ORIGINAL ARTICLE

Injectable Extracellular Matrix Hydrogels as Scaffolds for Spinal Cord Injury Repair

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Restoration of lost neuronal function after spinal cord injury (SCI) still remains a big challenge for current medicine. One important repair strategy is bridging the SCI lesion with a supportive and stimulatory milieu that would enable axonal rewiring. Injectable extracellular matrix (ECM)-derived hydrogels have been recently reported to have neurotrophic potential *in vitro*. In this study, we evaluated the presumed neuroregenerative properties of ECM hydrogels *in vivo* in the acute model of SCI. ECM hydrogels were prepared by decellularization of porcine spinal cord (SC) or porcine urinary bladder (UB), and injected into a spinal cord hemisection cavity. Histological analysis and real-time qPCR were performed at 2, 4, and 8 weeks postinjection. Both types of hydrogels integrated into the lesion and stimulated neovascularization and axonal ingrowth into the lesion. On the other hand, massive infiltration of macrophages into the lesion and rapid hydrogel degradation did not prevent cyst formation, which progressively developed over 8 weeks. No significant differences were found between SC-ECM and UB-ECM. Gene expression analysis revealed significant downregulation of genes related to immune response and inflammation in both hydrogel types at 2 weeks post SCI. A combination of human mesenchymal stem cells with SC-ECM did not further promote ingrowth of axons and blood vessels into the lesion, when compared with the SC-ECM hydrogel alone. In conclusion, both ECM hydrogels bridged the lesion cavity, modulated the innate immune response, and provided the benefit of a stimulatory substrate for *in vivo* neural tissue regeneration. However, fast hydrogel degradation might be a limiting factor for the use of native ECM hydrogels in the treatment of acute SCI.

Introduction

SPINAL CORD INJURY (SCI) is a devastating disorder that often results in permanent motor and sensory dysfunctions due to the inability of axons to regenerate in the hostile environment of the lesion.¹ Current therapeutic approaches are being combined to activate the intrinsic neuronal regeneration capacity, such as blocking axon growth-inhibitory factors, reducing excitotoxicity and the inflammatory response, administration of neurotrophic factors, or using various types of stem and progenitor cells.^{2,3} In addition to these approaches, tissue-engineered scaffolds play an important role in providing supportive substrates that contribute to replacing lost tissue and re-establishing damaged connections after SCI.^{4–7}

In terms of SCI repair, biomaterials, with their own intrinsic biological activity that would encourage endoge-

nous tissue repair without the need for additional bioactive molecules such as exogenous growth factors or peptides, may provide high treatment effectivity together with relative ease of application and scalable manufacturing potential.⁸

In contrast to artificial tissue-engineered materials that fail to mimic the complex structure and chemistry of the extracellular microenvironment seen *in vivo*, biological scaffolds composed of native extracellular matrix (ECM) represent structures very similar to those of the uninjured host tissue with advantages, such as a natural three-dimensional (3D) structure, biological activity promoting cell adhesion and proliferation, and biodegradability.⁹ The general concept of ECM scaffolds is based on the constructive remodeling process, in which a degradable biomaterial serves as a temporary inductive niche that is completely degraded and gradually

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replaced by anatomically appropriate and functional tissue as opposed to scar tissue.^{10,11}

Acellular tissue matrices have revealed an intrinsic ability to guide cells to differentiate into tissue-appropriate structures and phenotypes, and are also associated with positive *in vivo* host tissue remodeling and regeneration,^{12,13} degradation of ECM-evoked recruitment of endogenous stem and progenitor cells, and modulation of the innate immune response.^{14,15} After removal of cellular antigens, ECM scaffolds are considered biocompatible and nonimmunogenic even in allogeneic and xenogeneic settings. Currently, ECM scaffolds are being widely used for various tissue reconstructions, including heart valves, blood vessels, skin, bone, cartilage, trachea, lung, or peripheral nerves. A number of ECM scaffolds derived from a range of source species and tissues have also been approved by the FDA and commercially available for clinical use, for example, in wound healing, soft tissue repair, or heart valve replacement.^{10,11}

In contrast to the extensive research on ECM scaffolds used for the reconstruction of various tissues, there are only a few studies addressing biological scaffolds for the repair of SCI based on an acellular muscle scaffold,¹⁶ acellular sciatic nerve,¹⁷ or acellular spinal cord scaffolds.¹⁸ Nevertheless, the shape and conformation of such acellular scaffolds might be restrictive for bridging a chronic spinal cord lesion with an irregular cavity. Thus, in terms of suitability for clinical application, injectable *in situ* gelling hydrogels are more appropriate as these materials can easily conform to the lesion irregularity with minimal tissue damage during delivery.

To meet such requirements, tissue-specific injectable ECM hydrogels, prepared by decellularization of porcine brain, spinal cord (SC-ECM), and porcine urinary bladder (UB-ECM), have been recently described in terms of their composition, biomechanical properties, and neurotrophic properties.^{19,20} These materials proved to be advantageous for providing a supportive environment for the *in vitro* neural cell growth. However, experimentally, it is unknown whether these materials can be successfully used for SCI repair, either alone or in combination with various types of cells.

To evaluate the potential neuroregenerative properties of the central nervous system (CNS) and non-CNS-derived materials *in vivo*, this study examined the effects of ECM hydrogels based on SC-ECM and UB-ECM in the model of SCI.

The *in vitro* cell-adhesive properties and neurotrophic potential of the ECM hydrogels were studied on human mesenchymal stem cells derived from Wharton's jelly (hWJ-MSCs) and on dorsal root ganglia (DRG) explant culture, respectively. To evaluate the *in vivo* tissue compatibility, the ECM hydrogels were injected into the spinal cord hemisection in rats and histologically evaluated. The tissue-scaffold interactions were further investigated using real-time qPCR to determine changes in the messenger RNA (mRNA) expression of genes related to the inflammation and immune response, secretion of neurotrophic and growth factors, and astrogliosis.

Materials and Methods

Preparation of ECM hydrogels

The preparation of the ECM hydrogels was based on a previously described procedure.^{9,19,21} ECM samples were solubilized with 1.0 mg/mL pepsin in 0.01 N HCl (Sigma-

Aldrich, Steinheim, Germany) at a concentration of 10 mg ECM/mL and stirred at room temperature for 48 h to form a pregel solution (pH ~2). The pepsin-HCl ECM solution was neutralized to pH 7.4 with 0.1 N NaOH, isotonicity balanced with 10× phosphate-buffered saline (PBS), and diluted with 1× PBS to the concentration of 8 mg/mL. To form the hydrogel, the neutralized pregel was placed at 37°C for 45 min. The composition and biomechanical properties of the ECM hydrogels have been described in detail in Crapo *et al.*,⁹ Medberry *et al.*,¹⁹ and Wolf *et al.*²¹

Cell culture

Fresh human umbilical cord samples were collected from healthy full-term neonates after spontaneous delivery with the informed consent of the donors using the guidelines approved by the Institutional Ethics Committee at University Hospitals (Pilsen and Prague, Czech Republic). About 10–15 cm per umbilical cord were aseptically transported into sterile PBS (IKEM, Prague, Czech Republic) with antibiotic-antimycotic solution (Sigma-Aldrich) at 4°C.

After removal of blood vessels, the remaining tissue was chopped into small pieces (1–2 mm³) and transferred to 10-cm Nunc culture dishes (Schoeller, Prague, Czech Republic) containing the complete Alpha-Minimum Essential Medium (East Port, Prague, Czech Republic) supplemented with 5% platelet lysate (IKEM) and gentamicin 10 µg/mL (Sandoz, Prague, Czech Republic), and cultivated at 37°C and 5% CO₂ in a humidified atmosphere.

On day 10, the explants were removed from the culture dishes and the remaining adherent cells were cultured for 3 weeks or until 90% confluence. The medium was changed two times a week. Cells of the third passage were identified by flow cytometry (FACSaria™; Becton Dickinson, San Jose, CA) to confirm their purity. The following antibodies against human antigens were used: CD34, CD45, CD105 (Exbio, Vestec, Czech Republic); CD29, CD73, CD90, CD271, CD31, HLA-ABC, and CD235a (BD Pharmingen, San Jose, CA); CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Data analysis was performed using BD FACSDiva software.

In vitro cell growth and viability

In vitro cell growth on the surface of hydrogels was characterized using hWJ-MSCs cultures. Cell viability was determined after 1, 3, 7, and 14 days in culture using WST-1 reagent (Roche, Mannheim, Germany). Cells were cultured in 96-well plates (5000 cells/per well) coated with either UB-ECM or SC-ECM hydrogels (90 µL/well) before cell seeding. At the measured time points, 10 µL of WST-1 reagent was added to each well with 100 µL culture media and the plates were incubated for 2 h at 37°C. The absorbance was measured using a Tecan Spectra ELISA plate reader (Tecan Trading, Mannedorf, Switzerland) at 450 nm. Each type of hydrogel was seeded in triplicate; 14 independent experiments in three hydrogel batches were performed for each hydrogel type. Cell viability on the culture plastic was used as a control.

In 3D culture, cells were seeded within the hydrogels by adding concentrated cell suspension in PBS to neutralized liquid pregel solution for a final cell concentration of 2.5×10^6 cells/mL. Cells were thoroughly mixed in the pregel and 200 µL of the suspension was transferred inside the seeding

rings with a diameter of 0.8 cm (Scaffdex, Tampere, Finland) placed in the 24-well plates, and transferred at 37°C for 45 min to form a hydrogel around the cells. Following this, the seeding rings were removed, 1 mL of media was added, and the hydrogel discs were imaged after 4 h, 24 h, 4 days, and 7 days in culture to quantify gel contraction (more details in the Supplementary Data; Supplementary Data are available online at www.liebertpub.com/tea). The cell-seeded hydrogels used for *in vivo* experiments were placed into PBS and incubated at 37°C and 5% CO₂ in a humidified atmosphere for ~4 h before their implantation into the hemisection cavity.

The morphology of the cells on the hydrogels was examined by immunofluorescence staining for actin filaments. After fixation in 4% paraformaldehyde in 0.1 M PBS for 15 min, the cells were washed with 0.1 M PBS and stained with AlexaFluor 568 phalloidin (1:300); the nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye (all from Invitrogen, Paisley, United Kingdom).

DRG explant culture

Wistar rats (Velaz, Unetice, Czech Republic), aged 3–5 days, were used for DRG extraction. Briefly, their spinal cords were dissected and DRGs from low thoracic and lumbar parts were isolated, placed in cold Hank's Balanced Salt Solution without Ca²⁺/Mg²⁺ solution (Invitrogen, Waltham, MA), and cleaned of peripheral nerve processes.

DRG explants were then placed on SC-ECM ($n=5$) or UB-ECM ($n=4$) hydrogels in 24-well plates and cultured in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 0.5% NGF (50 ng/mL; PeproTech, Prague, Czech Republic), uridine (17.5 µg/mL; Sigma-Aldrich), and primocin (PeproTech) in humidified atmosphere at 37°C and 5% CO₂. The medium was changed every 3 days.

After 7 days of culture, DRGs were fixed with 4% paraformaldehyde in 0.1 M PBS for 10 min and subjected to immunocytochemical staining for neurofilaments (NF160, 1:200; Abcam, Cambridge, United Kingdom) and cell nuclei (DAPI, 1:1000). Imaging was performed using a Leica fluorescent microscope (Leica DMI 6000B) and TissueGnostics software (TissueGnostics GmbH, Vienna, Austria). The neurite extension area and the longest neurite length were determined using ImageJ software with the Neurite-J plug-in, as described by Torres-Espin *et al.*²²

Hydrogel injection into the SCI lesion

Male Wistar rats (250–300 g; Velaz) underwent a hemisection at the level of the 8th thoracic vertebra (Th8). The neutralized and isotonic balanced liquid pregel solution of SC-ECM and UB-ECM hydrogels (8 mg/mL) were acutely injected into the spinal cord defect after hemisection and allowed to gelate *in situ*, followed by histological evaluation 2, 4, and 8 weeks after implantation ($n=5$ per group, per time point).

The surgery was performed under adequate pentobarbital (PTB) anesthesia (60 mg/kg). The animals received local injections of mesocain (0.3 mL s.c. at the surgery site) in addition to general anesthesia, as well as gentamicin (0.05 mL i.m.; Sandoz) and atropine (0.2 mL, atropine solution 1:5) (both from BB Pharma, Prague, Czech Republic) injections.

First, a microsection of the skin was made at the level of Th8 spinal process using a scalpel. Then the laminectomy at Th8 was performed using rongeur, and the dura was incised with capsulotomy scissors. A 2 mm long spinal cord segment of the volume ~6 mm³ was dissected using delicate tissue scissors to generate a hemisection cavity. Then the dissected segment was removed using a small piece of cellulose and fine forceps. The aforementioned instruments were purchased from Medicon® (Tuttlingen, Germany). The dura mater was sutured with 10/0 monofilament nonresorbable thread (B Braun, Aesculap, Melsungen, Germany), and the hydrogels were injected into the cavity in a single injection using an Omnican® Insulin syringe for U-100 Insulin (Melsungen, Germany). The muscles and skin were sutured with 4/0 monofilament nonresorbable thread (4/0 Chirmax, Prague, Czech Republic), and the animals were housed, two rats in a cage, with food and water *ad libitum*.

In the control SCI group ($n=4$) the hemisection defect was filled with saline. In the animal group treated with SC-ECM hydrogel seeded with hWJ-MSCs ($n=4$), the hydrogels were implanted into the hemisection cavity. This animal group received a daily injection of the immunosuppressant cyclosporin A (10 mg/kg, intraperitoneally) (Sandimmune; Novartis, Basel, Switzerland), azathioprine (2 mg/kg, per orally) (Imuran; Aspen Europe GmbH, Bad Oldesloe, Germany), and methylprednisolone (2 mg/kg, i.m.) (Solu-Medrol; Pfizer, Puurs, Belgium) to prevent the rejection of the transplanted cells.

Of the 56 animals that underwent the hemisection, 4 animals died during the operation and 2 animals died 2–4 weeks after operation. All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), regarding the use of animals in research and were approved by the Ethics Committee of the Institute of Experimental Medicine, Academy of Sciences, Czech Republic (Prague, Czech Republic).

Hindlimb motor function between the first and fourth weeks post-SCI was recorded for the sham-operated control group, SC-ECM alone, and SC-ECM combined with hWJ-MSCs using the Basso–Beattie–Bresnahan (BBB) open field locomotor test²³ (Supplementary Data).

Tissue processing and histology

At 2, 4, and 8 weeks after hydrogel injection, the animals were deeply anesthetized with an intraperitoneal injection of overdose PTB and perfused with PBS followed by 4% paraformaldehyde in 0.1 M PBS. The spinal cord was left in the bone overnight, then removed and postfixed in the same fixative for at least 1 week. A 3 cm long segment of the spinal cord containing the lesioned site was dissected out and transferred to 10% and 30% sucrose. After freezing, the spinal cords were cryosectioned into 40 µm thick longitudinal sections. Hematoxylin–Eosin (H&E) and Masson's Trichrome staining was performed using the standard protocol.

For immunohistological analysis, the following antibodies were used: against neurofilament (NF160, 1:200), endothelial cells (RECA-1, 1:500), astrocytes (Cy3-conjugated mouse Glial Fibrillary Acidic Protein, GFAP, 1:200), all from Sigma-Aldrich; Schwann cells p75 (1:200), serotonin-positive axons (R-SERO, 1:100), oligodendrocytes (OSP, 1:1000), macrophages (ED1, 1:100), M1 macrophages (CD86, Cy5-

conjugated donkey anti-rabbit IgG-PerCp-Cy5,5, 1:2500), human mitochondria (MTCO2, 1:250), all from Abcam; and M2 macrophages (CD206, 1:250), axonal growth cone (GAP43, 1:100), all from Santa Cruz (Heidelberg, Germany). Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:200), Alexa Fluor 594-conjugated goat anti-mouse IgG (1:200) and Alexa Fluor 488-conjugated donkey anti-goat IgG (1:700) were used as secondary antibodies. The nuclei were visualized by using 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye. Fluorescent micrographs were taken using an AxioCam HRc Axioskop 2 Plus fluorescence microscope (Zeiss, Jena, Germany) and a LSM 510 DUO laser scanning confocal microscope (Zeiss).

For axonal and vessel analysis, multiple images across the entire lesion were taken using a 20× objective. Five images from each sample were selected and the total area of the axons (NF160 staining) and blood vessels (RECA staining) within the lesion area was outlined using ImageJ software (National Institutes of Health, Bethesda, MD) and divided by the area of the lesion to determine the percentage of the lesion that was occupied by new axons or vasculature.

Gene expression analysis

The expressions were studied using quantitative real-time reverse transcription polymerase chain reaction (qPCR) 2, 4, and 8 weeks after the surgery (in all groups $n=4$). RNA was isolated from paraformaldehyde-fixed frozen tissue sections using the High Pure RNA Paraffin Kit (Roche, Penzberg, Germany), following the manufacturer's recommendations. RNA amounts were quantified using a spectrophotometer (NanoPhotometer™ P-Class, Munchen, Germany). The isolated RNA was reverse transcribed into complementary DNA (cDNA) using the Transcriptor Universal cDNA Master (Roche) and a thermal cycler (T100™ Thermal Cycler; Bio-Rad, Hercules, CA).

The qPCR reactions were performed using cDNA solution, FastStart Universal Probe Master (Roche) and TagMan® Gene Expression Assays (Life Technologies), (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/tea). The qPCR was carried out in a final volume of 10 μ L containing 25 ng of extracted RNA. Amplification was performed on the real-time PCR cyclor (StepOnePlus™; Life Technologies). All amplifications were run under the same cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

All samples were run in duplicate, and a negative control was included in each array. Relative quantification of gene expression was determined using the $\Delta\Delta$ Ct method. Results were analyzed with StepOnePlus software. The gene expression level was normalized on *Gapdh* as a reference gene; control samples from unlesioned spinal cord tissue were used as a calibrator.

Statistical evaluation

The statistical significance of the differences in gene expression between the groups at three time points was determined using two-way repeated measurement analysis of variance (ANOVA) with a Student-Newman-Keuls *post hoc* pair-to-pair test. A one-way ANOVA was used for the comparisons of gene expression between groups with SC-ECM hydrogel at 4-week intervals and cell proliferation (SigmaStat 3.1; Systat Software, Inc., San Jose, CA).

Results

In vitro cell culture

The solubilized ECM matrix self-assembles from the pregel form into the hydrogel at 37°C and physiologic pH, as was described in Medberry *et al.*¹⁹ The biocompatibility and

FIG. 1. *In vitro* cell growth on extracellular matrix (ECM) hydrogels. Two-dimensional (2D) cell culture on (A) urinary bladder (UB)-ECM and (B) spinal cord (SC)-ECM hydrogels at 3 days. (C–F) Three-dimensional (3D) cell culture in UB-ECM at (C, D) 4 and (E, F) 24 h. Cells were stained for (A–F) phalloidin and (C–F) cell nuclei (DAPI). (G) Proliferation of human Wharton's jelly-derived mesenchymal stem cells (hWJ-MSCs) on ECM hydrogels using WST-1 assay. (H) Quantification of the highest neurite length and (I) neurite extension area of dorsal root ganglia explants on ECM hydrogels using Neurite-J plugin for ImageJ software. Scale bar: (A, B) 100 μ m, (C–F) 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 2. Representative Hematoxylin-Eosin staining of the spinal cord lesion (A–C) 2, (D–F) 4, and (G–I) 8 weeks after injection of (A, D, G) UB-ECM hydrogels; (B, E, H) SC-ECM hydrogels. (C, F, I) Represent a sham-operated control lesion. (D) The arrow shows the nondegraded part of the hydrogel. Scale bar: 500 μ m. Color images available online at www.liebertpub.com/tea

bioadhesive properties of ECM hydrogels were confirmed by the *in vitro* use of hWJ-MSCs in the two-dimensional (2D) and 3D cell cultures (Fig. 1). Flow cytometry was performed to detect cell purity, while CD markers were found positive for CD29, CD105, CD90, CD73, and HLA-ABC and negative for CD31, CD34, CD45, CD133, CD235a, and CD271. After seeding onto the ECM hydrogels, cells spread and proliferated on both hydrogel types (Fig. 1).

Cell proliferation was determined using the WST-1 assay after 1, 3, 7, and 14 days of the culture. Both ECM hydrogels showed comparable ability to support *in vitro* cell proliferation, which did not significantly differ from the control cell proliferation on tissue culture plastic (TCP). Cell viability increased until day 7, then, after reaching the confluency of the culture, decreased on both hydrogel types as well as on TCP (Fig. 1G).

When seeded in 3D culture (0.5 million cells per 0.2 mL), hWJ-MSC extended their lamellipodia within the hydrogels and formed a 3D network, as is shown in Figure 1C–F after 4 and 24 h. Rapid hydrogel contraction was observed already after 4 h, which progressed during the culture to at least 10% of the initial area after 7 days (Supplementary Fig. S1).

DRG explant culture on ECM hydrogels

DRG explant cultures were used to compare the neurotrophic properties of the CNS and non-CNS-derived ECM hydrogels. After 7 days of culture, neurites densely extended

from the DRG bodies (Supplementary Fig. S2). No significant differences were found between UB-ECM and SC-ECM in both examined parameters, total neurite extension area and longest neurite length (Fig. 1H, I).

Histological evaluation after ECM hydrogel injection

Both UB-ECM and SC-ECM hydrogels were injected into the cavity of the spinal cord hemisection and examined at 2, 4, and 8 weeks. The tissue response to the scaffolds was histologically evaluated by analyzing axonal ingrowth, vascularization, and infiltration of macrophages/microglia, astrocytes, and oligodendrocytes within the injury site.

At 2 weeks after injury, H&E staining of longitudinal spinal cord sections demonstrated that both hydrogel types were biocompatible with the surrounding host tissue and entirely filled the lesion cavity (Fig. 2A, B). The hydrogels were mostly degraded, but still detectable in the lesion area (Supplementary Fig. S3) and were densely populated among the host cells.

By 4 weeks postinjury, small areas of the original hydrogel were still present (Fig. 2D), while the newly formed tissue interconnected with the host tissue, bridging the lesion center. Macrophages massively infiltrated the periphery of the lesion where several small cysts developed due to the rapid degradation of the graft. A similar tissue response was found at 8 weeks, when the hydrogels had fully degraded, which was followed by further progression of cyst formation. In contrast

FIG. 3. Representative images of the spinal cord lesion (A–C) 2 and (D–F) 8 weeks after injection of SC-ECM hydrogels. Immunofluorescence staining for (A, B, D, E) neurofilaments (NF160), (C, F) astrocytes (GFAP), and (B, E) cell nuclei (DAPI, blue). Squares (A, D) are also shown under the higher magnification insets (B, E). (G) The effect of ECM hydrogels on the ingrowth of NFs. A significantly higher ingrowth of NFs was found in both the ECM hydrogel groups when compared to the control lesion at all time points. Scale bar: (A, C, D, F) 500 μ m; (B, E) 50 μ m. Color images available online at www.liebertpub.com/tea

to the tissue remodeling process observed in the lesion after hydrogel injection, large pseudocysts formed in the control sham-treated lesion (Fig. 2C, F, I).

To evaluate axonal ingrowth into the hydrogels, a neurofilament marker (NF160) was used (Fig. 3). Robust ingrowth

of NF-positive fibers into the hydrogel-treated lesion was observed from both the rostral and caudal stumps of the lesion, while dense infiltration of NFs was also found in the center of the lesion. Quantification analysis expressed the relative value of NF160 immunopositive area as a percentage

FIG. 4. Representative images of the spinal cord lesion at 2 weeks after injection of (A, B) UB-ECM and (C, D) SC-ECM hydrogels. (A–D) Immunofluorescence staining for blood vessels (RECA) and (B, D) cell nuclei (DAPI). Squares (A, C) are also shown under higher magnification insets (B, D). (E) An effect of ECM hydrogels on vascularization. A significantly higher ingrowth of blood vessels was found in both ECM hydrogel groups when compared to the control lesion at all time points. Scale bar: (A, C) 500 μ m; (B, D) 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 5. Representative immunofluorescence staining for (A, B) macrophages (ED1, green) and astrocytes (GFAP, red) in (A) UB-ECM at 2 weeks and (B) SC-ECM seeded with hWJ-MSCs at 4 weeks. Confocal micrographs of the staining for (C) serotonin-positive axons (5-HT, red) and blood vessels (RECA, green) in SC-ECM at 4 weeks; (D) serotonin-positive axons (5-HT, red), blood vessels (RECA, green), and cell nuclei (DAPI, blue) in SC-ECM seeded with WJ-MSCs at 4 weeks; (E) M1 macrophages (CD86, red) and M2 macrophages (CD206, green) in UB-ECM hydrogel at 2 weeks; (F) oligodendrocytes (OSP, green) and cell nuclei (DAPI, blue) in SC-ECM at 4 weeks; (G) neuronal growth cones (GAP 43, red) and cell nuclei (DAPI, blue) in SC-ECM at 4 weeks; (H) Schwann cells (p75, red) and cell nuclei (DAPI, blue) in SC-ECM at 4 weeks. The dotted line in (C, D, E, F, H) describes the border between ECM hydrogel and neural tissue (NT). Scale bar: (A, B) 500 μ m; (C, H) 100 μ m; (D–F) 50 μ m; (G) 25 μ m. Color images available online at www.liebertpub.com/tea

of the lesion area. The ingrowth of NFs was maximal at 2 weeks in both hydrogel groups and did not further increase at later time points. No differences in the NFs area were found between SC-ECM and UB-ECM hydrogels at all time points (Fig. 3G). Despite the isotropic structure of the ECM hydrogels, the ingrowing axons linearly bridged the SCI lesion while forming multiple bundles organized in the longitudinal direction along the spinal cord (Fig. 3B).

Astrocytes, evaluated by immunofluorescence staining for GFAP, did not migrate inside the lesion and thus served as a clear demarcation of the lesion area (Fig. 3C, F). Only a few astrocytic processes grew into the graft from the lesion border (Fig. 3F).

In terms of neovascularization, a number of blood vessels (RECA staining) grew into the hydrogel-treated lesions and formed a dense network (Fig. 4A–D). The area of blood vessels gradually increased with time, but no differences in blood vessel density were found between the UB-ECM and SC-ECM hydrogels at any time point (Fig. 4E).

The host tissue remodeling response was characterized by robust infiltration of CD68⁺ cells throughout the entire lesion area (Fig. 5A, B), which populated the hydrogels at all time points, and remained in the lesion site after the hydrogel had degraded. As is apparent from the staining for

M1 and M2 macrophages in Figure 5E, macrophages at the interface of the ECM hydrogel and the host tissue were predominantly of the M1 phenotype (CD86 staining), while M2 phenotype macrophages (CD206 staining) were mostly present within the hydrogel area.

Infiltration of serotonin-positive axons (Fig. 5C, D) was observed from the rostral part of the hydrogels, but these axons did not spread across the lesion. Infiltration of oligodendrocytes (OSP staining, Fig. 5F) within the lesion site indicated that myelination occurred in some of the regenerated axons. Newly sprouted axonal fibers were also detected using GAP43 staining (Fig. 5G). Numerous endogenous Schwann cells that migrated from the nerve roots were detected within the lesion site as well as in the surrounding tissue (Fig. 5H).

Histological evaluation of implanted ECM hydrogels combined with hWJ-MSCs

To evaluate the potential of ECM hydrogels as a cell vehicle, the SC-ECM hydrogels were mixed with hWJ-MSCs (0.5 million cells per 0.2 mL), and the cell-hydrogel constructs containing ~15,000 cells were acutely implanted into the hemisection cavity. Four weeks after

surgery, the grafts were densely infiltrated with endogenous tissue, while cysts had developed at the graft–tissue interface (Fig. 6A). Only very few surviving cells, positive for human mitochondria MTCO2 marker, were detected in the lesion (Fig. 6B). Transplanted cells did not further promote the ingrowth of NF-positive fibers or blood vessels. However, an increase in NF-positive fibers was found in those animal groups that received immunosuppression (Fig. 6F, G). As in the empty ECM hydrogels, M2 phenotype macrophages were mostly present within the hydrogel area (Fig. 6E).

Gene expression analysis induced by ECM hydrogels

Changes in the mRNA expression of genes related to inflammation (*Ptgs2*, *Ccl3*, *Ccl5*, *Il2*, *Il6*, *Il12b*), M1 macrophages (*Irf5*, *Cd86*, *Nos2*), M2 macrophages (*Mrc1*, *Cd163*, *Arg1*), growth factors (*NT-3*, *Fgf2*), axonal sprouting (*Gap43*), astrogliosis (*Gfap*), angiogenesis (*Vegfa*), and apoptosis (*Casp3*) were determined at 2, 4, and 8 weeks after hydrogel injection and compared to the control SCI lesion (Fig. 7, Supplementary Table S1).

The most profound host tissue response to the ECM hydrogels was observed 2 weeks after injury, when significant downregulation was found in the expression of *Fgf2*, *Cd163*, *Irf5*, *Ccl5*, and *Gap43* in both hydrogel groups, and of *Gfap* in the UB-ECM hydrogel group only, when compared to the control SCI lesion. At 4 weeks, no significant changes were detected between both hydrogel groups and the control group, except for a significant upregulation of *Arg1* in the UB-ECM hydrogel group compared to SC-ECM (Supplementary Table S2). A potential tissue-specific effect of SC-ECM was observed at 8 weeks, when significant upregulation of mRNA expression was detected for *NT-3*, *Fgf2*, *Irf5*, and *Casp3*. The expression of proinflammatory cytokines *IL-2*, *IL-6*, *Il12b*, and *Nos2* was undetectable in all groups.

The effect of hWJ-MSCs combined with SC-ECM hydrogels was determined 4 weeks after the scaffold implantation. The animals received immunosuppression to prevent rejection of the xenogeneic cells. Interestingly, the immunosuppression significantly decreased the mRNA expression of *Gfap* in both empty and cell-seeded hydrogels and of *Fgf2*, *Casp3*, *Ccl3*, and *Cd86* in empty hydrogels, when compared to the control lesion (Fig. 7, Supplementary

FIG. 7. Analysis of messenger RNA (mRNA) gene expression of several genes involved in inflammatory and reparative processes following spinal cord injury (SCI) treated with ECM hydrogels. The graphs show the log₂-fold changes in gene expression over intact spinal cord tissue. IS, animal groups that received immunosuppression. * $p < 0.05$, ** $p < 0.01$: Δ Ct values of ECM hydrogel versus control lesion. # $p < 0.05$, ## $p < 0.01$: Δ Ct values of SC-ECM hydrogel with IS versus SC-ECM. + $p < 0.05$, ++ $p < 0.01$: Δ Ct values of SC-ECM hydrogel with IS and hWJ-MSCs versus empty SC-ECM with IS.

Table S1). Moreover, a significant increase in the expression of *Vegfa* and *Gap43* was also found in cell-seeded hydrogels when compared to the empty hydrogels.

Discussion

In this study, we evaluated the *in vivo* neuroregenerative potential of two types of ECM hydrogels based on the CNS and non-CNS tissue, when injected into the spinal cord acutely after SCI. The ECM matrices were derived from porcine spinal cord and urinary bladder and processed into an injectable hydrogel form as was described previously.^{19,21} Regarding the different tissue sources used, SC-ECM and UB-ECM hydrogels were prepared using different decellularization methods and differed in their composition as well as in their physical and biological properties.^{19,21} Despite the lack of a native 3D ultrastructure from the source tissue, ECM hydrogels retain their biological activity and possess mechanical properties similar to that of soft neural tissue, with the advantage of injectability and *in situ* polymerization, which offer minimally invasive delivery techniques and facilitate the possibility of clinical translation.

When injected into the SCI, both hydrogel types were well integrated into the surrounding tissue, with persisting massive cell infiltration and neovascularization. A potentially important factor for tissue regeneration is tissue specificity

of the ECM hydrogel source. In this study, however, both studied materials proved to be advantageous for providing a supportive environment and revealed similar neurotrophic properties *in vitro* in the DRG explant culture as well as *in vivo* with regard to the ingrowth of NFs and neovascularization. These findings are consistent with those of a previous study, which showed no advantage of CNS-derived ECM materials versus non-CNS-derived ECM materials with respect to effects upon neural stem/progenitor cells.²⁴

Macrophages were the predominant infiltrating cells within the grafts that participated in the ECM degradation. As was shown previously, degradation of ECM scaffolds is essential for the constructive tissue remodeling process, by which a degradable biomaterial serves as a temporary inductive niche, which is gradually replaced by anatomically appropriate and functional tissue as opposed to scar tissue.^{11,25,26} Moreover, degradation of ECM scaffolds stimulates the release of matricryptic molecules; which possess a variety of bioactive properties, such as antimicrobial activity, angiogenic effects, as well as the recruitment of endogenous stem and progenitor cells.²⁶

In the present study, however, despite the fact that the lesion cavity was filled with endogenous cell-populated ECM hydrogels 2 weeks after their injection, further progression in matrix degradation at later time points was not followed by full neural tissue replacement, but rather

resulted in the formation of a dense network of tissue containing axons, blood vessels, and other neural tissue elements interrupted by a number of small cysts.

According to the gene expression analysis, *in vivo* ECM degradation was associated with a significant decrease in mRNA expression of markers for proinflammatory/M1 macrophages (*Irf5*) and regulatory/M2 macrophages (*Cd163*), inflammation (*Ccl5/RANTES*), as well as genes for growth factor *Fgf2*, astrogliosis (*Gfap*), and neuronal growth cones (*Gap43*). The expression of other markers related to immune response, such as *Cd86*, *Mrc1*, and *Ptgs2*, also decreased, but these changes were not found to be significant. Interestingly, these effects were detected during the early phase after injury, but decreased or even reversed at later time points, suggesting that ECM hydrogel degradation played a significant role in the transient modulation of the innate immune and tissue repair response.

Previous reports have shown increased numbers of M2 macrophages and more positive polarization toward an M2 phenotype associated with ECM *in vivo* degradation and the promotion of constructive tissue remodeling.^{15,27} Recent studies describe the M2 polarizing effects of ECM derived from several tissues.^{28,29} In the present study, the expression of genes related to both M1 and M2 macrophages decreased at 2 weeks, which reflects that both inflammatory as well as anti-inflammatory responses were inhibited after ECM hydrogel treatment. Nevertheless, according to positive staining for CD206, ECM hydrogel treatment led to spatial differences of macrophage distribution within the lesion, where M2 macrophages were mostly accumulated within the hydrogel, and M1 macrophages were in the surrounding tissue.

The ability of ECM hydrogels to promote *in vitro* cell growth and proliferation was examined using hWJ-MSCs. The hWJ-MSCs currently represent a promising cell type in regenerative medicine and are already being evaluated in various clinical trials, including SCI.³⁰ The *in vitro* 2D cell culture demonstrated that both types of ECM hydrogels promoted adhesion and proliferation of hWJ-MSCs.

However, when seeded in 3D culture, hWJ-MSCs triggered rapid gel contraction. This well-known phenomenon is characteristic for collagen gels seeded with fibroblasts that generate tension on the matrix during both extension and retraction of pseudopodia.³¹ A similar effect has also been described for ECM hydrogels derived from porcine dermis as well as urinary bladder seeded with fibroblasts.²¹

When used as a cell vehicle *in vivo* to fill the lesion cavity, the rapid gel contraction may then result in inhomogeneous scaffold distribution within the lesion. Moreover, when injected into the lesion, the ECM hydrogels may further contract over time as they are populated with various endogenous cells, such as fibroblasts or epithelial cells. Of note, a similar collagen concentration ($\sim 700 \mu\text{g}$ collagen/mg dry weight) as well as gel contraction rate was found for both SC-ECM and UB-ECM, while sulfated glycosaminoglycan concentration was higher for UB-ECM ($\sim 4 \mu\text{g}/\text{mg}$ dry weight) than for SC-ECM ($\sim 1 \mu\text{g}/\text{mg}$ dry weight).¹⁹

To evaluate ECM hydrogels for cell delivery, we prepared the cell seeded gels before their implantation into the lesions, which partly avoids the massive scaffold contraction within the lesion cavity. In spite of this, the inflammatory milieu of the acute lesion together with the massive infiltration of macrophages did not support cell survival. Furthermore, only

few cells were detected within the lesion 4 weeks after the implantation. On the other hand, due to the limited volume of the implanted scaffold, the total number of implanted cells within the hydrogel was relatively small ($\sim 15,000$). By increasing the number of implanted cells a higher *in vivo* cell survival rate could be achieved, however, an increased degree of gel contraction may result in the enhanced formation of dense cell bulks within the lesion cavity.

Notably, immunosuppression significantly promoted axonal ingrowth, decreased expression of *Gfap*, *Fgf2*, *Casp3*, *Ccl3*, and *Cd86*, and increased expression of *Vegfa*, which confirmed the neurotrophic effect of immunosuppressive agents.³²

In comparison with the synthetic nondegradable materials based on poly(2-hydroxyethyl methacrylate), which we previously developed and evaluated *in vivo*,^{33,34} ECM hydrogels are undoubtedly advantageous, in terms of their injectability, degradability, as well as their biological activity, which is able to modulate the immune response and stimulate vascularization and axonal ingrowth. At the same time, there are also two impediments that hinder the use of ECM hydrogels in their current form as optimal materials for CNS repair: (1) progressive hydrogel contraction in combination with fibroblast-like cells, such as MSCs and (2) rapid *in vivo* hydrogel degradation, which was too fast to be followed by full tissue reconstruction in the lesion cavity.

When applied immediately into the SCI, the neuroregenerative potential of ECM hydrogels might be burdened by the hostility of the acute SCI lesion due to the acute inflammatory response, which in turn may significantly influence the speed of hydrogel degradation and thus the character of tissue replacement.

Remarkably, acute lateral hemisection, which we used to evaluate the feasibility of ECM hydrogels in CNS repair, is the least invasive and devastating SCI model. On the other hand, hemisection is a case of partial lesion with a high rate of spontaneous recovery and a high risk of inconsistencies in the injuries from one animal to the next, which might lead to misinterpretation of the behavioral evaluation.^{35,36}

Further investigation using a subacute or chronic compression SCI model together with systematic functional evaluation is, therefore, the next step in establishing the link between *in vivo* biological properties of the ECM hydrogel in acute and chronic SCI.

To slow degradation, chemical crosslinking of the ECM hydrogel may offer longer scaffold persistence within the lesion and thus provide more time to complete the tissue remodeling. However, recent studies suggest that degradation of the ECM scaffold is an essential component of a rapid constructive remodeling response. Moreover, crosslinking of the ECM may reduce or eliminate the amount of cellular infiltration into the implant or even cause a foreign body reaction.¹⁵ Alternatively, hydrogels composed of concentrations $>8 \text{ mg/mL}$ as used in the present study may slow the degradation process.

Conclusions

This study evaluated the *in vivo* function of two types of ECM hydrogels derived from decellularized porcine spinal cord and urinary bladder tissues as scaffolds for SCI repair. Both ECM hydrogels showed significant immunomodulatory

and neuroregenerative effects and provided the substrate for tissue bridging after SCI. Further studies concerning the optimization of hydrogel degradation time as well as the analysis of the ability to restore neuronal function after SCI in combination with a suitable cell type are needed to consider the potential of ECM hydrogels for clinical translation.

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Disclosure Statement

No competing financial interests exist.

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