vinyl alcohol aqueous-GVA. After the immunohistochemical procedures, each section was evaluated using light microscopy for immunostaining for TGF-β1 and bFGF in the endoneurium, perineurium, epineurium, and perivascular regions of the nerve in all groups.

Appropriate tissue sections used for positive and negative controls of each primary antibody were also labelled.

2.4. Evaluation of immunostaining

All sections were analysed using a light microscope (Olympus BX40, Tokyo, Japan) for assessment of immunostaining of TGF-β1 and bFGF in the anastomotic region of the transected sciatic nerve. Immunoreactivity of the sections was graded independently by researchers blinded to the experimental conditions on the following semiquantitative scale. The selection of the area for the analysis was based on availability of sufficient neural tissue for immunohistochemical scoring. The degree of positive staining for TGF-β1 and bFGF was evaluated by scoring intensity on a scale of 1–4 (I: none, mild, moderate, and strong), and distribution on a scale of 1:4 (D: none, focal, patchy, and diffuse). Tissues with I × D less than or equal to 4 were considered 'weakly positive', those with I × D greater than 4 were designated 'strongly positive', and those with no immunoreactivity were designated as 'absent'.[36]

3. Results

3.1. Transforming growth factor-β1

In all control groups (intact control, vehicle, melatonin, and sham operation groups), the immunohistochemical staining for TGF-β1 was weakly positive in the endoneurium and perineurium of the anastomosed sciatic nerve, but strongly positive in the epineurium (Fig. 1A). Strongly positive immunoreactivity was also observed in the epineurium in the pinealectomy and the pinealectomy plus vehicle group (Fig. 1B). In animals receiving melatonin after pinealectomy, there was weakly and strongly positive immunoreactivity in the epineurium and perineurium, respectively (Fig. 1C). Within the resolution of the semiquantitative assessment, immunoreactivity for TGF-β1 in the endoneurium, perineurium, epineurium, and perivascular regions in all groups is shown in Table 1.

3.2. Basic fibroblast growth factor

In general, an increased number of immunoreactive cells for bFGF was detected in the epineurial and perivascular areas of the anastomosed sciatic nerve in all groups. Strongly positive immunoreactivity was observed in all

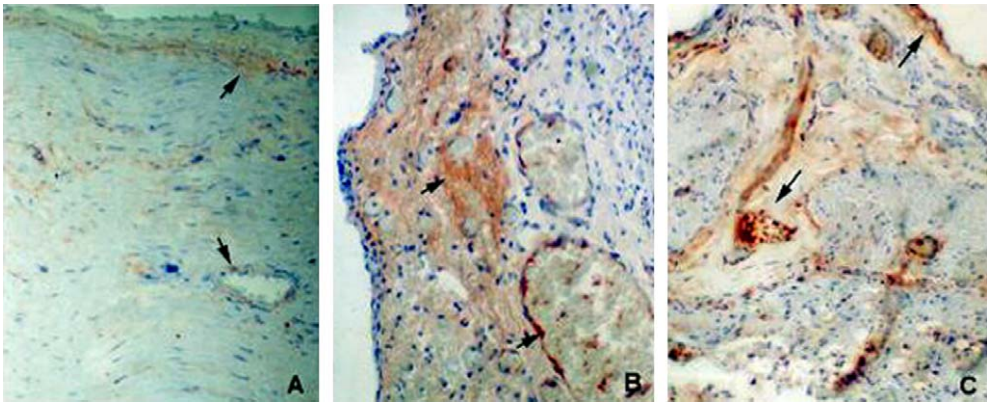

Fig. 1. Immunostaining (arrows) for transforming growth factor (TGF)-β1 in the anastomotic region of the sciatic nerve in, (A) normal animals in the control group, (B) pinealectomised animals in the pinealectomy plus vehicle group, and (C) animals receiving melatonin treatment after pinealectomy in the pinealectomy plus melatonin group. (A) original magnification, ×126; (B, C) original magnification, ×63. This figure is available in colour on www.sciencedirect.com.

Table 1
Immunoreactivity for TGF-β1 in the endoneurium, perineurium, epineurium, and perivascular regions of anastomosed sciatic nerve[a]

Group	Endoneurium	Perineurium	Epineurium	Perivascular region
Intact control	Absent	Absent	Strong	Weak
Vehicle	Absent	Absent	Strong	Weak
Melatonin	Weak	Weak	Strong	Weak
Sham operation	Absent	Absent	Strong	Weak
Pinealectomy	Weak	Weak	Strong	Weak
Pinealectomy plus vehicle	Weak	Weak	Strong	Strong
Sham operation plus vehicle	Absent	Absent	Strong	Weak
Pinealectomy plus melatonin	Weak	Strong	Weak	Strong

[a] Quantification of immunoreactivity is described in the Materials and methods section - absent, weakly positive, or strongly positive.[36]

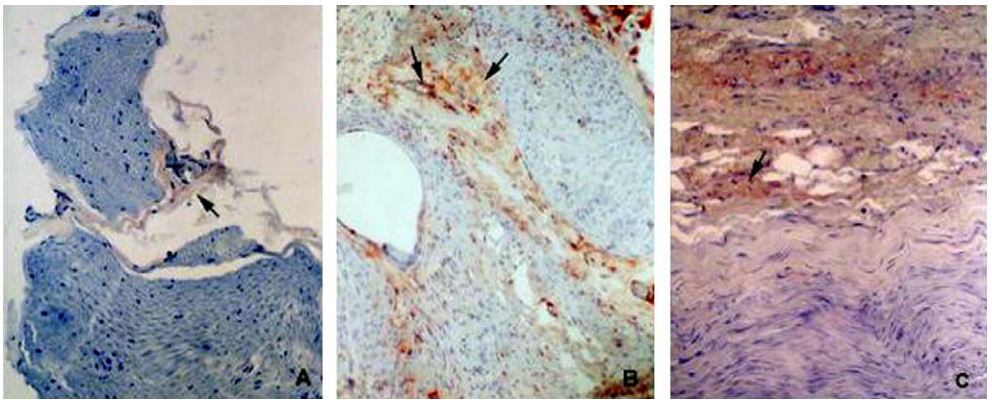

Fig. 2. Immunostaining (arrows) for basic fibroblast growth factor (bFGF) in the anastomotic region of the sciatic nerve in, (A) normal animals in the control group, (B) pinealectomised animals in the pinealectomy group, and (C) animals receiving melatonin treatment after pinealectomy in the pinealectomy plus melatonin group. (A) original magnification, ×252; (B, C) original magnification, ×126. This figure is available in colour on www.sciencedirect.com.

Table 2
Immunoreactivity for bFGF in the endoneurium, perineurium, epineurium, and perivascular regions of anastomosed sciatic nerve[a]

Groups	Endoneurium	Perineurium	Epineurium	Perivascular region
Intact control	Absent	Absent	Weak	Weak
Vehicle	Absent	Absent	Weak	Weak
Melatonin	Weak	Weak	Weak	Strong
Sham operation	Absent	Absent	Weak	Weak
Pinealectomy	Weak	Weak	Strong	Strong
Pinealectomy plus vehicle	Weak	Weak	Strong	Strong
Sham operation plus vehicle	Absent	Absent	Weak	Weak
Pinealectomy plus melatonin	Weak	Weak	Strong	Strong

[a] Quantification of immunoreactivity is described in the Materials and methods section - absent, weakly positive, or strongly positive.

pinealectomised animals (pinealectomy, pinealectomy plus vehicle, and pinealectomy plus melatonin groups) and staining was weakly positive in both the control groups and the melatonin treatment group (Fig. 2A-C). Immunoreactivity for bFGF in the endoneurium, perineurium, epineurium, and perivascular regions in all groups is shown in Table 2.

4. Discussion

Axotomy or crush of a peripheral nerve is a commonly encountered clinical problem and in patients with a transected peripheral nerve, surgical suture anastomosis with or without autologous nerve grafts is the treatment most likely to allow successful regrowth of nerve fibres from the proximal nerve segment. Unfortunately, this repair technique often results in neuroma formation and unsatisfactory functional outcome despite dramatic advances in microsurgical techniques.[1,2,5–8]

There has been extensive investigation to develop methods to restore function after peripheral nerve damage. Cellular and molecular mechanisms involved in nerve regeneration and degeneration, must be further elucidated to provide successful regeneration of the injured nerve. The present study was undertaken to test the hypothesis that cytokines such as TGF-β and bFGF have a role in the production of scar tissue in the damaged peripheral nerve and that pineal melatonin has an effect on this process. The semiquantitative results of this study suggest that exogenous application of melatonin following sciatic nerve

section has an indirect effect to reduce collagen accumulation at the anastomosis of a transected sciatic nerve.

Recently, it has been shown that a variety of growth factors have an active role in collagen synthesis by fibroblasts.[24,28–30,32,33,37] Although there are at least three, and up to five, isoforms of TGF-β with cross-regulation and auto-induction responsible for scar formation in mammals, the best described of these is TGF-β1.[14,23,24,26,29,30,32,33,38,39] TGF-β1 has been shown to cause excessive collagen production and scar formation by increasing the mRNA levels of collagen type I.[4,24,28,40] In injury to a peripheral nerve, TGF-β1 levels increase and remain elevated until regenerating nerve fibres have crossed to the distal end of the transected nerve.[14] In the literature, there are conflicting theories of the functional roles of growth factors in the peripheral nerve regeneration process. Some authors report that proliferation of Schwann cells and production of extracellular matrix occurs as a result of interactions between Schwann cells and growth factors, including TGF-β1 and bFGF.[14,26,27,31] In contrast, others suggest that this effect may be inhibitory on nerve regeneration depending on the presence of other growth factors.[32,39] Ide et al[41] have shown that exogenous application of bFGF stimulates axonal regeneration and has a trophic effect on myelination in the central nervous system. The role of growth factors in the neural regeneration process awaits further clarification.

In clinical practice, the optimal procedure for surgical anastomosis remains unclear, despite the fact that transected peripheral nerve axons have a high regeneration potential.[1,2,5–8,42] Recently, it has been hypothesised that recovery of a peripheral nerve depends on a critical balance between Schwann cell regeneration and scar tissue formation.[42] Therefore, the TGF-β family cytokines are likely to be key substances for therapeutic manipulation.[26,43,44] Interestingly, Shah et al demonstrated that scarring in cutaneous wounds was reduced using isoform-specific blocking antibodies for TGF-β1 and TGF-β2, due to a decrease in the number of fibroblasts.[44] Additionally, melatonin has been found to reduce the collagen content of wound tissue due of its anti-proliferative effect.[10–13] Cunnane et al[9] reported that in pinealectomised rats, the tissue collagen content was increased due to melatonin deprivation. Later, Drobnik and Dabrowski showed a low wound collagen content in animals receiving melatonin at a dose of 30 μg/100 g body weight after pinealectomy, suggesting a reversing effect of melatonin on increased collagen content caused by pineal gland removal.[12] In our study, we also employed exogenous melatonin at the same dose in the pinealectomised rats to evaluate the regulatory action of the pineal gland on collagen synthesis. Our results support such an action of melatonin on tissue collagen content.

In this study, the staining intensity of TGF-β1 and bFGF in different tissue planes of the peripheral nerve was experimentally evaluated using immunohistochemical techniques in a pinealectomised rat model. To the best of our knowledge, this is the first study to demonstrate both TGF-β1 and bFGF immunohistochemically in the peripheral nerve of rats receiving melatonin after pinealectomy, demonstrating their potential roles in collagen production and peripheral nerve regeneration. In an earlier similar study, we reported strongly positive immunoreactivity for type I and III collagen in all connective tissue planes of the anastomosed sciatic nerve, particularly in the epineurium, in the control groups of rats, but there was weakly positve immunostaining in rats receiving melatonin after pinealectomy.[34] In the present study, it is noteworthy that immunostaining for TGF-β1 and bFGF was most intense in the epineurium in animals that underwent pinealectomy, while no or weakly positive immunoreactivity was observed in animals in the control groups and the melatonin treatment group. Overall, these immunohistochemical findings suggest inhibition of excessive fibroblast proliferation and collagen synthesis by reduction of TGF-β1 overexpression by melatonin. The intense immunostaining for bFGF may stimulate angiogenesis.[45] The increased staining of bFGF in both pinealectomised animals and those receiving melatonin replacement following pinealectomy is consistent with the stimulating effect of melatonin on the neural regeneration process.

The experimental groups were small in this preliminary study due to the expense of immunohistochemistry. To confirm the results, we suggest that TGF-β1 and bFGF immunostaining should be confirmed by additional molecular biology approaches, such as Western blotting, in situ hybridisation, quantitative PCR or Northern analysis in further experimental studies.

In summary, TGF-β1 and bFGF immunostaining was altered in the anastomotic region of the sciatic nerve in rats following pinealectomy and exogenous melatonin administration compared to controls. These results provide evidence for the critical role of these cytokines for collagen accumulation and neuroma formation at the anastomotic site, and a potential beneficial effect of melatonin on nerve regeneration by reducing collagen accumulation at the region of microsurgical nerve repair. Thus, the pineal hormone melatonin may be a novel treatment option for damaged peripheral nerves. Further studies are, however, needed to confirm this effect.

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