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Chemically extracted acellular muscle: A new potential scaffold for spinal cord injury repair

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Abstract: Extracellular matrix is the gold standard for tissue regeneration. In this study, we directly made the extracellular matrix of the tissue or organ into scaffold for spinal cord injuries, a strategy that is seldomly tried in spinal cord engineering. The aim of this study was to determine if the chemically extracted acellular muscle could be a potential scaffold for spinal cord injury. The chemically extracted acellular muscle was implanted in the lateral hemisected adult rat thoracic spinal cord. Control rats were similarly injured. After 1 and 4 weeks, scaffold integration and biocompatibility, axon sprouting, and myelination were evaluated. The chemically extracted acellular muscle scaffolds were found to be well integrated with the host tissue. Sprouting axons grew into the full length of the scaffold in a strikingly parallel and linear fashion. A few remyelinated axons were also detected in the scaffolds. The tracing results in another six rats showed that labeled fibers entered the chemically treated muscle grafts. Furthermore, there were no apparent quantitative differences in the ED-1 and glial fibrillary acidic protein positive cells between groups. Neuron counting showed more surviving neurons in the acellular muscle treated group than those of the injured only group. Vascularization of the grafts was also confirmed. These findings clearly demonstrated that chemically extracted acellular muscle grafts provided useful biomatrices to enhance axon sprouting in the injured spinal cord. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 100A: 578–587, 2012.

Key Words: acellular scaffold, spinal cord injury, nerve tissue engineering, nerve sprouting

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INTRODUCTION

Spinal cord injury (SCI) often leads to permanent functional deficits due to failed axonal regeneration at the injury site and to the atrophy and/or retrograde death of axotomized neurons.[1,2] Despite the lack of spontaneous regeneration after SCI, several experimental strategies have been recently attempted and all have demonstrated significant axonal growth in rodent SCI models. Such approaches include the provision of supportive substrates,[3,4] providing neurotrophic factors to injured axons[5,6] and neutralizing inhibitory proteins at the site of injury.[7,8] Within these strategies, bridging strategies are essential to provide physical substrate allowing axons to grow across the lesion site, because cavity formation and glial scar are important obstacles impeding regeneration after SCI.

In recent years, various synthetic and naturally derived materials have been used to construct spinal cord scaffold.[9–11] These researches aim to mimic native extracellular matrix that is the gold standard for tissue regeneration and have provided promising results toward improving the function of injured nervous tissue. However, because these approaches have not provided the ultimate nerve regeneration scaffold, the approaches reported previously require further improvements to get higher similarity with extracellular matrix, such as improvement in providing stimulatory cues[12] and optimization on scaffold internal architecture design.[13] Furthermore, these scaffold materials have their respective disadvantages, such as degradation products that are unfit for cell survival and axonal regeneration; much poorer physical or biological characteristics; and low adhesiveness of cells. In contrast, acellular scaffolds that are biologic scaffolds derived from decellularized tissues and organs can effectively retain the structural and functional proteins of extracellular matrix and their original three-dimensional (3D) distribution. Till date, acellular scaffolds

Additional Supporting Information may be found in the online version of this article.
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have been successfully used to substitute and repair skin,[14] bladder,[15] urethra,[16] small bowel,[17] cardiac valve,[18] blood vessel,[19] skeletal muscle,[20] and even much more complex organs such as heart[21] and lung.[22] However, until now, few acellular scaffolds have been tried for spinal cord repair.

In the present investigation, we have tried to use the acellular muscle scaffold as bridging scaffold for SCI, which can promote axonal regeneration in peripheral nerves.[23] This is because (i) the basal lamina of acellular muscle contains axon growth-promoting molecules, such as laminin and fibronectin, and thus, acellular muscle can provide the molecular guides for axonal regeneration; (ii) the basal laminae of muscle fibers are long cylinders, similar to endoneural tubes, running parallel to one another, and thus, they could theoretically provide physical guidance for regenerating axons; (iii) acellular materials have very low immunogenicity, potentially allowing allografting or perhaps even xenografting; and (iv) the acellular muscle is easy to prepare and inexpensive. All these characteristics raise the possibility that the acellular muscle might be used in the repair of SCI.

MATERIALS AND METHODS

Animals and research design

Male Wistar rats were purchased from the experimental animal center of Jilin University. Animals were housed in a standard cage and kept under standard laboratory conditions (temperature at 22 ± 2°C; 12–12 h light/dark cycle). All experiments were approved by the local Ethics Committee for Animal Research at Jilin University and performed in accordance with international standards for animal welfare. All surgical procedures were performed under anesthesia produced by intraperitoneal injection with 10% chloral hydrate.

Twenty-four adult Wistar male rats (250–350 g) were randomly assigned to the two main groups: (a) chemically extracted acellular muscle graft and (b) lesion only. These two groups were all subdivided into two subgroups of six animals each to be sacrificed on days 7 and 28. All animals underwent spinal cord lateral hemisection. The treatment consisted of application of chemically extracted acellular muscle graft into the lesion gap which was left empty in the lesion group. Histological examinations and ED-1 staining for quantification of macrophages/microglia were performed on all groups. In animals surviving until day 28, Holmes' silver stain and neurofilament staining were used to detect axonal sprouts. The sprouting axons in the grafts were quantified. Given the dramatic axonal sprouting induced by the chemically extracted acellular muscle, we decided to ascertain other potential benefits of the chemically extracted acellular muscle for grafting. Myelin basic protein (MBP) staining was chosen to detect myelinated nerve fibers. The biocompatibility of chemically extracted acellular muscle graft with host tissues was further tested by analyzing responses of astrocytes, neuron survival, and neoangiogenesis at the fourth week. To further test the origin of the sprouting axons in the chemically treated muscle, fluororuby (FR) tracing was used in another set of six rats treated with the chemically extracted acellular muscle for 4 weeks.

TABLE I. Preparation of Chemically Extracted Acellular Muscle Graft

Reagent[a]	Time (days)
Distilled water	2
3% solution of Triton X-100 in Tris-buffered salts solution	2
Distilled water	2
0.1% solution of sodium dodecyl sulfate (SDS) in Tris-buffered salts solution	2
Phosphate-buffered saline	1

[a] The volume of each reagent was at least 30 times greater than the volume of the muscle samples.

Preparation of acellular muscle grafts

Wistar rats were killed by intraperitoneal overdose with 10% chloral hydrate (60 mL/kg). The paravertebral muscles were excised, and bundles were longitudinally harvested. The preparation of the chemically extracted acellular muscle was based on a previously described procedure.[24] Briefly, the muscle segments were treated with a series of detergent baths (Table I) consisting of Triton X-100 and sodium dodecyl sulfate (SDS; all reagents from Sigma). All steps were performed at 37°C with constant stirring. Following this, all samples were then sterilized using ampicillin (50 mg/mL) and stored in phosphate buffered saline (PBS) at 4°C until use.

After processing, longitudinal cryostat sections of the chemically extracted acellular muscle were stained with hematoxylin and eosin and examined by light microscopy.

Spinal cord hemisection and transplantation procedures

Lateral SCI was induced as previously described.[25,26] Male Wistar rats weighing 250–350 g were anesthetized with 10% chloral hydrate solution (3.5 mL/kg). A laminectomy was carried out to expose the thoracic spinal cord at T10. Iridectomy scissors were used to make a right-sided hemisection of the spinal cord at the T10 level, followed by the removal of a 2-mm block of the right-sided spinal cord tissue. To ensure that all lateral spinal tissue had been severed, a fine surgical blade was used to repeatedly scrape along the ventral surface of the vertebral canal in the lesional area. In the lesion only group, the lesional cavity was left empty. In the treatment group, immediately after completing the injury and achieving hemostasis, a graft with its longitudinal axis parallel to that of the spinal cord was carefully implanted into the cavity. To stabilize the scaffold within the spinal cord, a piece of silicone sheeting was placed over the scaffold and dorsal surface of the cord. The surgical wound was closed layer by layer in all animals. To prevent infection, rats received daily subcutaneous ampicillin (100 mg/kg) and gentamicin (12 mg/kg) for a period of 1 week.

Tissue processing

At the designated time points, animals were intracardially perfused with 4% paraformaldehyde in PBS. The region of the spinal cord containing the injury and adjacent portions were removed and postfixed overnight in the same fixative. Then the tissue was soaked overnight in 15% sucrose followed by 30% sucrose, and cut into 16-μm thick coronal sections using a cryostat [19]. Every seventh section was taken for hematoxylin and eosin staining.

Immunofluorescence and immunohistochemistry

The sections were immersed in phosphate-buffered saline(PBS) containing 0.1% Triton X-100 for 30 min. Nonspecific antibody binding was blocked with 10% normal goat serum in PBS containing 5% bovine serum albumin (BSA) for 1 h. Primary antibodies including ED-1(a mouse monoclonal antibody specific for microglia/macrophages,1:100, Serotec), glial fibrillary acidic protein (GFAP, a mouse monoclonal antibody specific for astrocytes ,1:100, Labvision), neurofilament (a rabbit polyclonal antibody specific for neurofilament in the neuronal axons,1:400, Millipore), and MBP (a mouse monoclonal antibody specific for the myelin membrane in the central nervous system, 1:100,Chemicon) were applied for 12 h at 4°C. Cy3-conjugated secondary antibodies (goat anti-mouse, 1:400, Jackson Immunoresearch Labs) or fluorescein isothiocyanate-conjugated secondary antibodies (goat anti- rabbit, 1:200, Jackson Immunoresearch Labs) were used to visualize primary antibody binding. The primary antibody was replaced by PBS for negative controls. After staining, the sections were observed under a fluorescence microscope using appropriate filters.

For immunohistochemistry, the water-absorbed sections were immersed in 3% (v/v) hydrogen peroxide for 10 min. After washed with PBS, the sections were immersed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 30 min, and incubated with serum blocking solution (10% normal goat serum in PBS containing 5% BSA) for 1 h. Primary antibodies including ED-1(a mouse monoclonal antibody specific for microglia/macrophages, 1:100, Serotec) and S100 antibody (a rabbit polyclonal antibody specific for S100 protein, 1:100, Boster, China) were applied for 12 h at 4°C. After wash with PBS, the samples were treated with MaxVision™ HRP-Polymer anti-Mouse/Rabbit Reagent (Maxim, China) at 25°C for 15 min, and visualized with 3,3-diaminobenzidine-plus substrate kit (maxim, China) for 5 min.

Silver staining of axons

For detection of newly formed axons (axonal regrowth) entering the biomatrices or the lesion gap, sections from animals which had survived 4 weeks were fixed in 10% formalin followed by 70% ethanol and stained by Holmes' silver stain,[27] an axon specific staining method.

Tracing experiments

To further test the origin of the regenerating axons in the chemically treated muscle, fluororuby tracing was used in another set of six rats treated with the chemically extracted acellular muscle for 4 weeks. One week before termination of the experiment, after anesthetization, the rats underwent laminectomy to expose the thoracic spinal cord at T8. A total of 1.8 μL of 10% FR (10.000 MW; Molecular Probes) were injected into the spinal cord 0.5 mm lateral to the dorsal median sulcus bilaterally, with 0.3 μL each at the depths of 0.5, 1, and 1.5 mm from the pia matter by a 1-μL microsyringe. Seven days after labeling, the spinal cord extending 5 mm rostral and 5 mm caudal to the center of the injury site was taken out and sectioned as described above.

Visualization of blood vessels

Four weeks after transplantation, sections from the chemically treated muscle group were stained for alkaline phosphatase, an enzyme present in endothelial cells of capillaries.[28] The slices were washed in PBS and then incubated for 2 min with the substrate-solution (fast 5-bromo-4-chloroindolyl-phosphate/nitro blue tetrazodium chloride, dissolved in its special buffer). The sections were then washed in PBS followed by ethanol dehydration, cleaned in xylene, and mounted in synthetic resin.

Quantification of sprouting axons, myelinated axons, macrophages/microglia, astrocytes, and neurons

The method for quantification of macrophage/microglia and astrocytes in spinal cord sections was developed from protocols described elsewhere.[29,30] Positive stained cells were counted inside the lesion and in the adjacent white matter. In the lesion group, cells were counted in tissue adjacent to the central cavity; in the graft-treated cords, cells were counted in tissue adjacent to but not including the graft area. For macrophages and microglias, all positive cells in six microscopic high-power fields (×400) for each section were counted. Five sections of each spinal cord at every 112 μm interval were separately analyzed and averaged. The mean value from the combined data of all six animals in each subgroup was calculated. For astrocytes, positive cells were quantified by determining GFAP positive area as a percentage of the total image area per microscopy field with Image-Pro Plus, v. 6.0, and calculated in a similar way with the quantification of macrophage/microglia.

Quantification of axons that had grown within the graft was performed using methods described elsewhere.[12,31] Briefly, two Holmes' silver stained sections were randomly selected from each animal. The total number of axons penetrating the scaffold was quantified at six microscopic fields within each section at a 400× magnification. These quantified fields were distributed within three regions of each scaffold: the rostral region (200–400 μm from rostral host-implant interface), middle region (between 600 μm from rostral and 600 μm from caudal host–implant interface), and caudal region (200–400 μm from caudal host-implant interface). The mean number of axons obtained from two sections of each animal was defined as the number of axons. The number of axons of six animals in each group was calculated.

To quantify the myelinated axons in the chemically treated muscle, two MBP positive stained sections from each animal were randomly chosen. Six microscopic fields at a magnification of 400 times with most myelinated axons in

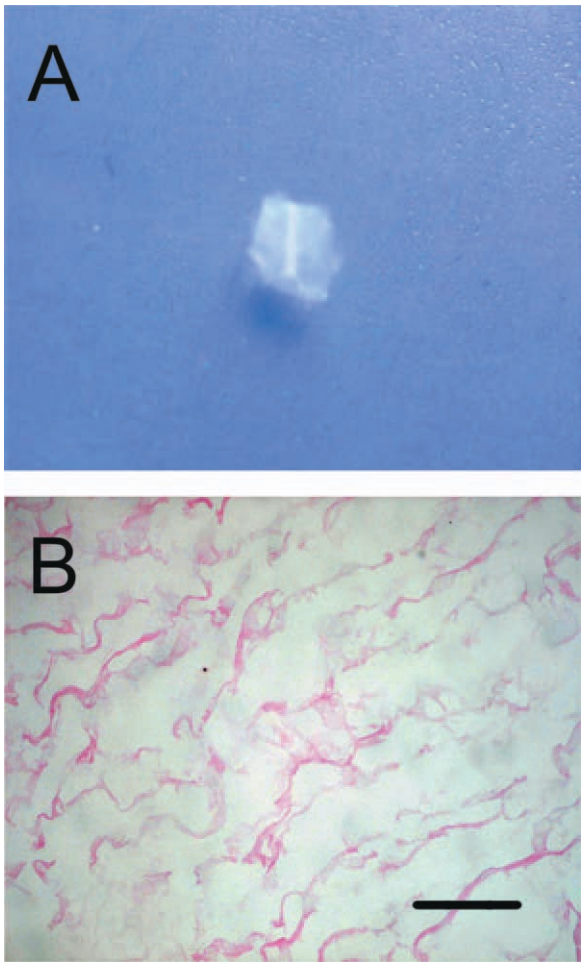

FIGURE 1. Chemically extracted acellular muscle scaffold. (A) Photograph showing a chemically extracted acellular muscle scaffold. Note the transparent appearance of the scaffold. (B) Longitudinal section of a chemically extracted acellular muscle scaffold, hematoxylin and eosin staining. The figure shows that all cellular constituents have been removed in chemically extracted acellular muscle leaving parallel tubules of extracellular matrix. Scale bar, 125 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

each section were counted and averaged. The mean number of axons observed in two sections from each animal was defined as the number of axons. The number of axons in six animals from the treatment group was calculated.

For the quantification of neurons that survive 4 weeks after SCI, six hematoxylin and eosin or silver-stained sections were used, about 200 μm apart and running cross the gray matter. All the neurons within the area including and extending 200 μm beyond the lesion site in each section were counted. The mean value from the combined data of all six animals in each group was calculated.

All values are expressed as mean ± SD. Differences between groups were analyzed by student's *t*-test. A p value of <0.05 was considered statistically significant.

RESULTS

Gross and histologic appearance

Following chemical extraction, the acellular muscle appeared ivory, translucent, and displayed soft properties [Fig. 1(A)]. In the chemically extracted acellular muscle, the myoplasm and other cellular constituents were completely removed from all muscle fibers, leaving uniaxial linear channels arranged in a parallel pattern [Fig. 1(B)].

After transplantation, inspection of acellular muscle scaffold implanted sites revealed that lesion cavities were completely filled with scaffolds (Fig. 2). The surface of the scaffold was cohesive with the surface of the spinal cord, and there was no sign of scaffold extrusion. At the microscopic level, histological results were consistent with gross images (Fig. 3). Connectivity between the stumps was obtained. There was no scar tissue at both ends of the implants. Scaffolds were infiltrated by numerous host cells, such as ED-1 and S100 positive cells (Supporting Information Figs. 1 and 2), and the implants integrated well with neural tissue from both proximal and distal stumps.

Biocompatibility of acellular muscle with spinal cord

The biocompatibility of the scaffold was assessed by analyzing responses of macrophages/microglia, astrocytes, neuron survival, and neoangiogenesis surrounding or within the scaffolds. The ED-1 positive stain showed that the numbers of macrophages/microglia in the parenchymal tissue around the cavity or graft area were slightly different between the groups at 1 and 4 weeks (Fig. 4). However, the differences were not statistically significant. GFAP staining results showed that reactive astrocytes were present in all subjects at the host/lesion interface. This GFAP reactivity at the host/lesion interface did not differ qualitatively from animals of control group (Fig. 5). It is noteworthy that some astrocytes penetrated into the scaffold in a linear arrangement parallel with long axis of the scaffold. The staining for alkaline phosphatase and the hematoxylin and eosin staining demonstrated that

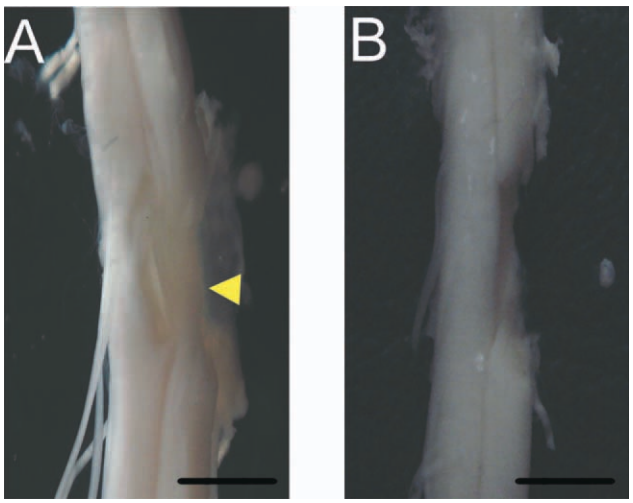

FIGURE 2. Photographs showing ventral views of the spinal cord with (A) or without (B) chemically extracted acellular muscle scaffold at 4 weeks after implantation. For the lesion control group, there is a large cavity formed in the spinal cord. In contrast, the cavity is completely filled with the scaffold in the acellular muscle treated group. The triangle points to the graft area. Scale bars, 2 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

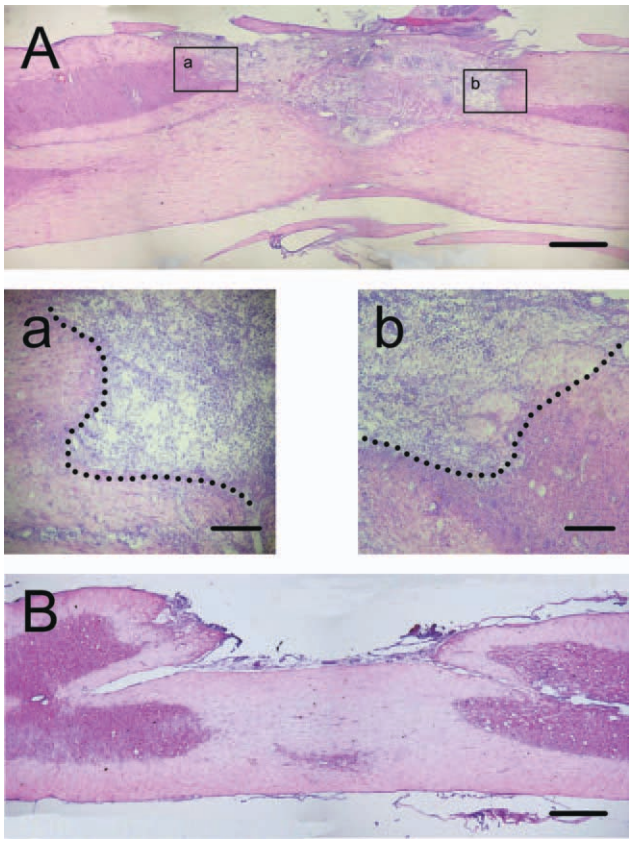

FIGURE 3. Hematoxylin/eosin (H&E) staining of coronal sections from each group. A, (a), and (b): Images of a section from the chemically extracted acellular muscle treated group. The figures show that the chemically extracted acellular muscle forms a tissue bridge between the stumps of hemisected spinal cord. The scaffold is infiltrated by numerous host cells, and integrates well with the host spinal cord. There is no fibrous scar at the interface between the scaffold and spinal cord. The broken lines mark the borders between the spinal cord and graft. (B): Image of a section from the lesion control group. Scale bars: (A) and (B), 625 μm; (a) and (b), 125 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

there were many blood vessels in the implants (Fig. 6 and Supporting Information Fig. 3). Neuron counting showed more surviving neurons in the acellular muscle treated group than those of the injured only group (Table II).

Sprouting fibers after implantation of acellular muscle scaffolds

The effect of acellular muscle scaffolds on neural fiber sprouting following SCI was evaluated by silver stain analysis. As a control for the silver staining, we performed the staining at day 0 after transplantation. And the results showed that no sprouting axons grew into the scaffolds (Supporting Information Fig. 4). Four weeks after operation, the Holmes' silver stain results showed that many sprouting axons grew into the acellular muscle scaffolds, which were apparent throughout the complete length of the scaffolds (Fig. 7). To further confirm the results of the silver stain, we performed the immunofluorescence staining of neurofilament. And the results showed that there were many axons penetrating into the scaffolds (Fig. 8). The numbers of sprouting axons were statistically different between the two groups (Fig. 9). More strikingly, the sprouting axons were distributed in a linear manner (Figs. 7 and 8).

The remyelination of the sprouting axons were visualized by MBP positive staining. The results suggested that some remyelination of ingrown axons had taken place inside the chemically extracted acellular muscle graft (Fig. 10), although the number of myelinated axons was few (only 6.2 $\pm$ 4.0 per 400$\times$ microscopic field).

The sprouting axons after SCI may be derived from both the spinal cord and the nerve roots.[32] To further test if the chemically extracted acellular muscle can promote the axonal sprouting of spinal cord itself, fluororuby tracing method was used. Results showed that fluororuby-labeled

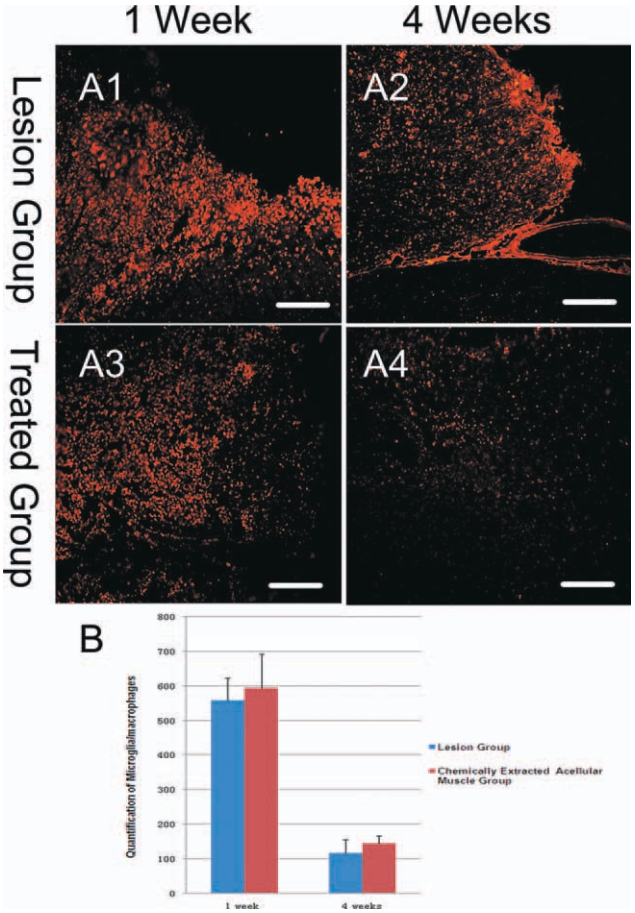

FIGURE 4. ED-1 immunoreactivities in each group. (A1, A2) Images of a section from the lesion control group at 1 week and 4 weeks postsurgery. (A3, A4) Images of a section from the chemically extracted acellular muscle treated group at 1 week and 4 weeks postsurgery. The pictures show the ED-1 positive cells in the spinal cord adjacent to the scaffold. (B) A quantitative comparison of the ED-1 positive macrophages/microglia ($n = 6$). There is no statistically significant difference in the numbers of ED-1 positive cells between the groups at 1 week and 4 weeks postsurgery. Scale bars: 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

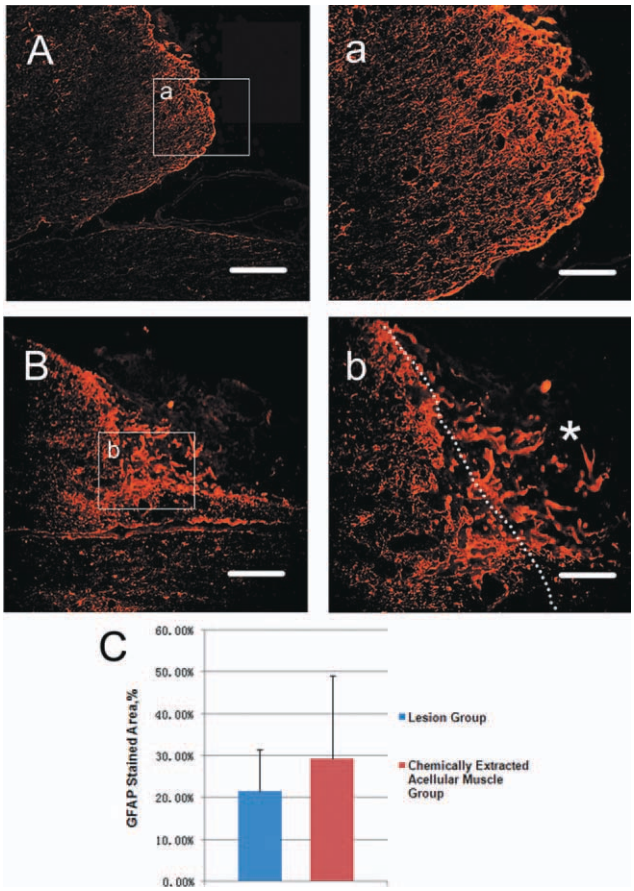

FIGURE 5. GFAP positive astrocyte responses in each group. A and a: GFAP-positive cells in the lesion control group. B and b: GFAP-positive cells in the chemically extracted acellular muscle treated group. The asterisk indicates the graft area. C: A comparison of GFAP-positive area between each group ($n = 6$). There is no statistically significant difference in the GFAP-positive area between the groups. Scale bars: (A) and (B), 250 μm; (a) and (b), 125 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

axons were observed in the acellular muscle scaffolds (Fig. 11).

DISCUSSION

In this study, we tried the strategy that directly made the extracellular matrix of the tissue or organ into scaffold for spinal cord injuries. The results showed that the chemically extracted acellular muscle scaffold was compatible with spinal cord tissue. The current study also determined that after SCI, the chemically extracted acellular muscle scaffold could promote axonal growth in a distinctly organized and linear fashion. This distributed fashion may help to guide sprouting axons into the spinal cord distal to the lesion site and ultimately result in effective reinnervation of target neurons.

The interaction between the implanted scaffold and the host tissue is an important aspect in using any biomaterials in medicine. The cavities or cysts, which are caused by acute trauma and form physical obstacles for axon regeneration, structure rebuilding, and return of function, should be filled and bridged by the scaffolds to restore continuity of the spinal cord. The host cells should be able to migrate and infiltrate into the scaffold to support axonal regeneration. In this study, the acellular muscle scaffolds do not have fibrous tissue encapsulation at the interface between the scaffold and spinal cord, and they integrate well with the spinal cord. The cavities or cysts are totally filled and bridged by the scaffolds. Numerous host cells, including ED-1 and S100 positive cells, infiltrate and uniformly distribute in the scaffolds demonstrating the ability of acellular muscle to promote cellular migration into the lesion site. This differs from many types of synthetic scaffolds, such as agarose,[12,33] alginate,[34] poly lactic-*co*-glycolic acid[35] and poly D,L-lactic acid (PLA),[36] which leave relatively large cavities at the interface between the scaffold and spinal cord, or prevent host cellular migration resulting into the failure of host cell/tissue bridge formation.

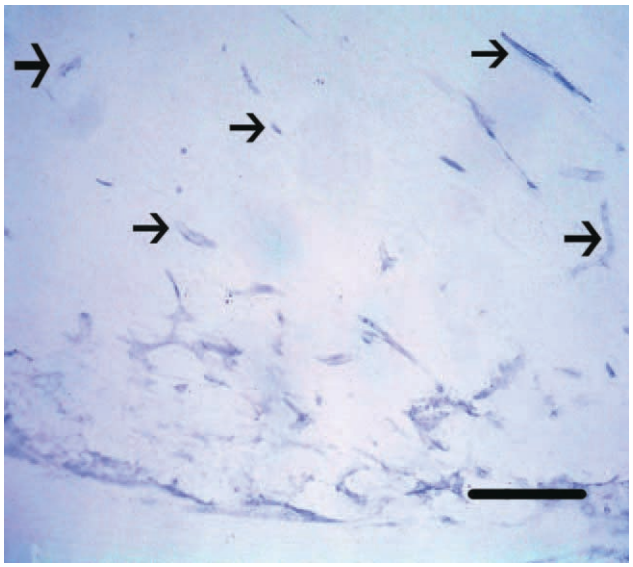

FIGURE 6. Capillaries visualized by staining for alkaline phosphatase in the chemically extracted acellular muscle on the 28th postoperatively day. The picture shows the capillaries in the graft area. The arrows indicate the blood vessels. Scale bar, 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The data of this study also show that the acellular muscle scaffolds have good biocompatibility with the host tissue. The transplantation of acellular muscle does not evoke upregulation of astrocytes, microglia and macrophages. Both reactive astrocytes and activated microglia are characteristic for the foreign-body reaction after transplantation in spinal cord. In addition, the results of neuron counting show that the scaffold does not cause additional damage to the spinal cord and provides some degree of neuronal protection. All

TABLE II. Number of Neuron Survival ($\bar{x} \pm$ SD)

Groups	Neurons/slide
Chemically extracted acellular muscle	333.47 ± 80.24*
Lesion	285.47 ± 83.16

* $p < 0.05$, versus lesion group.

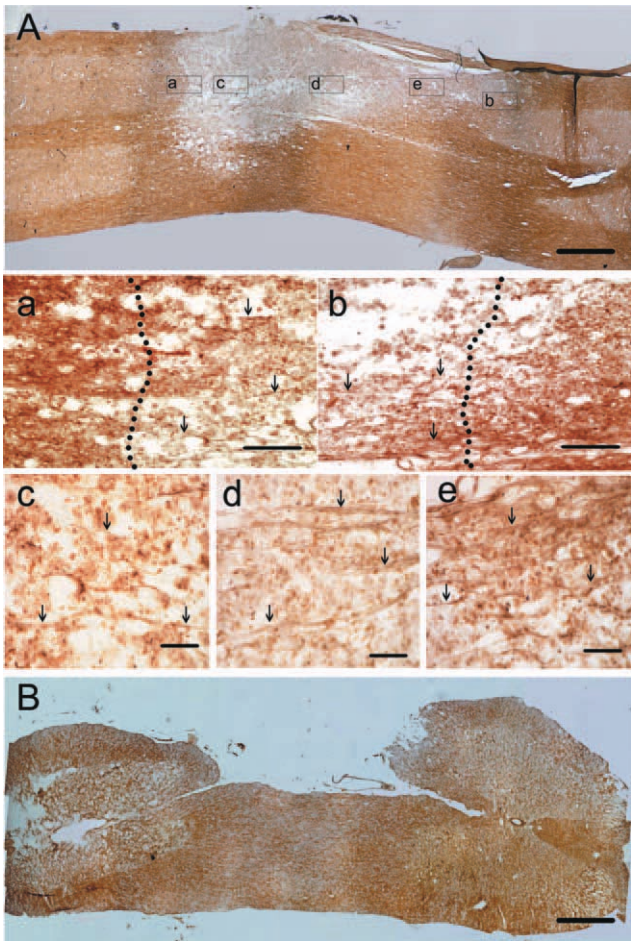

FIGURE 7. Sprouting axons 28 days after surgery, Holmes' silver stain. A, a–e: Photomicrographs of a coronal section of the spinal cord, including the chemically extracted acellular muscle implant, 4 weeks after the implantation. The figures show that sprouting axons are present in the rostral, middle, and caudal area of the scaffold. These axons are distributed in a strikingly parallel and linear manner. The arrows indicate the sprouting axons in the graft. B: A photomicrograph of a coronal section in the lesion control group. The figure shows that there are no axons in the lesion cavity. Scale bars: (A) and (B), 625 μm; (a) and (b), 125 μm; (c), (d), and (e), 62.5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

these demonstrate that acellular muscle can be safely used in the treatment of spinal cord injuries. The results shown here are consistent with previous studies demonstrating that acellular extracellular matrix scaffolds are tolerated well by allogeneic recipients.[28] In addition, the acellular muscle are well vascularized after transplantation. This result raises the possibility that the acellular muscle scaffold may be applied in relatively long distance repair of spinal cord injuries.

Following SCI, the local environment within the SCI area is intensely inhibitory to axonal regeneration. Then one goal of spinal cord engineering is to investigate a permissive environment for spinal cord regeneration by providing a combination of physical and chemical guidance cues for extending neurites. Unlike some scaffolds, such as poly-β-hydroxybutyrate[37] and PLA,[38] which may need adding additional stimulating molecules or cells to promote axonal regeneration, acellular muscle itself, can promote axonal regeneration. It has been reported that acellular muscle can promote the axonal regeneration of peripheral nerve.[24] The walls of acellular muscle contain laminin, fibronectin, and collagen.[23] Laminin and fibronectin are well known to enhance regeneration of peripheral and central axons.[39] Several synthetic scaffolds just incorporate them to enhance the axonal regeneration-promoting effect.[10] Collagen is a major component of the extracellular matrix and can provide physical support for cellular proliferation.[40] It has been reported that collagen fibers can promote the axonal regeneration and functional recovery after SCI.[41] In acellular muscle, these extracellular matrix molecules naturally combined with each other in a 3D distribution which may provide physical support and chemical guidance for regenerating axons. This study shows that the sprouting axons can grow through the

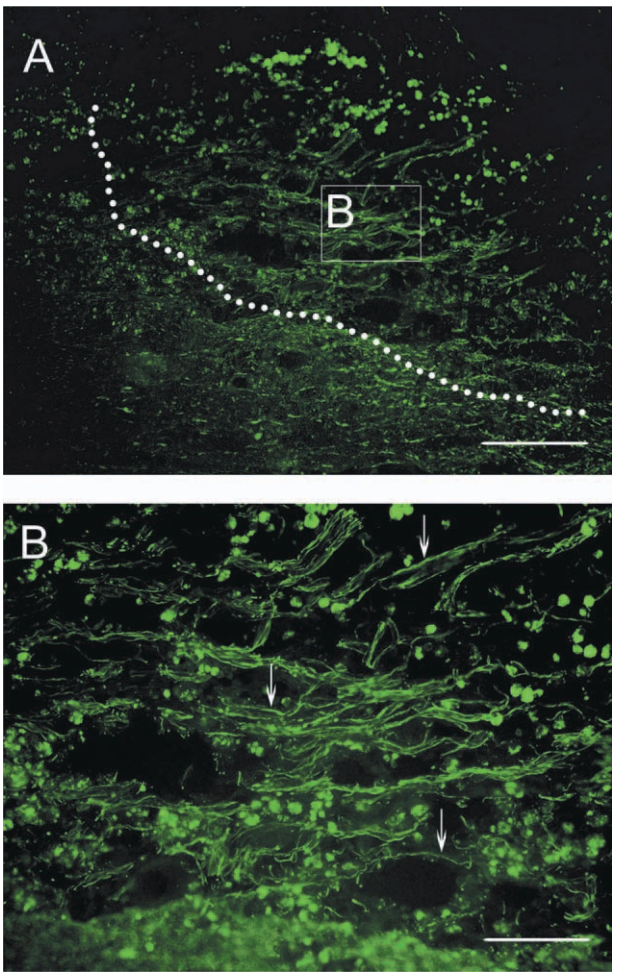

FIGURE 8. Neurofilament positive axons in the chemically extracted acellular muscle 28 days after surgery. Immunofluorescence was performed by using the anti-neurofilament and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. The broken line marks the border between the spinal cord and graft. The arrows indicate the neurofilament positive axons in the graft. The cell body-like structures are autofluorescent cells. Scale bar: (A), 250 μm; (B), 125 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

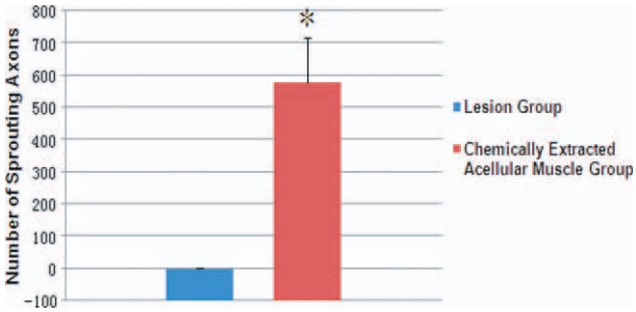

FIGURE 9. The number of sprouting axons of each group ($n = 6$). *Significant difference in the numbers of regenerating axons ($p < 0.05$, t test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

whole length of acellular muscle after SCI, indicating that acellular muscle scaffold can create a permissive environment for spinal cord regrowth. And the degree of their axonal sprouting is similar to that in some other extracellular matrix scaffolds, such as fibronectin[42] and collagen fibers.[43] In addition, the silver staining results showed that the axons from both proximal and distal spinal cord could sprout into the scaffold. However, we did not find that the fluororuby-labeled axons in the scaffolds were continuous with axons in the distal spinal cord. Because we cannot exclude the possibility that fluororuby-labeled axons in distal spinal cord originated from the spared spinal cord, it need further experiments to test if the axons can leave the graft and re-enter the spinal cord.

The main rationale for the treatment of SCI is to promote the regeneration of injured axons and to guide regenerated axon to reinnervate the previous target neurons in the spinal cord distal to the lesion site, as a result, to gain the function restoration. However, although several experimental strategies, such as the provision of neurotrophic factors to injured axons,[5] providing supportive substrates,[3] and neutralizing inhibitory proteins at the site of injury,[7] have recently demonstrated that axonal sprouting can in fact be significantly augmented, the growth is generally highly disorganized, preventing reconnecting of disrupted axon pathways with their target neurons. Within the skeletal muscle, muscle cells are internally coaxially aligned. As a result of decellularization, the acellular muscle has naturally multiple linear channels which can provide topographical guide for sprouting axons. Our results show that the sprouting axons in acellular muscle are arranged in a linear pattern, indicating that the acellular muscle scaffolds can guild the

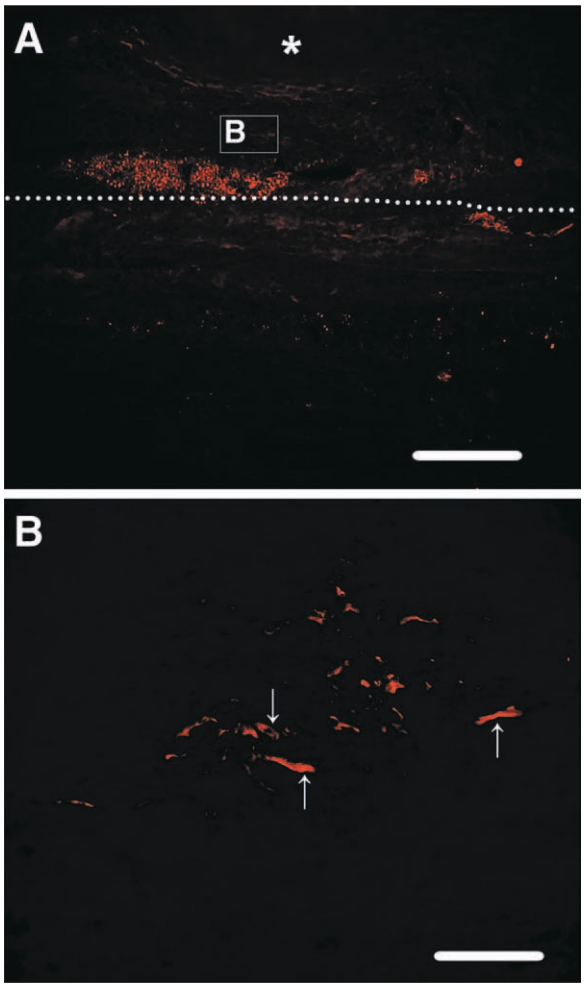

FIGURE 10. Sprouting myelinated nerve fibers in the chemically treated muscle 4 weeks after transplantation, MBP positive staining. A few myelinated nerve fibers are present in the chemically extracted acellular muscle graft. The broken line marks the border between the spinal cord and graft. The asterisk indicates the graft area. The arrows point to the myelinated nerve fibers in the graft. Scale bar: (A), 250 μm; (B), 62.5 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

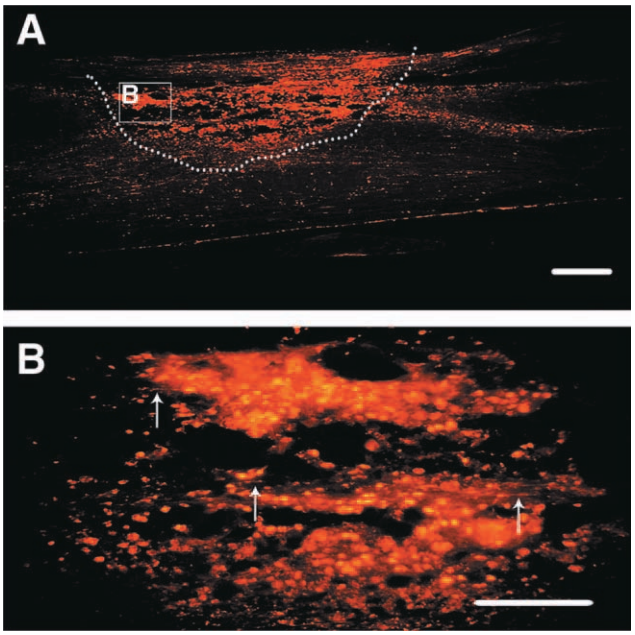

FIGURE 11. Fluororuby-labeled sprouting spinal axons 4 weeks after chemically extracted acellular muscle transplantation. The broken line marks the border between the spinal cord and graft. The arrows indicate the labeled axons in the graft. The cell body-like structures are autofluorescent cells. Scale bar: (A), 625 μm; (B), 125 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sprouting axons in a controlled direction. This may help for the reconnection of regenerated axons with their original target neurons.

Several additional properties make the acellular muscle scaffold useful for the treatment of spinal cord injuries. The scaffolds are soft and flexible. The fabrication is easy and the preparation costs are low. Based on the reduced immunogenicity of acellular scaffold, there is the possibility that acellular muscle could be used as allografts.

There is room to improve the acellular muscle scaffold for the treatment of SCI. In this study, we did not find the apparent functional recovery in chemical extracted acellular muscle treated group 4 weeks after transplantation (data not shown).This may be partly because only a few myelinated axons formed in the scaffold.[44] The formation of few myelinated axons may be related to lack of sufficient proliferation and differentiation of oligodendroglial progenitors.[45] This problem can be overcomed by combining with other myelin formation-promoting strategies, such as providing a source of neurotrophic protein.[46] In addition, this kind of strategy may further improve the capacity of acellular muscle scaffold to promote the axonal regeneration after SCI.

CONCLUSIONS

Taking all these observations together, chemically extracted acellular muscle provides a potential scaffold for spinal cord repair, which combines several key features required for axonal regeneration in the central nervous system.

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