Enhancement of perineurial regeneration and repair of nerve function by novel polysaccharide-derived hydrogel

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Introduction
Adhesion and perineurial scarring represent major causes of treatment failure after peripheral nerve surgery. A novel carboxymethylcellulose (CMC)-derived hydrogel, in which phosphatidylethanolamine (PE) was introduced into the carboxyl groups of CMC has been developed. The effects of CMC-PE hydrogel in preventing peripheral scarring and early restoration of nerve function were reported.

The perineurium represents a continuum with the pia-arachnoid in the central nervous system and surrounds nerves within the epineurium throughout the peripheral nervous system. The function of the perineurium is to protect the nerve against mechanical stretching and to maintain the homeostasis of the nerve by acting as a diffusion barrier, called the blood-nerve barrier. Bunge et al. conducted a lineage analysis of perineurial cells using beta-galactosidase as a cell marker and demonstrated that perineurial cells originate from fibroblasts but not from Schwann cells.

Recently, several papers have reported constitutive expression of tenascin-C(TN-C) by perineurial cells. Although TN-C is diffusely expressed during neurogenesis in the peripheral nervous system, only the perineurium continues to express small splice variants after birth. Therefore, it is possible that small variant TN-C can be used to track perineurial cells during perineurial regeneration.

Myofibroblasts are specialized fibroblasts that share characteristics with smooth muscle cells expressing smooth muscle actin (α-SMA). Recent papers demonstrated that myofibroblasts predominantly produce a large splice variant of TN-C.

The present study investigated the effects of CMC-PE hydrogel for enhancement of perineurial regeneration after extensive internal neurolysis of rat sciatic nerve by immunolabeling of TN-C and α-SMA.

Materials and Methods
All experimental protocols and animal maintenance procedures used in this study were approved by the Animal Ethics Research Committee of Nagoya University. A total of 72 sciatic nerves from 36 Lewis rats (body weight, approximately 250 g each) were used. Animals were anesthetized by intraperitoneal injection of 5% pentobarbital. Operative microscopy, sciatic nerves were dissected from the surrounding tissues. Both the epineurium and perineurium were carefully removed by cutting circumferentially and stripping distally for 15 mm, taking care to minimize damage to the axons. Nerves were randomly assigned to one of the following groups: Control group (n=24), operated but no treatment; CMC-PE group (n=24), operated and treated with 0.5 mL of CMC-PE hydrogel; Sham group (n=24), in which just skin and muscle fascia were dissected then immediately repaired.

Nerves in each group were harvested and kept immersed in 4% paraformaldehyde overnight for histologic and immunologic evaluation (n=6 from each group at days 2, 7, 14, 21, and 28). The specimens were embedded in paraffin and cut into 4-μm sections that were stained with hematoxylin and eosin (HE).

Immunohistochemical studies were performed with monoclonal mouse anti-TN-C antibody clones 4F10TT (IBL, Gunma, Japan) and 4C8MS (IBL). 4C8MS specifically recognizes the alternative splicing sites, whereas 4F10TT reacts with constitutive sites of TN-C molecules. Sections on slides were incubated with either rabbit polyclonal antibodies (1 μg/mL), 4F10TT (2 μg/mL) or 4C8MS (5 μg/mL) overnight at 4°C and subsequently with peroxidase-conjugated anti-mouse or anti-rabbit IgG Fab’ (1:500; MBL, Nagoya, Japan) for 1 hour. After washing, diaminobenzidine/H2O2 solution was used to visualize antibody binding. The sections were then lightly counterstained with hematoxylin to facilitate orientation. The monoclonal antibody of TN-C (4F10TT) specifically recognizes the EGF-like domain of TN-C, therefore the antibody can be detected in all TN-C isoforms. On the other hand, the monoclonal antibody of TN-C (4C8MS) specifically recognizes domain B in FNIII repeats of TN-C. Therefore, small variant TN-C is tracked by subtracting the immunolabeling of TN-C (4C8MS) from TN-C (4F10TT).

Myofibroblasts were labeled by a direct immunoperoxidase method with anti-α-SMA antibody (M 0851; Dako Japan, Kyoto, Japan).

Results
In the sham group, the expression patterns of TN-C and α-SMA were normal. TN-C (4F10TT) was expressed at the perineurium and around the vessel walls. In contrast, TN-C (4C8MS) and α-SMA was expressed only at vessel walls.

Two days after operation, in Control group and CMC-PE group, TN-C (4F10TT) was overexpressed in the endoneurium, especially in the outer part of the fascicles (Fig. 1a,2a). TN-C (4C8MS) was expressed at low levels in the outer part of the nerve. However, α-SMA was expressed only at the vascular wall of the capillary vessels and the expression pattern was the same as in the sham group.

At 7 days in Control group, TN-C (4F10TT) was overexpressed in the outer part, particularly at the edge of the nerve fascicles (Fig. 2b). TN-C (4C8MS) and α-SMA were expressed with low levels at the edge of the nerve fascicles. Contrary in CMC-PE group, TN-C (4F10TT) was expressed at the perineurium like original perineurium (Fig. 2b0). TN-C (4C8MS) and α-SMA were expressed with low levels at the perineurium.

At 20 days in Control group, TN-C (4F10TT) was expressed in the periphery of the nerve fascicles incompletely, and was not expressed in the nerve fascicle (Fig. 1c). On the contrary in CMC-PE group, TN-C (4F10TT) was expressed completely surrounding the endoneurium (Fig. 2c). There was no appreciable difference in expression pattern of TN-C (4C8MS) and α-SMA in terms of spatial distribution or intensity compared with CMC-PE group.

At 42 days in both Control group and CMC-PE group, TN-C (4F10TT) were expressed in the surrounding nerve fascicles and appeared to be an original perineurium (Fig. 1d, 2d). TN-C (4C8MS) and α-SMA were expressed only at the vascular wall and expression patterns matched that observed for the sham group.

Discussion
Regenerative process of the perineurium was enhanced by the CMC-PE hydrogel treatment after microsurgical resection of epi-perineurium in rat sciatic nerve. Regenerated perineurium was expressed TN-C (4F10TT) but not α-SMA. Therefore, as to regenerative process of the perineurium, participation of myofibroblast is unlikely. Perineurium is regenerated by TN-C positive intraneurale fibroblasts. CMC-PE hydrogel enhances the regeneration of the perineurium therefore, early restoration of nerve function were emerged.

Fig. 1 Control group

Fig. 2 CMC-PE group

References
1) Novel polysaccharide-derived hydrogel prevents perineural adhesions in a rat model of sciatic nerve adhesion.

2) Perineurium originates from fibroblasts: demonstration in vitro with a retroviral marker.