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A rolled sheet of collagen gel with cultured Schwann cells: Model of nerve conduit to enhance neurite growth

Eiko Goto, Masahiro Mukozawa, Hideki Mori, and Masayuki Hara*

Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan

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We prepared a rolled sheet of collagen gel with cultured mouse Schwann cells (SCs) as a nerve conduit (a medical device for neurosurgeons to repair an injured peripheral nerve). PC12 cells and dorsal root ganglion (DRG) cells were used as neuronal cells for evaluating the neurite growth-promoting activity of the device. As a control, we compared the rolled device with a rod device. Those neuronal cells inoculated at the terminal part of the rolled device migrated into the central part along the inter-layer space of the collagen gel layer, and then differentiated into neurons, extending many neurites for 3–12 days in culture. Significantly, this migration of neuronal cells into the device and their subsequent neurite growth was not observed in the absence of the SCs. We conclude that our rolled sheet of collagen gel with SCs was well designed and very effective to promote neurite growth, and is a promising candidate for the nerve conduit.

[**Key words:** Nerve conduit; Neurite growth; Collagen; Alginate; Schwann cell; Dorsal root ganglion; PC12]

Migration of neuronal cells and neurite growth in the developing nervous system are controlled by extracellular matrices, cell adhesion molecules, and diffusive growth factors (neurotrophic factors). Guidance of the neurites is controlled by attractants and repellents, such as netrin and semaphorin, respectively.

If the gap in the injured peripheral nerve is very short, the proximal and distal stump can be directly sutured by neurosurgical operation. However, injury with a longer gap cannot be repaired by this method. Autograft is another treatment strategy available to the patient that shows relatively good results in clinical records (1). But it is harmful to take another part of the peripheral nervous system out of the patient's body. A cellular graft involving the removal of antigenic cellular components prepared from cadaveric nerve tissues or muscle tissues has been investigated as a substitute for autografts but is still not in clinical application (2, 3). Various types of nerve conduits (sometimes called as nerve guides) have also been studied in the neurosurgery and biomaterials research field, and have been developed to repair the nerve injury (4-14).

The purpose of using a nerve conduit is to support the re-growth of axons along the damaged nerve fibre to the target organ. It is also important to keep the space for the proper re-growth of axons from being blocked by scar tissues formed after the inflammation. We have an interest in the activity of Schwann cells (SCs), cells that are key players in the regeneration of peripheral nerves.

A nerve conduit can be classified into three categories based on composition: (i) a device composed of biomaterials, (ii) a device composed of biomaterials and neurotrophic factors (e.g. NGF), and (iii) a device composed of biomaterials and cultured cells (e.g. SCs). The conduit promotes the regeneration of the injured nerve by (i) blocking the invasion of fibroblasts that form scar tissue, and keeping space for the re-growth of axons, (ii) supplying the neurotrophic factors (e.g. NGF) that promote regeneration, and (iii) secreting the extracellular matrices (ECMs) suitable for re-growth of axons. SCs have been reported to contribute to (ii) and (iii).

The tube conduit filled with collagen gel containing viable SCs can be prepared. However, it is difficult to supply enough oxygen and nutrients, because the gel limits their diffusion. We prepared the rolled device model of the collagen gel sheet with cultured SCs as a confluent state. The model kept the viability of SCs in culture and efficiently enhanced the neuronal cell migration and neurite growth from the neurons.

MATERIALS AND METHODS

Materials and chemicals ICR mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). PC12 cells were obtained from RIKEN Cell Bank (RCB-0009, Tsukuba, Japan). Dulbecco's modified Eagles Medium (DMEM low glucose, cat. No. D6046), horse serum (HS, cat. No. H-1270), L-15 medium (cat. No. L5520), forskolin (cat. No. F6886), cytosine-D-arabinofulanoside (Ara C, cat. No. C1768), and poly-D-lysine (cat. No. P7786) were obtained from Sigma (St. Louis, MO, USA). Porcine skin collagen solution (cat. No. 309-31595), collagenase (cat. No. 031-17601), and sodium alginate (cat. No. 190-0991) were obtained from Wako Chemical Industry Ltd. (Osaka, Japan). Fetal bovine serum (FBS, cat. No. GIBCO 26140-087), streptomycin/penicillin (cat. No. GIBCO 15240-062), Ham's F-12 (cat. No. GIBCO 11765), MEM (cat. No. GIBCO 11095), N-2 supplement (cat. No. GIBCO 17502-048), Dulbecco's modified Eagles Medium (DMEM high glucose, cat. No. GIBCO 11995), trypsin solution (cat. No. GIBCO 15090), Alexa Fluor 568 F(ab')2 fragment of goat anti-rabbit IgG (H+L) (cat. No. A21069), nerve growth factor (2.5S-NGF, 13257-019), and FITC-goat anti-mouse IgG

* Corresponding author. Tel./fax: +81 72 254 9842.
E-mail address: hara@b.s.osakafu-u.ac.jp (M. Hara).

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(H + L) (cat. No. Zymed 62-6511) were obtained from Invitrogen (Carlsbad, CA, USA). Anti-S100 protein rabbit polyclonal antibody (cat. No. Z0311) was obtained from Dako (Glostrup, Demark). Anti-neuronal class III β-tubulin (Tuj 1) mouse monoclonal antibody (cat. No. MMS-435P) was obtained from Covance (NJ, USA). Other chemicals in the reagent grade are used in the experiments.

Cell culture PC12 cells were cultured for proliferation at 37 °C under 5% CO_2 in LG-DMEM medium containing low glucose DMEM, 5% (v/v) FBS, 5% (v/v) HS and 1% (w/v) streptomycin-penicillin. Medium was changed once every 2 days. NGF was added in the culture medium as a final concentration of 50 ng/ml to induce the differentiation of cells into neuron-like morphology.

Isolation of SCs was carried out as follows. A 2-day-old male ICR mouse was killed, sterilized with 70% (v/v) ethanol, and then fixed on a plate for dissection. After muscles and connective tissues were removed from the lower limb, sciatic nerves (approximately 15 mm long) were taken and immersed within a dish (6 cm diameter) containing ice-cold solution of phosphate buffered saline without calcium ions (PBS(−)). Connective tissues were carefully removed again from the sciatic nerve by tweezers under stereomicroscopic observation. Samples of the sciatic nerve were collected from the littermate (9–14 mice), dissected with scissors, transferred to a centrifuge tube, and then washed by centrifugation for 5 min at 500 rpm. After the addition of 3 ml of 1 mg/ml collagenase, the sample was incubated for 40 min at 37 °C, and then incubated for another 20 min after addition of trypsin (final concentration of 0.08%(w/v). Following this, cells were suspended by gentle mixing after addition of 3 ml H-G DMEM. Cells were collected by centrifugation for 5 min at 1200 rpm, suspended in 10 ml H-G DMEM and then cultured in a 0.01(w/v) PDL-coated dish of 0.90 mm diameter. Ara C was added at a concentration of 10 μM the following day to selectively kill the proliferating fibroblasts. The majority of fibroblasts died approximately 2–3 days later. Cells were washed with H-G DMEM, and then cultured in the H-G DMEM containing 10 μM forskolin to produce a confluent culture of SCs. Medium was changed twice in a week. SCs passaged several times were used in the experiments.

Isolation of dorsal root ganglions (DRGs) was carried out as follows. A 4-week-old male ICR mouse was killed, sterilized with 70% (v/v) ethanol, and then fixed on the plate for dissection. Most of the internal organs, were removed from the ventral side. After head, upper limbs and lower limbs were removed the body was washed in ice-cold L-15 medium. Vertebras were opened carefully under a stereomicroscope using fine scissors so as not to damage the spinal cord. After the spinal cord appeared, DRGs were removed from the both sides of the spinal cord by manipulation using fine tweezers. Approximately 50 DRGs were collected from a single mouse. Connective tissues were carefully removed from the DRGs in ice-cold L-15 medium. Cells were dissected, collected by centrifugation for 5 min at 500 rpm, and treated for 1 h in 2 ml of L-15 medium containing 1 mg/ml collagenase. After addition of 3 ml L-15 medium, cells were dispersed in the medium, collected by centrifugation for 5 min at 1200 rpm, and suspended in the F-12 medium for use in our experiments.

Device model of the rolled collagen gel layer with SC Device models with SCs were prepared as follows. The procedure to prepare the collagen gel sheet with SCs was carried out similar to a method previously reported, with some minor modifications (15). We used citrate to chelate calcium ions instead of using ethylenediaminetetraacetic acid (EDTA) because of its lower toxicity. One milliliter of 1% (w/v) sodium alginate solution was spread on the inner dish of a cell culture insert (Falcon cat. No. 353091, Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a porous membrane (pore size 3 μm) on the bottom. After the excess amount of the viscous solution was removed by aspiration, the inner dish was immersed in the 0.1 M $CaCl_2$ solution for 2 min at room temperature. The solution was permeated across the membrane from the outer side and formed a thin calcium alginate gel layer on the upper surface of the membrane. Five milliliter of the solution consisting of 1% (w/v) collagen, DMEM, and PBS(−) for adjustment of pH (pH = approximately 7) was poured onto the inner dish and incubated for 24 h at 37 °C to prepare the collagen gel layer. The surface of the gel was coated with 50 μg/cm^2 laminin. SCs (1×10^5 cells) were inoculated on the collagen gel layer, and then cultured in the H-G DMEM medium to be in a confluent state. When the inner dish carrying the collagen gel layer with SCs was immersed into the 0.2 M sodium citrate solution, the thin calcium alginate gel layer on the porous membrane was dissolved. The SCs/collagen gel layer could then be easily detached from the membrane, and was rolled up by manual manipulation with a flat-tip tweezer for use as a device model. An aliquot of the neutralized collagen solution was used as a glue to fix the rolled shape. Cells from 5 DRGs suspended in 10 μl medium were inoculated on the edge of the device model in the experiment for the observation of neurite growth. That procedure was carried for the experiments in Fig. 3.

For a more quantitative evaluation of the promoting effect on neurite growth, we prepared another type of the device model with a square collagen gel layer for the experiments in Figs. 4 and 5. We made an inner dish of aluminium (17 mm × 17 mm × 15 mm in length, depth, height, respectively) with a filter paper (cat. No. 14372, Whatman, Maidston, Kent, UK) on the bottom. The rolled device model was prepared as described above. Another rod-shaped device model consisting of a collagen gel containing 1×10^5 SCs was used as a control to compare the design of roll shape and rod one. The gel was prepared in a container (7 mm in diameter × 20 mm in length). Either 5×10^3 cells of PC12 or neuronal cells from 2 DRGs were embedded in a small short cylinder-shaped collagen gel (3-5 mm in length × 6 mm in diameter). The gel was attached to the edge of the device model with a collagen gel as glue to ensure the quantitative inoculation of neurons. Fig. 1 schematically shows the principle of our experiment to prepare the rolled device and the rod device.

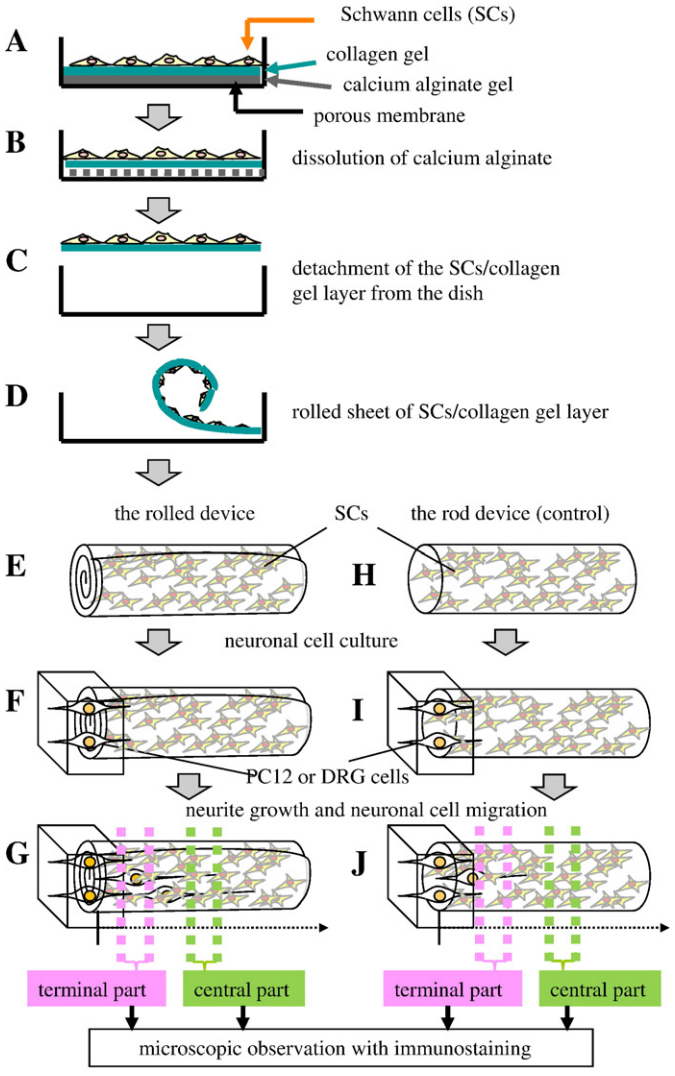

FIG. 1. Schematic representation of the protocol to prepare the rolled device (A–G), and the rod device as a control (H–J).

Coating with laminin was omitted in the procedure described above because we wanted to compare the efficacy between the devices of different shapes: the rolled device and the rod device, in the experiments from Figs. 4 and 5. If we coated the laminin on the surface of the collagen gel in the rolled device, it was difficult to prepare the rod device with the same amount of laminin. The device model was characteristic because the confluent SCs on the thin collagen gel sheet was rolled to be a tube-like structure. We thought that the design would enable the migration of neurons into the depth of the device easily, due to the inter-layer space between the collagen gel.

We did experiments described above using a commercial cell culture insert to prepare the round collagen gel sheet with SCs in order to prepare the rolled sheet according to the procedure previously reported (15). We prepared the square collagen gel sheet with SCs as an improved device model for more quantitative evaluation of the device model to enhance the neurite growth. Another technical problem was how to fix the number of cells inoculated on the device model, because the cells can be easily detached from the collagen gel and run out. PC12 cells were embedded in a collagen gel of the short circular cylinder shape, and attached to the edge of the rolled sheet. This procedure ensured that the number of cells inoculated on the device was fixed.

Microscopy Cells cultured on the 0.001% (w/v) PDL-coated cover slip in a 24-well culture plate was washed three times with PBS(+), fixed with 4% (w/v) paraformaldehyde (PFA) in PBS(−), and washed three times more. Cell membranes were made permeable for 5 min in PBS(−) containing 0.1% (w/v) Triton X-100. Cells were washed three times with PBS(−) and blocked in the presence of PBS(−) containing 0.01% (w/v) Triton X-100 and 10% (v/v) normal goat serum for 1 h. After the sample was washed three times with PBS(−), the primary antibody (either anti-S100 protein rabbit polyclonal antibody (1:300), or anti-βIII tubulin mouse monoclonal antibody (1:500)) was incubated with the sample overnight at 4 °C. After the sample was washed three times with PBS(−), the sample was incubated with the secondary