Bridging A 20mm Rat Sciatic Nerve Gap Using An Undifferentiated Bone Marrow-derived Mesenchymal Stem Cell-laden Conduit Containing Vessels And Decellularized Allogenic Basal Lamina.

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Introduction: Although autologous nerve grafting is considered the gold standard approach in treating severed peripheral nerve injuries, it is associated with some inevitable drawbacks, including a limited source of donor nerves and donor-site morbidity. An artificial nerve conduit is one of the possible alternative treatment options. Our previous studies demonstrated successful axonal regeneration through a vessel-containing tube (VCT) supplemented with undifferentiated bone marrow stromal cells (uBMSCs) in a rodent [1] and a canine model. In the present study, we investigated whether adding thermally decellularized allogenic basal lamina (DAB) to the tube would yield even better axonal regeneration.

Methods: DABs were prepared from sciatic nerves of Dark Agouti (DA) rats (n = 15). To confirm the decellularization and the preservation of the basal lamina, hematoxylin and eosin (H&E) staining and immunohistochemistry for laminin were performed on sections of one DAB. Twenty-millimeter defects in Lewis rat sciatic nerves were bridged using one of the following four different nerve conduits: (1) a silicone tube containing a DAB (Group V-, n = 5); (2) a silicone tube containing a DAB and retrograde vessels accompanying the sural nerve (D/VCT) (Group V+, n = 5); (3) a D/VCT in which isogenic ex vivo expanded uBMSCs were implanted (uBMSC-laden D/VCT) (Group D+, n = 19); and (4) a VCT supplemented with uBMSCs (uBMSC-laden VCT) (Group D-, n = 16). No immunosuppressant was used in the perioperative period of any group. To evaluate neovascularization of the DABs that were implanted in VCTs, immunohistochemical staining for RECA-1 was performed on the sections of the DABs in the midportion of the tubes in Group V+ (n=5) and Group V- (n=5) at 5, 7, 14, 21, and 28 days. To assess the immunogenicity of the DABs in VCTs, the sections of the DABs in the distal portion of the tube in Group V+ (n=4) underwent H&E staining and immunohistochemical staining for CD8 at 7, 14, 21, and 28 days. In this experiment, 20-mm gaps in Lewis rat sciatic nerves were bridged with an unprocessed DA rat sciatic nerve segment (as a positive control, n=4). The nerves that regenerated through the conduits in Group D+ (n = 16) and Group D- (n = 16) were assessed via electrophysiological, morphometric, and wet muscle weight measurements at 12 and 24 weeks. The amplitude and motor nerve conduction velocity (MNCV) of the compound muscle action potentials (CMAPs) detected in the pedal adductor muscles were calculated under anesthesia. The ratios of the CMAP amplitude and MNCV of the operated limb to those of the contralateral healthy limb were used to express the CMAP amplitude and MNCV in each rat, respectively. The total number of myelinated axons (MAN), the nerve fiber diameter (NFD), and the myelin thickness (MT) were measured on transverse sections of the distal portion of the regenerated nerves. The wet muscle weight (WMW) of tibialis anterior muscles was also measured. The ratio of the
WMW of the operated limb to those of the contralateral limb was calculated. In three rats in Group D+ (n = 3), uBMSCs prepared from a transgenic LEW rat expressing green fluorescent protein (GFP) were implanted. These three rats underwent immunohistochemistry for GFP, glial fibrillary acidic protein (GFAP), and S-100 protein (S-100) at 6 weeks.

**Results:** H&E staining revealed that there were almost no nuclei in the DAB, and immunohistochemical staining demonstrated the preservation of laminin in the DAB. In Group V+, RECA-1-positive cells were observed in the DAB around the inserted vessels at 1 week, and these had spread diffusely in the DAB at 4 weeks. In contrast, in Group V-, only a few positive cells were observed exclusively in the peripheral region of the DAB at 4 weeks (Figure 1). In Group V+, H&E staining and immunohistochemistry showed a much smaller number of cells, including CD8+ cells, compared with unprocessed fresh allogenic nerve grafting at 2 and 4 weeks (Figure 2). Electrophysiological studies revealed a significantly higher amplitude (0.10 ± 0.06 vs 0.04 ± 0.03, p < 0.05 at 12 weeks; and 0.69 ± 0.17 vs 0.11 ± 0.09, p < 0.05 at 24 weeks) and higher MNCV ratios (0.50 ± 0.17 vs 0.38 ± 0.24, p = 0.41 at 12 weeks; and 0.63 ± 0.09 vs 0.55 ± 0.08, p = 0.13 at 24 weeks) in Group D+ compared with Group D- at both 12 and 24 weeks. The histomorphometric analysis revealed that Group D+ had a significantly greater MAN (3370 ± 1253 vs 852 ± 435, p < 0.05 at 12 weeks; and 4650 ± 722 vs 2324 ± 1035, p < 0.05 at 24 weeks), a larger MT (0.58 ± 0.04 μm vs 0.45 ± 0.09 μm, p < 0.05 at 12 weeks; and 0.63 ± 0.08 μm vs 0.49 ± 0.04 μm, p < 0.05 at 24 weeks), and a greater NFD (3.47 ± 0.37 μm vs 2.89 ± 0.36 μm, p < 0.05 at 12 weeks; and 3.88 ± 0.53 μm vs 3.09 ± 0.27 μm, p < 0.05 at 24 weeks) than did Group D- at both time points. The mean value of the WMW ratio in Group D+ was significantly superior to that detected in Group D+ at both time points (0.40 ± 0.07 vs 0.22 ± 0.09, p < 0.05 at 12 weeks; and 0.69 ± 0.06 vs 0.29 ± 0.07, p < 0.05 at 24 weeks).

Six weeks postoperatively, some of the GFP-positive cells were also immunopositive for S-100 and GFAP.

**Discussion:** The results of the current study indicated that DABs implanted in VCTs were completely decellularized and provided immunologically tolerated basal lamina scaffolds, which were revascularized in the early posttransplantation phase and allowed some of the uBMSCs that had been implanted into the D/VCT to differentiate into Schwann cell-like cells. Several recent studies have demonstrated that uBMSCs produce various kinds of growth factors and cytokines that facilitate axonal regeneration [2,3].

Cells, growth factors, and scaffolds are considered as three critical factors in tissue engineering. We believe that providing vascularity is another important factor for successful tissue regeneration. Our artificial nerve conduit included these four factors, which were assumed to promote nerve regeneration in uBMSC-laden D/VCTs bridging a long nerve gap compared with uBMSC-laden VCTs.

**Significance:** Our novel nerve conduit, uBMSC-laden D/VCT, is expected to be one of the promising options for peripheral nerve repair, without the use of immunosuppressants.
Figure 1. Revascularization of the DABs.
Immunohistochemistry for RECA-1 was performed on transverse sections of the midportion of the conduits in Group V+ (a and b) and Group V− (c). The area surrounded by a yellow dotted line is a DAB. Scale bar, 100 μm.

Figure 2. Assessment of immunogenicity (H&E staining).
The longitudinal sections of the distal portion of the conduits in Group V+ were stained with H&E. (a and c) The sections of the unprocessed fresh allogenic nerve collected 2 and 4 weeks after implantation served as a positive control. (b and d) Scale bar, 100 μm.
Figure 3. The regenerated nerve in the distal portion of the conduit. The transverse sections of the regenerated nerves that were stained with toluidine blue are shown. Scale bar, 100 μm.