Effect of progesterone on recovery from nerve injury during leg lengthening in rats

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We investigated the effect of progesterone on the nerve during lengthening of the limb in rats. The sciatic nerves of rats were elongated by leg lengthening for ten days at 3 mm per day. On alternate days between the day after the operation and nerve dissection, the progesterone-treated group received subcutaneous injections of 1 mg progesterone in sesame oil and the control group received oil only. On the fifth, tenth and 17th day, the sciatic nerves were excised at the midpoint of the femur and the mRNA expression level of myelin protein P0 was analysed by quantitative real time polymerase chain reaction. On day 52 nodal length was examined by electron microscopy, followed by an examination of the compound muscle action potential (C-MAP) amplitude and the motor conduction velocity (MCV) of the tibial nerve on days 17 and 52. The P0 (a major myelin glycoprotein) mRNA expression level in the progesterone-treated group increased by 46.6% and 38.7% on days five and ten, respectively. On day 52, the nodal length in the progesterone-treated group was smaller than that in the control group, and the MCV of the progesterone-treated group had been restored to normal.

Progesterone might accelerate the restoration of demyelination caused by nerve elongation by activating myelin synthesis.

Lengthening of limbs is frequently employed to treat discrepancy in length. Although lengthening techniques have improved, complications of the peripheral nerves are still occasionally reported. Patients may suffer from pain, dysaesthesia or hyperaesthesia and, in more serious cases, permanent motor palsy and sensory loss. Paley¹ reported four cases of motor palsy in 60 limbs which were subjected to lengthening by the Ilizarov technique.² The frequency of nerve injury was high in lower limb lengthening in achondroplastic patients.^{3,4} The elongated nerve has been reported to show demyelination or axonal degeneration which correlates with the distance and/or speed of lengthening. The lengthened nerve also shows electrophysiological abnormalities resulting from mechanical injury and ischaemia.⁵⁻⁷ We have investigated the morphological changes in peripheral nerves in femoral lengthening in adult rats. Almost all sciatic nerve fibres showed paranodal demyelination at a lengthening speed of 3 mm per day.⁸⁻¹¹ We have also reported that the peripheral nerve recovers from paranodal demyelination by synthesising myelin sheath, resulting in increases in internodal length.^{8,9} We hypothesised that injury of the peripheral nerve can be prevented during limb lengthening by activating myelin synthesis.

It has been shown that steroid sex hormones regulate important functions in neuronal development and regeneration, thus giving them the name 'neurosteroids'.¹²⁻¹⁵ Previous investigations have shown that progesterone, pregnenolone and their reduced or sulphated metabolites promote synthesis of myelin in peripheral nerves.¹⁶⁻¹⁸ Therefore, we predicted that progesterone will aid recovery of the peripheral nerve.

In order to test our hypothesis, we quantified P0 (myelin protein zero) mRNA. This allowed us to determine whether progesterone activates the synthesis of myelin in rat models of femoral lengthening. The myelin-specific protein P0, a major structural protein that constitutes more than 50% of proteins present in the peripheral myelin sheath,¹⁹ has been used as a marker of myelination.²⁰⁻²² Through histological and electrophysiological studies we investigated whether progesterone exerts any effect on an elongated nerve.

Materials and Methods

A total of 50 male Wistar rats aged 13 weeks and weighing between 380 g and 430 g were maintained according to the guidelines of the Ethics Committee of the University of Tsukuba.

Surgical procedure. Under anaesthesia induced by intraperitoneal sodium pentobarbital (40 mg per kg body-weight), the right femur was exposed and osteotomised at its mid-point. An external fixator was applied across the gap and the wound closed. Over the course of ten days, beginning from the day after the operation, the sciatic nerve was elongated indirectly by lengthening the femur at a rate of 3 mm per day. Sodium pentobarbital (20 mg per kg body-weight) was injected at each adjustment of the fixator. The pin sites were redressed daily. Femoral lengthening of 30 mm was maintained until nerve dissection.

Medication. The rats were divided randomly, using the toss of a coin, into two groups. From the day after the operation until nerve dissection, the progesterone-treated group received subcutaneous injections of 1 mg progesterone suspended in 250 μ l sesame oil on alternate days. The control group received subcutaneous injections of 250 μ l of sesame oil.

Morphological analyses. At day 52 after the initiation of nerve lengthening, five rats in each group were killed with an overdose of sodium pentobarbital and the left ventricle was perfused with 3% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). The sciatic nerves were excised at the mid-point of the femur. These specimens were immersed in the above buffered fixative for one hour, post-fixed in 2% osmium tetroxide for three hours, dehydrated in a graded ethanol series, and embedded in Epon 812 (TAAB Laboratories Equipment Ltd, Aldermaston, United Kingdom). Semi-thin transverse sections (1 μ m thick) were cut and stained with toluidine blue for light microscopy observations. Longitudinal ultra-thin sections (70 nm to 80 nm thick) were mounted on copper grids. After staining with lead citrate and uranyl acetate, the sections were observed under an electron microscope (H7000; Hitachi, Tokyo, Japan); 50 nodes of Ranvier were examined for each animal, and the nodal length was measured.

RNA extraction and reverse transcription. At five, ten and 17 days after the initiation of nerve lengthening, five rats from each group were killed with an overdose of sodium pentobarbital and both sciatic nerves were similarly excised. The total RNA was isolated and purified from 30 mg of each sample using an RNeasy Protect Mini kit (Qiagen, Hilden, Germany), as described previously.⁹ The reaction products were stored at -20°C until further PCR analysis.

Quantitative RT-PCR analysis. The quantity of mRNA was determined using a PRISM 7700 sequence detection system (Applied Biosystems, Foster City, California) with TaqMan standard curve methods²³ with a two-step PCR protocol at 95°C for ten minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute, as described previously.¹¹ TaqMan probes for rat P0 were as follows: forward primer, 5'-TCTTTTACCTGGCGCTACCAG-3'; reverse primer, 5'-GTTGACCCTTGGCATAGTGGA-3'; and TaqMan probe, 5'-Fam-AAGGAGGCCGAGATGCCATTTCAATC Tamra-

3', which amplified a 73 bp region of rat P0 mRNA (nucleotides 218 to 290; NCBI accession no. K03242). To account for the variation in quality of mRNA between samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed as an internal control. The primers and TaqMan probe for GAPDH were produced by Applied Biosystems (TaqMan Rodent GAPDH Control Reagents, VIC Probe).

Electrophysiology. At 17 and 52 days after the initiation of lengthening, the motor conduction velocity (MCV) and the compound muscle action potential (C-MAP) amplitude were used to assess neural function. The five rats from each group were laid on a hot plate and their rectal temperature was monitored. The nerve was stimulated at the ischial tuberosity and at the entrance of the extensor digitorum longus muscle of the tibial nerve. The recording was carried out at the gastrocnemius muscle. The stimulus used consisted of a square-wave pulse of 0.1 ms duration delivered by an isolator at an amplitude that was adjusted to that of the supramaximal stimulus. C-MAP was amplified, digitised, recorded, and analysed using a computer with MacLab (ADInstruments, Castle Hill, New South Wales, Australia). C-MAP amplitude was quantified by its peak-to-peak amplitudes in the tibial nerve. The conduction velocity was assessed in combination with the interelectrode distance and negative peak latency.

Statistical analysis. All data are presented as the mean $\pm$ SD. Two-factorial ANOVA²⁴ and Fisher's PLSD²⁴ were used to evaluate differences in the relative quantities of mRNA, MCV and C-MAP between the progesterone-treated group and the control group. Student's *t*-test for unpaired data was used to evaluate differences in nodal length between the two groups. A *p*-value < 0.05 was considered to indicate statistical significance.

Results

Morphological analyses. Light microscopic observations of transverse sections at 52 days after the initiation of lengthening showed no significant differences in either axon diameter or thickness of the myelin sheath between the two groups (Fig. 1). There was no myelin debris or endoneurial oedema in either group. The electron microscopic analysis of longitudinal sections on day 52 showed that the mean nodal lengths were 5.43 μ m in the progesterone-treated group and 6.29 μ m in the control group. Almost all fibres in both groups showed nodal lengths of 1 μ m to 5 μ m (Fig. 2a), but some had nodal lengths of more than 10 μ m (Fig. 2b), which indicates demyelination. The percentages of fibres with nodal lengths of more than 10 μ m were 7.14% (SD 1.35%) in the progesterone-treated group and 14.29% (SD 3.18%) in the control group. The number of fibres with a long nodal length in the progesterone-treated group was significantly lower than in the control group (*p* = 0.045).

Quantitative analysis of P0 mRNA. In order to examine the effects of progesterone on the myelin synthesis of elongated nerves, we analysed the changes in the level of mRNA

Fig. 1a

Fig. 1b

Transverse sections of sciatic nerves on day 52 observed by light microscopy a) progesterone-treated group and b) control group. No significant differences were evident in either axon diameter or thickness of myelin sheath between the two groups. Scale bars: 10 μ m.

Fig. 2a

Fig. 2b

Longitudinal sections of sciatic nerves on day 52 observed by electron microscopy. Nodal lengths (the distance between the arrow heads) of almost all the fibres were between 1 μ m to 5 μ m in both groups. Figure 2a shows a fibre with a nodal length of 2.27 μ m in the progesterone-treated group. There were twice as many fibres with nodal lengths of more than 10 μ m in the control group as in the progesterone-treated group; figure 2b, 13.3 μ m.

expression of P0, a major myelin glycoprotein, by quantitative RT-PCR analysis. GAPDH mRNA was used as the internal control; no significant differences in GAPDH mRNA expression level were observed between the two groups at any time. We calculated the relative quantities of P0 mRNA by dividing the values of P0 mRNA by that of GAPDH mRNA. No significant difference in the relative quantity of P0 mRNA were noted in the control group at any time during the experiment.

Five days after the initiation of lengthening, the relative quantity of P0 mRNA in the progesterone-treated group was 35.64 (SD 7.54), and that in the control group was 24.31 (SD 8.74). The level of expression of P0 mRNA in the progesterone-treated group increased significantly by

46.6% compared with the control group (0%, $p = 0.048$; Fig. 3). On day ten, the relative quantity of P0 mRNA was 37.55 (SD 9.27) in the progesterone-treated group and 27.07 (SD 9.62) in the control group. There was no significant difference, although P0 mRNA expression in the progesterone-treated group increased by 38.7%. P0 mRNA expression in the progesterone-treated group increased transiently between day five and day 17. At 17 days the level of expression in the control group increased, but there was no significant difference between the two groups.

Electrophysiology. At day 17 after the initiation of lengthening, the mean MCV of both groups decreased, 15.40 m/s (SD 5.3) in the progesterone-treated group and 20.25 m/s

(SD 11.9) in the control group. At 52 days, the mean MCV of the progesterone-treated group increased to 34.93 m/s (SD 8.6), but that of the control group was 15.98 m/s (SD 6.0). A significant difference was observed between the two groups at 52 days ($p = 0.016$; Fig. 4a). The mean C-MAP amplitude in the progesterone-treated group was 11.0 mv (SD 2.09) on day 17, and 15.8 mv (SD 1.42) on day 54, and that in the control group was 6.76 mv (SD 1.10) on day 17 and 8.77 mv (SD 1.03) on day 54. At both time points, the amplitude of C-MAP in the progesterone-treated group was higher than in the control group, with significant differences (Fig. 4b).

Discussion

The level of expression of P0 mRNA in the progesterone-treated group increased significantly on day five, whereas in the control group it had only begun to increase on day 17. Remyelination in an elongated nerve might have started earlier in the progesterone-treated group, resulting from the promotion of myelin synthesis by progesterone. There is evidence that progesterone accelerates myelin formation in rat Schwann cell neuron co-culture.¹² The nodal length of the progesterone-treated group was smaller than in the control group on day 52. In our previous study, we observed teased fibres of rat sciatic nerve. In normal nerve, less than 5% of fibres have shown paranodal demyelination.⁸ In this study, the proportion of fibres with a large nodal length was about 7% in the progesterone-treated group and 14% in the control group. The incidence of demyelination in the progesterone-treated group was half of that in the control

Fig. 3

Bar chart showing changes in P0 mRNA expression level with time, determined by quantitative RT-PCR analysis. On day five, the relative quantity of P0 mRNA in the progesterone-treated group was 35.64 (SD 7.54), and that in the control group was 24.31 (SD 8.74). P0 mRNA expression level in the progesterone group increased with significant difference ($p = 0.048$). In the progesterone-treated group it was increased transiently to 17 days. On day 17, P0 mRNA expression level in the control group increased, but there was no significant difference between the two groups.

group. This suggests that the progesterone-treated group made a good recovery from the demyelination caused by nerve elongation. Furthermore, the amplitude of C-MAP of the progesterone-treated group was higher than in the con-

Fig. 4a

Fig. 4b

a) On day 17, the mean motor conduction velocity (MCV) of both groups was decreased: 15.40 m/s (SD 5.3) in the progesterone-treated group and 20.25 m/s (SD 11.9) in the control group. On day 52, the mean MCV of the progesterone-treated group increased to 34.93 m/s (SD 8.6), but that of the control group was 15.98 m/s (SD 6.0). A significant difference was observed between the two groups on day 52 ($p = 0.016$). b) Mean compound muscle action potential (C-MAP) amplitudes in the progesterone-treated group were higher than in the control group on days 17 and 54, with statistically significant differences.

trol group on days 17 and 54, and the MCV of the progesterone-treated group was faster on day 52. This indicates that functional recovery in this group was better than in the control group. These results suggest that progesterone might accelerate the rescue of electrophysiological abnormalities caused by nerve stretching.

The pathological change caused by a gradual elongation of a nerve is generally demyelination.^{5,6,8} In recent studies, we have demonstrated that peripheral nerves can initially recover from demyelination by synthesis of myelin during gradual elongation under appropriate conditions.⁸⁻¹⁰ Interactions between axons and Schwann cells are important for myelin formation,²⁵ and the initiation of myelination and the thickness of the myelin sheath are regulated by specific properties of axons.²⁶ Elongation of a nerve may cause some changes in both nodal structure and the lamellar form of the myelin sheath created by slippage in the localisation of the node or the increase in nodal length. These changes may affect components of the axonal and extracellular matrix and stimulate myelin synthesis in Schwann cells. There probably is a limit to the capacity of restoration of demyelination. Thus disorders of a peripheral nerve may occur when the speed of lengthening or distance are excessive. However, it might be possible to prevent these disorders by promoting myelin synthesis, as shown in this study.

Several neurotrophic factors, such as insulin-like growth factors,²⁷ neurotrophin (NT)-3,²⁸ and brain-derived neurotrophic factor (BDNF),^{28,29} as well as neurosteroids, are regulators of myelination. Among them, progesterone is already in therapeutic use in the field of obstetrics. Although side effects, including pseudopregnancy symptoms such as nausea or transient fever occasionally occur, the safety of this therapy is established. In recent studies, several effects of progesterone on the nervous system were observed and the molecular mechanisms underlying these effects have been investigated. Progesterone is locally synthesised by Schwann cells.¹⁶ They also have an intracellular receptor for progesterone, which functions as an autocrine signalling molecule.³⁰ Progesterone may promote myelination at the transcriptional level by activating the expression of genes coding for the transcription factors (Krox-20) of myelin proteins.³¹ It has been reported that progesterone promotes myelination in regenerating nerves or cultures of Schwann cells.^{11,29,32,33} Progesterone also rescues segmental demyelination induced by ageing by stimulating P0 gene expression.¹⁶ In addition, progesterone promotes the proliferation of Schwann cells and neural progenitor cells.^{14,17} These studies have shown the many actions of progesterone in the central and peripheral nervous systems.

In this study we have demonstrated that progesterone might have a recovery effect on the nerve injury which occurs during limb lengthening. This finding may have other important clinical implications. One is in the treatment of demyelinating polyneuropathies, such as the Guillain-Barré syndrome or diabetic neuropathy, and the

other is in the repair of nerve gaps by gradual elongation of the nerve.³⁴⁻³⁶ The development of this therapy that can prevent the side effects of long-term medication might encourage the use of progesterone as a neurotherapeutic agent.

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