Cauda Equina-Derived Extracellular Matrix for Fabrication of Nanostructured Hybrid Scaffolds Applied to Neural Tissue Engineering

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Extracellular matrix (ECM) components have become important candidate materials for use as neural scaffolds for neural tissue engineering. In the current study, we prepared cauda equina-derived ECM materials for the production of scaffolds. Natural porcine cauda equina was decellularized using Triton X-100 and sodium deoxycholate, shattered physically, and made into a suspension by differential centrifugation. The decellularization procedure resulted in the removal of >94% of the nuclear material and preserved the extracellular collagen and sulfated glycosaminoglycan. Immunofluorescent staining confirmed the presence of collagen type I, laminin, and fibronectin in the ECM. The cauda equine-derived ECM was blended with poly(l-lactide-co-glycolide) (PLGA) to fabricate nanostructured scaffolds using electrospinning. The incorporation of the ECM increased the hydrophilicity of the scaffolds. Fourier transform infrared spectroscopy and multiphoton-induced autofluorescence images showed the presence of the ECM in the scaffolds. ECM/PLGA scaffolds were beneficial for the survival of Schwann cells compared with scaffolds consisting of PLGA alone, and the aligned fibers could regulate cell morphologic features by modulating cellular orientation. Axons in the dorsal root ganglia explants extended to a greater extent along ECM/PLGA compared with PLGA-alone fibers. The cauda equina ECM might be a promising material for forming scaffolds for use in neural tissue engineering.

Introduction

THE REPAIR AND RECONSTRUCTION of damaged nerves remain a clinical challenge. Neural tissue engineering provides an alternative medical approach to conventional transplantation.¹ The selection of biomaterials is important for the generation of scaffolds for neural tissue engineering. Numerous synthetic and natural biomaterials have been used to construct scaffolds, including poly(l-lactide-co-glycolide) (PLGA),² poly(ϵ -caprolactone),³ poly-L-lactic acid (PLLA),⁴ collagen,⁵ gelatin,⁶ silk fibroin,⁷ and chitosan.⁸ However, synthetic biomaterials lack biocompatibility and biomimetics; naturally derived biomaterials are often needed to improve their physical and biological properties.^{9,10} Scaffolds comprising both strong biodegradable synthetic materials and the natural immunologically neutral extracellular matrix (ECM) might help to overcome these obstacles.^{11,12} This combinatorial approach has yielded some success in encouraging Schwann cell (SC) growth, neurite elongation from the dorsal root ganglion (DRG),

neurite outgrowth, and functional recovery. Examples include the addition of a composite collagen and hyaluronan hydrogel to a poly(L-lactic acid)-co-poly(ϵ -caprolactone) (PLCL) scaffold,¹³ blending collagen with PLGA or PCL to produce a scaffold using electrospinning,^{14,15} the synthesis of a core-shell and laminin blended nanofibers incorporating PLCL,¹⁶ coupling laminin onto PLLA nanofibers using covalent binding, physical adsorption, or blended electrospinning procedures,¹⁷ or modifying the surface of a poly- β -hydroxybutyrate scaffold with fibronectin, laminin, or collagen.¹⁸

The ECM is a multifunctional complex of proteins and proteoglycans. The fundamental role of the ECM is to influence cell proliferation, differentiation, adhesion, and migration.¹⁹ Biologic scaffolds comprising ECM components are used commonly in tissue engineering with cartilage,²⁰ the myocardium,²¹ the musculofascia,²² and peripheral nerves.²³ During nerve regeneration, the nerve tissue ECM consisting of many macromolecules, such as collagen, laminin, fibronectin, and other nerve factors deposited among cells, forms

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the microenvironment for the growth of SCs, which play an important role in nerve regeneration.^{24,25}

Natural neural ECM might be one of the best choices of material for scaffolds in neural tissue engineering. Scaffolds have been produced successfully from decellularized peripheral nerves after the removal of cellular antigens and the preservation of most of the structural and functional proteins that constitute the ECM.^{23,26} Unlike peripheral nerves, the cauda equina is not surrounded by the epineurium and endoneurium. The perineurium of the cauda equina consists of only a single or double layer of thin sheath cells.²⁷ After decellularization, the cauda equina ECM might retain more basement membrane components, which are effective for regenerating axons.²⁸ However, few studies have investigated the use of the cauda equina-derived ECM for peripheral nerve repair.

The aim of the current study was to prepare the cauda equina-derived ECM and assess the feasibility of its use to construct scaffolds for neural tissue engineering. The ECM was prepared from natural porcine cauda equina by chemical extraction and differential centrifugation, and the structure and composition of the ECM were analyzed. The hybrid ECM/PLGA scaffolds with random and aligned fibers produced by electrospinning were compared with PLGA-alone scaffolds. The properties of the scaffolds were examined using scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and contact angle and multiphoton microscopy. SCs and DRGs were seeded on hybrid scaffolds to understand the effect of incorporating cauda equina ECM components.

Materials and Methods

Preparation of the cauda equina ECM

Cauda equina segments harvested from 150-kg pigs were immersed in distilled water and incubated with 3% (v/v) Triton X-100 (Sigma) with shaking for 2 h at 25°C. They were then agitated for 2 h in 4% sodium deoxycholate (Sigma) in distilled water. After decellularization, the tissue segments were rinsed in sterile phosphate-buffered saline (PBS) solution, homogenized under a powerful shearing force using a tissue disintegrator (Ultra-Turrax; IKA) in distilled water to form a suspension slurry, and centrifuged by differential centrifugation (Beckman Allegra X-22R) for 10 min at 1500 rpm using an F0850 rotor. The suspended microparticles were separated using precipitated macroparticles and centrifuged for another 10 min at 3000 rpm, followed by 10 min at 6000 rpm. Next, the precipitates were centrifuged for 30 min at 9000 rpm to collect the cauda equina ECM precipitates. The precipitates were rinsed with sterile PBS solution, diluted in a 3% (w/v) suspension, and then transferred into a freeze dryer and lyophilized for 48 h under a vacuum. The ECM powder was preserved in sterile glassware at 4°C.

Evaluation of the structure and composition of the cauda equina ECM

To observe the morphological features of the cauda equina ECM, a 3% (w/v) ECM suspension was smeared onto microscope slides, dehydrated in a graded alcohol series, and dried at room temperature. The samples were then sputter coated with gold and observed using SEM (Carl Zeiss).

The DNA content in the ECM was quantified by digesting comminuted ECM in papain solution (125 µg/mL papain; Sigma), 5 mM EDTA, 5 mM cysteine, and 1 M NaCl in PBS at 60°C for 48 h on a shaker. The samples were centrifuged (10,000 rpm, 5 min) and the pellet discarded. Residual DNA in the lysates was quantified using a Quant-iT PicoGreen kit (Invitrogen) according to the manufacturer's instructions. The samples were read on a TBS-380 minifluorometer (Turner Biosystems). Analyses were controlled for tissue weight and performed using native tissue and decellularized ECM.

The sulfated glycosaminoglycan (sGAG) contents of native and decellularized cauda equina were determined using a 1,9-dimethylene blue (DMB) dye-binding assay.²⁹ Papain-digested supernatants (20 µL) were transferred to a microtiter plate and DMB dye (Sigma; 200 µL, 16 µg/mL) was added. The sGAG content was quantified by measuring the optical density (OD₄₉₀). The amounts of sGAG were calculated against a standard curve for chondroitin sulfate (Sigma).

The collagen contents of native and decellularized cauda equina were measured using a hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The amount of total collagen content was calculated using a collagen:hydroxyproline ratio of 1:0.134.

For histology and immunohistochemistry, cauda equina ECM precipitates were mounted using an optimal cutting temperature compound (Tissue-Tek Miles). Cryosections (10 µm thick) were cut, placed on microscope slides, left to dry, and then fixed in acetone for 30 min at room temperature. The degree of decellularization was evaluated by staining the comminuted nuclei with Hoechst 33258 after a brief wash in PBS (Molecular Probes).

Immunofluorescent staining was used to detect specific ECM proteins, including collagen type I, laminin, and fibronectin. Briefly, decellularized cauda equina tissues were cut into eight 1 cm pieces, fixed with 4% paraformaldehyde for 10 min, washed thrice with PBS for 5 min, and incubated with mouse antitype I collagen (1:200 dilution), mouse antilaminin (1:200), and rabbit antifibronectin (1:200; all from Sigma) antibodies overnight at 4°C. They were then incubated with FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Shiankexing) secondary antibodies for 60 min and washed thrice with PBS for 5 min. Finally, the color was developed using diaminobenzidine tetrahydrochloride, and 10 images were captured from each sample using a microscope.

The synthesis of random and aligned cauda equina ECM/PLGA nanofiber scaffolds

PLGA and ECM at a weight ratio of 70:30 were dissolved in hexafluoro-2-propanol (HFP) (Sigma) and stirred for 24 h at room temperature to create a 7% (w/v) solution. Pure 7% (w/v) PLGA and 4% (w/v) ECM solutions were prepared by dissolving PLGA and ECM in HFP and stirring at room temperature for 24 h. The solution was electrospun from a 1-mL syringe containing a needle with a diameter of 0.4 mm and volumetric flow rate of 3 mL/h. A high voltage (20 kV) was applied to the tip of the needle when the fluid jet was ejected. Coverslips (15 mm) were secured to a grounded aluminum collector disc, and a target rotating at 200 and 2500 rpm was used to generate random and aligned fibers, respectively.

Characterization of the scaffolds

The morphological features of the scaffolds were studied under an AURIGA Cross Beam FIB/SEM station (Carl Zeiss) at an accelerating voltage of 10 kV after sputter coating with gold. The diameters of the electrospun fibers were calculated from SEM images using ImageJ software (National Institutes of Health). The distribution and orientation of the aligned fibers were analyzed by calculating the relative angle of individual fibers from $+90^\circ$ to -90° , where 0° was taken as the axial direction. At least 60 fibers were measured for each preparation.

The hydrophilic/hydrophobic nature of the electrospun nanofibrous scaffolds was assessed by measuring the sessile drop water contact angle using a video contact angle system (OCA15plus; Dataphysics). Distilled water was used to form the drops.

FTIR studies involved compressed films containing KBr pellets with an FTIR spectrophotometer (Spectrum One). All spectra were recorded in absorption mode at 4-cm intervals and a wavelength range of 4000–450 cm.

Multiphoton microscopy and second harmonic generation (SHG) imaging were performed using an upright microscope (BX61WI; Olympus) and an FV1000-MPE laser scanning microscope system. ECM structure-dependent autofluorescence and SHG were induced at a wavelength of 840 nm. All images were processed using the system software (Olympus FV1000 Viewer). Multiphoton images are shown in green to illustrate the fluorescent properties of the fibers.

Cell and DRG culture

SCs were harvested from the sciatic nerves of Sprague-Dawley neonatal rats using a previously established protocol.³⁰ SCs were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 2 μ M forskolin (Sigma), and 10 ng/mL heregulin- β -1 (PeproTech) in a 75-cm² cell culture flask at 37°C in a humidified atmosphere containing 5% CO₂; the culture media were changed every 3 days. PLGA and ECM/PLGA aligned and random nanofibrous scaffolds on 15-mm coverslips were placed in 24-well plates. The specimens were sterilized by irradiation for 12 h with Co⁶⁰, washed thrice with PBS, and immersed in the culture medium overnight before seeding. Cells were seeded on scaffolds at a density of 1×10^4 cells/well and left in the incubator to facilitate cell growth. Cells were incubated for 7 days until fixation for further analysis.

DRGs were harvested from rat pups on postnatal day 1. The nerve roots were removed, and the DRGs were seeded on random and aligned PLGA and ECM/PLGA nanofibrous scaffolds. The fibers were covered with DMEM/F12 1:1 media supplemented with 10% FBS and 50 ng/mL nerve growth factor. DRGs were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and the culture media were changed once every 3 days. DRGs were incubated for 7 days and then fixed and used for further analysis.

Cell proliferation assay

Cell proliferation was evaluated using a cell counting kit (CCK-8 kit; Dojindo Laboratories) to determine the number of viable cells in culture. After 2, 4, and 7 days of culture,

the media were discarded and ~ 500 μ L serum-free DMEM/F12 and 50 μ L CCK-8 solution were added to each well sequentially. They were then incubated at 37°C for 3 h to form a formazan product. The supernatants were then transferred to a 96-well plate, and the optical density (OD₄₅₀) was measured using a microplate reader (Beckman) to represent the metabolic activity of the cells present in the scaffolds.

Cell morphology

The morphology of *in vitro* cultured cells grown on PLGA and ECM/PLGA aligned and random nanofibrous scaffolds was examined. After 7 days of culture, the scaffolds were processed for SEM. Specifically, they were rinsed twice with PBS, fixed in 2.5% glutaraldehyde for 3 h, rinsed in DI water, and dehydrated using increasing concentrations of ethanol (50%, 70%, 90%, and 100%) twice for 20 min each. After a final wash with 100% ethanol, the samples were treated with hexamethyldisilazane (HMDS). The HMDS was air-dried by placing the samples in a fume hood. Finally, the scaffolds were sputter coated with gold and observed using SEM.

The orientation of the cells was approximated by measuring the orientation of the long axis of an elongated cell using ImageJ software. The orientation angles were binned every 10° , and the frequency distribution was plotted between 0° and 180° , with 0° representing the vertical direction of the image.

Immunocytochemistry

SCs and DRGs were grown on electrospun nanofibrous scaffolds for 7 days and processed for immunocytochemistry. Cells and DRGs were fixed with 4% paraformaldehyde (Sigma) at room temperature for 30 min and then washed with PBS. Cells were further incubated with antibodies against S100 (Abcam; 1:100 diluted in PBS, mouse IgG), and DRGs were incubated with NF200 (Abcam; 1:100 diluted in PBS, mouse IgG) at 4°C overnight. The next day, cells and DRGs were washed with PBS and incubated with the corresponding secondary antibodies (phycoerythrin-conjugated goat anti-mouse IgG; Invitrogen) for 60 min at room temperature in the dark. After a third rinse in PBS, the nuclei were counterstained with Hoechst 33258 for 5 min. After a final wash in PBS, cells and DRGs were visualized under a fluorescence microscope (Olympus BX-50), and the images were recorded digitally and processed using Image-Pro Plus (Media Cybernetics).

Statistical analyses

All quantitative data were analyzed and expressed as means $\pm$ standard deviations. Statistical analyses were performed using one-way analysis of variance (ANOVA). Comparisons between two means were analyzed using Tukey's test, and $p < 0.05$ was considered to indicate statistical significance.

Results

Characterization and biochemical composition of the ECM

Figure 1 shows the simplified protocol used to obtain the ECM from porcine cauda equina. Decellularization of the

FIG. 1. Schematic showing the procedure used to prepare porcine cauda equina-derived extracellular matrix (ECM). Segments of the cauda equina were immersed in distilled water and decellularized with 3% (v/v) Triton X-100 and 4% (w/v) sodium deoxycholate. The decellularized cauda equina was shattered physically and prepared into a 3% (w/v) suspension after differential centrifugation. The suspension was transferred into a freeze dryer and lyophilized for 48 h under a vacuum. The ECM powder was preserved in sterile glassware at 4°C. Color images available online at www.liebertpub.com/tea

cauda equina material effectively removed the cellular contents, as demonstrated by Hoechst tissue staining and a PicoGreen assay. Nucleic acid staining of the cauda equina-derived ECM (Fig. 2B) was lower compared with native tissue sections (Fig. 2A). Quantitative PicoGreen analysis of the residual DNA content demonstrated that >94% of DNA had been removed from the decellularized tissue (Fig. 3A). The native cauda equina tissue had 897.4 ± 64.5 ng/mg ECM DNA, which was reduced to 45.2 ± 7.6 ng/mg after decellularization. An SEM image of the ECM revealed that it had

a nanofibrous structure with a variably porous ECM microenvironment (Fig. 2C).

The preservation of the ECM proteins in the native tissue is a major goal in the development of ECM material. Immunofluorescent staining of the cauda equina-derived ECM showed preserved collagen I (Fig. 2D), laminin (Fig. 2E), and fibronectin (Fig. 2F) with similar distributions in the decellularized ECM material. Figure 3B and C shows the results of collagen and sGAG quantification in the native and decellularized cauda equina tissues. A significant

FIG. 2. Characterization of the composition and structure of the cauda equina ECM. Cell nuclei were visible in native (A), but not in decellularized (B) cauda equina tissue after Hoechst 33258 staining. Scale bar = 200 μ m. (C) Scanning electron microscopy (SEM) of the cauda equina ECM. Scale bar = 1 μ m. Immunofluorescent staining revealed that collagen type I (D), laminin (E), and fibronectin (F) were present in the decellularized cauda equina tissue. Scale bar = 200 μ m. Color images available online at www.liebertpub.com/tea

FIG. 3. Analysis of DNA and the biochemical components in the native and cauda equina ECM. (A) DNA quantification revealed lower concentrations of DNA in the cauda equina ECM compared with native tissues. Collagen (B) and sulfated glycosaminoglycan (sGAG) (C) quantification in the native and decellularized cauda equina tissues showed a significant increase in collagen content, but a reduction in the sGAG content. $n=5$; $*p<0.001$.

FIG. 4. Characterization of the morphology of the electro-spun nanofiber scaffolds. SEM micrographs of cauda equina-derived ECM nanofiber scaffolds (A, B); scale bar = 1 μm in (A) and 2 μm in (B). Random (C) and aligned (D) poly(l-lactide-co-glycolide) (PLGA) nanofiber scaffolds; scale bar = 1 μm. Random (E) and aligned (F) ECM/PLGA nanofiber scaffolds; scale bar = 1 μm. (G) Diameters of the PLGA and ECM/PLGA nanofiber scaffolds. (H) Histogram of the fiber angle of aligned PLGA and ECM/PLGA nanofiber scaffolds.

FIG. 5. Chemical characterization of the electrospun scaffolds. Fourier transform infrared spectroscopy spectra for PLGA and ECM/PLGA nanofiber scaffolds.

increase in collagen content was observed after decellularization, which might be due to the loss of cellular materials in the cauda equina ECM. However, the sGAG content was reduced in the decellularized ECM material.

Morphology and chemical characterization of the electrospun nanofibers

An SEM image of the electrospun cauda equina-derived ECM revealed a fibrous mat with fibers arranged in a random orientation (Fig. 4A, B). However, fiber breakage occurred frequently because of the low solution viscosity. Pure cauda equina ECM was less suitable for the production of

aligned nanofibers. By adding PLGA, a greater degree of alignment was obtained (Fig. 4F). For the aligned ECM/PLGA scaffolds, 53% of the fibers varied within $0^\circ \pm 10^\circ$, and for the aligned PLGA scaffolds, 46% of the fibers varied within $0^\circ \pm 10^\circ$ (Fig. 4H).

SEM micrographs of PLGA and ECM/PLGA nanofibrous scaffolds revealed the formation of porous, nanoscaled fibrous structures under controlled parameters. Randomly oriented PLGA and ECM/PLGA nanofibrous scaffolds had mean fiber diameters of 263 ± 60 and 182 ± 82 nm, respectively (Fig. 4G). Random and aligned PLGA and ECM/PLGA nanofibers did not differ in diameter. PLGA scaffolds were highly hydrophobic with a mean contact angle of $117^\circ \pm 8^\circ$. However, ECM/PLGA scaffolds were more hydrophilic and had a mean contact angle of $67^\circ \pm 5^\circ$.

The FTIR spectra for the PLGA scaffold showed absorbance peaks at 749 cm^{-1} (CH-bend), 1093 and 1185 cm^{-1} (C–O stretch), 1384 and 1456 cm^{-1} (CH-bend), 1759 cm^{-1} (C=O ester), and 2998 and 2947 cm^{-1} (CH_3 stretch) (Fig. 5). A broad peak at 3535 cm^{-1} illustrates the vibration of the OH terminal groups.³¹ The ECM/PLGA nanofibrous scaffolds showed common protein bands at $\sim 1650\text{ cm}^{-1}$ (amide I) and 1540 cm^{-1} (amide II) corresponding to the stretching vibrations of the C=O bond and the combination of the bending of the N–H bond and stretching of the C–N bond, respectively.³²

The PLGA and ECM/PLGA scaffolds were examined using multiphoton-induced autofluorescence and SHG microscopy (Fig. 6). The PLGA scaffolds shown did not emit much fluorescence, as shown in Figure 6B. However, the fibers in the ECM/PLGA scaffold fluoresced because of the collagen content of the ECM (Fig. 6D). Multiphoton imaging demonstrated that most fibers in the ECM/PLGA scaffolds were a true hybrid of the base materials.

FIG. 6. Verification of the ECM architecture in the electrospun scaffolds. Multiphoton microscopy of electrospun PLGA nanofiber scaffolds in reflection mode (A) and fluorescence (B). ECM/PLGA nanofiber scaffolds in reflection mode (C) and fluorescence (D). Color images available online at www.liebertpub.com/tea

FIG. 7. Effect of the cauda equina ECM on cell proliferation. The proliferation of Schwann cells (SCs) cultured on PLGA and ECM/PLGA nanofiber scaffolds was quantified using a CCK-8 kit and compared with control cultures grown on TCP. $n=8$; $*p<0.05$, $†p<0.01$, $‡p<0.001$. A, aligned nanofibers; R, random nanofibers.

Cell proliferation on the scaffolds

The proliferation of SCs was higher on ECM/PLGA than PLGA nanofibrous scaffolds (Fig. 7). On day 7, the cell proliferation was 27% higher on ECM/PLGA compared with PLGA random nanofibers and 25% higher on aligned ECM/PLGA nanofibers compared with PLGA. Therefore, ECM/PLGA nanofibrous scaffolds were more suitable substrates than PLGA nanofibers for SC proliferation.

SC morphology and adherence on the scaffolds

SCs were seeded on PLGA and ECM/PLGA scaffolds for 7 days. SEM revealed that the cells were oriented along the direction of both PLGA and ECM/PLGA aligned nanofibers and were clustered around the fibers in a longitudinal manner (Fig. 8B, D). For both PLGA and ECM/PLGA random nanofibers, cells were oriented in different directions relative to the nanofibers (Fig. 8A, C). A similar phenomenon was found in S100-stained images (Fig. 9). To quantify the directional bias, cell orientation was plotted as a frequency distribution (Fig. 10). The results suggested that cell orientation did not exhibit any significant bias on the random scaffolds. On the aligned scaffolds, 56% of cells on PLGA scaffolds varied between 70° and 80° , whereas 45% of cells on ECM/PLGA scaffolds varied between 170° and 180° . In addition, SCs on the ECM/PLGA nanofibrous scaffolds showed bipolar extensions and spindle-shaped cells, whereas those on PLGA nanofibrous scaffolds showed bipolar processes with rounded and flattened cells.

Neurite outgrowths from DRGs on aligned scaffolds

DRGs were seeded on PLGA and ECM/PLGA nanofibers to quantify neurite outgrowth. NF200-positive axons extended parallel to the direction of the aligned fibers. However, axons extended along the ECM/PLGA fibers to a greater extent than along the PLGA fibers (857 ± 124 vs. $314 \pm 82 \mu\text{m}$, $p<0.001$) (Fig. 11). Axons extended along the aligned fibers were longer than those along the random fibers (data not shown). This is consistent with several previous studies.^{33–36}

FIG. 8. Characterization of the morphology of SCs seeded on the electrospun nanofiber scaffolds using SEM. (A) Random PLGA nanofiber scaffolds. (B) Aligned PLGA nanofiber scaffolds. (C) Random ECM/PLGA nanofiber scaffolds. (D) Aligned ECM/PLGA nanofiber scaffolds.

FIG. 9. Characterization of the morphology of SCs seeded on the electrospun nanofiber scaffolds using immunofluorescent staining. (A) Random PLGA nanofiber scaffolds. (B) Aligned PLGA nanofiber scaffolds. (C) Random ECM/PLGA nanofiber scaffolds. (D) Aligned ECM/PLGA nanofiber scaffolds. Samples were fixed and stained with S100 to visualize the actin cytoskeleton (green) and Hoechst 33258 to visualize the nuclei (blue). The alignment direction is indicated by the arrows. Color images available online at www.liebertpub.com/tea

Discussion

Tissue engineering is an alternative to the traditional treatments for peripheral nerve regeneration. One of the fundamental concepts that guide the development of tissue-engineered scaffolds is the selection of the biomaterial for the scaffold.³⁷ Natural neural ECM contains a large amount of biological information that can affect cell adhesion, migration, proliferation, differentiation, and gene expression; therefore, it is a superior material in neural scaffolds.³⁸ However, several recent studies have used parts of the ECM, rather than the major ECM component, to construct neural scaffolds.^{17,39–41} In the present study, we developed an ECM biomaterial from the cauda equina that retained a biochemical composition consistent with natural neural ECM. The cauda equina ECM was blended with PLGA to

synthesize scaffolds by electrospinning. ECM/PLGA scaffolds highly supported the axonal growth of the DRG and maintained healthy SC morphological features compared with PLGA-alone scaffolds, suggesting that the cauda equina ECM could be used as a scaffold material to prepare tissue-engineered nerve grafts and promote peripheral nerve regeneration.

In the current study, we produced the cauda equina-derived ECM using chemical extraction and differential centrifugation. A critical feature for producing the ECM is to reduce the cellular materials to prevent undesirable immune rejection. The efficiency of decellularization of the cauda equina was quantified using a PicoGreen assay and Hoechst 33258 staining. The cauda equina ECM, in this study, had significantly less residual DNA compared with the native cauda equina, with <50 ng DNA/mg ECM dry weight for decellularized cauda equina, suggesting that the cauda equina ECM was decellularized effectively.⁴² Because the retention of the ECM components was a central goal, an important finding was the detection of collagen, sGAG, and other proteins such as fibronectin and laminin in the derived ECM. Insoluble ECM molecules, such as collagen, laminin, and fibronectin, are important for the development and growth of axons.⁴³ Collagen plays an important role in the formation of the nerve tissue matrix. Collagen I also induces and improves the regeneration of peripheral nerves.^{44,45} Laminin is the major component of the basement membrane; it has neurite-promoting activity, stimulates SC mitosis, and plays a critical role in peripheral nerve regeneration.^{46,47} Fibronectin plays an important role in modulating SC migration and neurite outgrowth from the DRG.⁴⁸ The cauda equina-derived ECM retains significant biological components that might be useful for nerve regeneration. The cauda equina-derived ECM has a fibrous architecture with variable porosity, which provides the microarchitecture required for the cellular infill.

Electrospinning has been used successfully for generating fibers for tissue engineering that mimic ECM-like properties, such as nanoscale dimensions, high porosity,

FIG. 10. Effect of fiber alignment on cell orientation. The frequency distribution of the SC orientation was measured using ImageJ.

FIG. 11. Effect of the cauda equina ECM on neurons extending from the dorsal root ganglia. (A) PLGA nanofiber scaffolds. (B) ECM/PLGA nanofiber scaffolds. Samples were stained with NF200 (green). (C) Quantification of neurite outgrowth on ECM/PLGA and PLGA nanofiber scaffolds. $n = 15$; $*p < 0.001$. Color images available online at www.liebertpub.com/tea

interconnected pores, and a large surface area.⁴⁹ The cauda equina ECM was blended with PLGA to fabricate scaffolds using electrospinning to explore the feasibility of using the cauda equina ECM to construct scaffolds for neural tissue engineering. The cauda equina ECM was electrospun successfully to synthesize the nanofiber scaffolds. However, scaffolds containing ECM alone are a complex mixture of nonliving materials, including a set of proteins that dissolve and lose their structure when introduced into the cell culture medium. In addition, the ECM solution has low viscosity and is less suitable for producing oriented nanofibers. Therefore, we blended PLGA with the ECM before electrospinning. PLGA has been used extensively for tissue engineering because of its high tensile strength, nontoxic nature, and biodegradability; however, its hydrophobic characteristics are undesirable for *in vitro* cell culture.⁵⁰ By combining the ECM with PLGA before electrospinning, the attachment of cells onto the fibers in the hybrid scaffolds was possible without further modification. The results from the contact angle measurements provided evidence of increased hydrophilicity in the ECM/PLGA scaffolds. SEM of the nanofibrous scaffolds revealed that scaffolds were topographically functionalized with aligned and random nanofibers in both types, and the fiber size was polydispersed. The directions of neurite outgrowth and cellular elongation were parallel to those of aligned nanofibrous scaffolds; several studies have shown that this phenomenon of contact guidance plays an important role in peripheral nerve regeneration.^{49,51} For the aligned ECM/PLGA scaffolds, 53% of the fibers were well oriented, and ~45% of the SCs on these scaffolds were aligned moderately in an oriented manner along the nanofibers. The growth of SCs on the aligned nanofibers provides good guidance cues for cell growth and axonal extension and therefore better nerve

repair. FTIR analysis of ECM/PLGA random fibers showed common protein bands at $\sim 1650 \text{ cm}^{-1}$ (amide I) and 1540 cm^{-1} (amide II), which confirmed the presence of the ECM in the hybrid material. Multiphoton imaging also confirmed the presence of the ECM and demonstrated that most fibers in ECM/PLGA scaffolds were true hybrids of the base materials.

SCs are the primary structural and functional cells that play a crucial role in peripheral nerve regeneration. Therefore, it is important to understand the *in vitro* biocompatibility between the cauda equina ECM and SCs. SC proliferation on day 7 was 27% higher on ECM/PLGA than PLGA random nanofibers and 25% higher on ECM/PLGA than PLGA aligned nanofibers. This suggests that the cauda equina ECM was more suitable for SC growth than the PLGA nanofibrous scaffolds. SEM of SCs on day 7 of culture on the nanofibrous scaffolds showed normal cellular morphological features on the ECM/PLGA nanofibers, whereas cells were rounded and flat on PLGA-alone scaffolds. This suggests that the cauda equina ECM might favor normal SC morphological features. To examine the adhesion, spread, and growth of neural tissue-derived cells on the cauda equina ECM scaffolds, DRGs were cultured on ECM/PLGA scaffolds. Neurite outgrowth occurred within the DRGs and attached to the ECM/PLGA scaffolds, and there was a greater extension of axons in DRG explants compared with PLGA-alone fibers. This suggests good biocompatibility between the cauda equina ECM and peripheral nerve tissues. The cauda equina-derived ECM contains a large amount of biological information that influences cell adhesion, proliferation, and differentiation. Therefore, it can enhance neuronal outgrowth. As such, blending the cauda equina ECM with PLGA provided a nanofibrous scaffold with novel biocompatibility, bioactivity, and cellular affinity for neural tissue engineering.

Conclusions

ECM biomaterials have become important candidate materials as neural scaffolds for tissue engineering because of their role in cell proliferation, differentiation, adhesion, and migration in biological processes. The cauda equina-derived ECM yields a diverse biochemical composition consistent with natural neural ECM. Biomimetic cauda equina ECM/PLGA scaffolds synthesized using electrospinning possessed a nanostructure that could be well oriented. Compared with PLGA-alone scaffolds, composite scaffolds possessed good hydrophilicity. ECM/PLGA scaffolds had a good cellular affinity for adhesion and proliferation. They also supported SC migration and maintained healthy cellular morphological features. The extension of the axons in DRG explants was greater along ECM/PLGA compared with PLGA-alone fibers. Therefore, cauda equina-derived ECM material might have potential applications in neural tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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