Video Article

Experimental Strategies to Bridge Large Tissue Gaps in the Injured Spinal Cord after Acute and Chronic Lesion

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Abstract

After a spinal cord injury (SCI) a scar forms in the lesion core which hinders axonal regeneration. Bridging the site of injury after an insult to the spinal cord, tumor resections, or tissue defects resulting from traumatic accidents can aid in facilitating general tissue repair as well as regenerative growth of nerve fibers *into* and *beyond* the affected area. Two experimental treatment strategies are presented: (1) implantation of a novel microconnector device into an acutely and completely transected thoracic rat spinal cord to readapt severed spinal cord tissue stumps, and (2) polyethylene glycol filling of the SCI site in chronically lesioned rats after scar resection. The chronic spinal cord lesion in this model is a complete spinal cord transection which was inflicted 5 weeks before treatment. Both methods have recently achieved very promising outcomes and promoted axonal regrowth, beneficial cellular invasion and functional improvements in rodent models of spinal cord injury.

The mechanical microconnector system (mMS) is a multi-channel system composed of polymethylmethacrylate (PMMA) with an outlet tubing system to apply negative pressure to the mMS lumen thus pulling the spinal cord stumps into the honeycomb-structured holes. After its implantation into the 1 mm tissue gap the tissue is sucked into the device. Furthermore, the inner walls of the mMS are microstructured for better tissue adhesion.

In the case of the chronic spinal cord injury approach, spinal cord tissue - including the scar-filled lesion area - is resected over an area of 4 mm in length. After the microsurgical scar resection the resulting cavity is filled with polyethylene glycol (PEG 600) which was found to provide an excellent substratum for cellular invasion, revascularization, axonal regeneration and even compact remyelination *in vivo*.

Video Link

The video component of this article can be found at http://www.jove.com/video/53331/

Introduction

A traumatic injury to the spinal cord not only leads to the loss of axons but it further results in tissue defects which hinder any regenerative responses (for review see [1,2]). Spinal cord tissue is often lost through secondary degeneration leading to cyst formation or holes in and around the lesion area. Most experimental therapeutic interventions focus on incomplete spinal cord damages like partial transection, crush or contusion injuries with a remaining rim of healthy tissue. For complete injuries like total transections resulting from traumatic accidents or surgical interventions, like tumor resections, only very limited treatment options are available today[3,4]. After complete transection, mechanic tension of the tissue results in spinal stump retraction, leaving a small gap in the spinal cord. Most strategies focus on filling this gap with tissue, cells or matrices[5,6].

Here, a different strategy is presented, namely re-adaptation of the separated stumps using a novel microconnector device[7]. In order to readapt the two stumps, mechanical force has to be applied as a slight negative pressure to accomplish this (**Figure 1**). The mechanical microconnector system (mMS) is a multi-channel system of polymethylmethacrylate (PMMA) with honeycomb-shaped holes (**Figure 1A**) and provided with an outlet tubing system. It is implanted into the tissue gap resulting from complete spinal cord transection in the rat (**Figure 1C**). One tube can be connected to a vacuum pump to apply negative pressure to the mMS (**Figure 1D**). The pressure pulls the disconnected spinal cord stumps into the honeycomb-shaped holes of the mMS, which have microstructured walls to hold the tissue in place when pressure is released (**Figure 1B**). The tubing can be left intact after surgery and attached to an osmotic minipump in order to infuse substances into the lesion core (**Figure 1E-F**).

Besides an acute transection of the spinal cord another type of complete lesion results from surgical removal of a spinal tumor or a solid chronic lesion scar leading to large tissue gaps of several millimeters, which cannot be overcome by the mMS so far. The majority of patients with spinal cord trauma suffer from chronic injuries. In these patients, a fully developed scar occupies the lesion core. The surgical removal of the

lesion scar is a concept for treatment which is currently investigated after experimental SCI[8,9]. While the resection procedure itself can be performed without causing considerable additional damage, the resulting tissue gap needs to be bridged with a suitable matrix which allows and promotes the regeneration of tissue and, in the specific case of spinal cord injuries, the regeneration of nerve fibers to maintain and promote locomotor functions. It was found that low-molecular weight polyethylene glycol (PEG 600) is a very suitable material for this purpose. Its lack of immunogenicity and the very low viscosity allow smooth integration into the surrounding tissue. Insertion of the biopolymer alone promotes the invasion of beneficial cells, including endothelial cells, peripheral Schwann cells, and astrocytes, and - very importantly - the regeneration and elongation of axons of descending and ascending fiber tracts as well as their ensheathment by compact myelin[8]. These regenerative responses were found to be accompanied by long-lasting functional improvements. The combination of resection of scar tissue and subsequent implantation of PEG 600 presents a safe and simple, yet very efficient means of bridging substantial spinal cord tissue defects.

Protocol

Institutional guidelines for animal safety and comfort were adhered to, and all surgical interventions and pre- and post-surgical animal care were provided in compliance with the German Animal Protection law (State Office, Environmental and Consumer Protection of North Rhine- Westphalia, LANUV NRW).

1. Complete Transection of the Thoracic Spinal Cord of Female Wistar Rats (220 - 250 g)

1. **Preparation of the Spinal Cord**
    1. Use isoflurane inhalation anesthesia (2 - 3% of isoflurane in O_2/NO_2 at a ratio of 1:2) and bolus injection of Carprofen (subcutaneously [s.c.] 5 mg/kg). The combination of carprofen and the opioid buprenorphine (0.02 mg/kg s.c.) is recommended. Start surgery when eye lid reflex to light touch with a cotton bud and paw withdrawal reflex to pinching stimulus with forceps are no longer observed.
    2. Place the animal on a heating blanket at 37 °C to maintain body temperature during surgery and put eye ointment on eyes to avoid dryness while under anaesthesia.
    3. Shave the animal's back and prepare the skin with a skin disinfectant.
    4. Cut the skin at the midline along the thoracic vertebrae for 4 cm with a surgical blade and open it. From this step on, use sterilized surgical instruments for all procedures (autoclaved or immersion-sterilized).
    5. Retract the muscles above the thoracic vertebrae using a small muscles clamp.
    6. Remove the spinous processes at thoracic level 8 (Th8) and Th9 with a bone rongeur by cautiously clipping small pieces of bone until the vertebral bones are flat.
    7. Use anatomical forceps to lift the vertebral column at spinous process Th7 and use a rongeur to clip small pieces of vertebral bone from caudal to rostral until a laminectomy is performed at Th8 and Th9. Expose the dura mater without damaging it by carefully removing only few and very small pieces of vertebral bone at a time.
    8. Clamp the backbone by 2 stabilization clamps at spinous processes Th7 and Th10, lift the animal to uncouple breathing movements from the vertebrae.

2. **Complete Spinal Cord Transection at Thoracic Level 8/9**
    1. Lift the dura mater with fine forceps, cut the dura mater with fine eye scissors in transversal direction.
    2. Hold the lateral cut end of the dura mater with fine forceps and insert a spinal hook into the subarachnoidic space between dura mater and arachnoidea. Avoid damage of the meninges by not pricking into the pia or dura mater with either forceps or spinal cord hook.
    3. Slowly rotate the hook to place it all along the spinal cord tissue, taking care not to prick into the dura (pia is still intact).
    4. Lift the spinal cord for approximately 1 - 2 mm upwards, until a gap is seen at the ventral side between spinal cord tissue and dura.
    5. Insert fine eye scissors into the space between dura and pia and cut the spinal cord while the spinal hook is left in place.
    6. Lift the two stumps of the spinal cord with two forceps and visually ensure complete transection.
    7. For control-lesioned animals and animals with a chronic injury, close the dura by interrupted sutures with monofilament non-adsorbable 9.0 threads.
    8. For chronic lesions follow part 1.6.

3. **mMS Implantation**
    1. Place mMS above the injury site with the two tubes lying on each lateral side of the vertebra and lower it into lesion cavity.
    2. Suture tubes to the muscles at the side of the vertebra with non-resorbable 4-0 thread to ensure stabilization of the mMS. Ensure a fixation of the mMS using forceps during this step.
    3. Remove the mMS connector pin by cutting with a pair of fine scissors.
    4. Close the dura above the mMS and suture it with 9.0 threads.
    5. Attach one tube to the vacuum pump, and seal the other by clamping.
    6. Apply gentle negative pressure to the mMS by a vacuum pump *via* the open tube, sucking the spinal cord stumps into the mMS lumen. Apply the negative pressure for several minutes (at maximum for 10 min) and monitor by sensors (250 - 350 mbar).
    7. Cut the tubes close to the mMS and remove the tubes. Continue with Step 1.6.

4. **Resection of Spinal Cord Tissue Including the Chronic Lesion Scar at Week 5 after Initial Injury**
    1. Follow steps 1.1.1 - 1.1.5.
    2. Identify scar tissue by brown-yellow appearance and stiff tissue on top of the spinal cord. Gently remove superficial layers of scar tissue by holding with fine forceps and cutting with fine scissors. With this method, re-open the site of laminectomy to expose the tissue containing the spinal cord injury area. Stop preparation when the dura suture is visually identified.
    3. Clamp the backbone by 2 stabilization clamps at spinous processes Th7 and Th10, and then elevate the animal to uncouple breathing movements from the vertebrae.
    4. With a small paperboard ruler measure the spinal cord area which is to be resected (length: 4 mm) and mark the edges of this respective tissue area with transverse incisions to allow the subsequent removal of tissue.

5. Remove scar tissue *via* a combination of cutting and aspiration. Take out the tissue that has been separated from the spinal cord with the transverse incisions. Use gentle aspiration whenever it is sufficient to allow the removal of the tissue. In addition, whenever the stiff texture of the scar tissue makes tissue aspiration too difficult, use fine scissors to cut and remove this tissue.
6. Insert a piece (approximately 5 mm x 5 mm x 5 mm cube) of hemostatic gelatin sponge into the tissue gap until bleeding subsides. The gelatin sponge will shrink in size as soon as it is soaked with liquid.

5. **Implantation of PEG 600**
   1. Prepare 1 ml of pure undiluted PEG 600 for injection by heating to 37 °C.
   2. Remove gelatin sponge.
   3. Insert sufficient amount (approximately 5 - 7 µl) of PEG into the gap using a 10 µl syringe or a 10 µl pipette.
   4. Carefully cover the area with a piece (approximately 5 mm x 4 mm) of sealant/dura replacement.
   5. Fix the sealant to surrounding muscle tissue with a few drops of tissue glue. Place the sealant on top of the PEG-filled resection gap. To prevent slippage of the sealant, use small drops of tissue glue to fix the corners of the sealant to the surrounding muscle tissue. Note: Avoid leakage of the tissue glue onto the spinal cord tissue!

6. **Closing of Tissue and Postoperative Care**
   1. Suture muscles and skin layer for layer with interrupted sutures with braided adsorbable 4-0 threads
   2. Inject 2 × 2.5 ml of sodium chloride (NaCl [0.9%]) at 36 °C s.c. for rehydration after the surgery. A single injection of 5 ml NaCl would stretch the animal's skin and might cause unnecessary harm to the animal. Do not leave animal unattended until full consciousness is regained.
   3. Inject daily carprofen (s.c. 5 mg/kg) for at least 2 days after the surgery. House animal in single cage during the first 2 days, afterwards in groups of 2 - 3.
   4. Apply antibiotic treatment (daily oral administration of enrofloxacin) for the first post-operative week.
   5. Do manual bladder voiding at two to three times per day by gently stroking over the belly of the animal from rostral to caudal. Be careful not to move the vertebral column of the animal and don't lift the animal at the tail. Manually void the completely spinalized animal's bladder daily during the whole survival time. Care must be taken to place food pellets and water bottles in a height which can be reached by the animals without standing on their hindpaws. Although being impaired in hind quarter function, the animals move and explore the cages actively immediately after surgery. It is recommended to keep the rats in sociable groups.
   6. For chronic lesions leave a survival time of the animals of five weeks before scar resection. Follow steps 1.4.

Representative Results

Tissue Preservation, Axonal Regrowth and Functional Benefit of mMS Implantation after Acute Complete Transection of the Spinal Cord

It is demonstrated that the acute implantation of the mMS stabilized the completely transected spinal cord stumps and decreased shrinkage of the tissue (**Figure 2A *versus* B**). As visualized by Trichrome staining in sagittal sections, the green connective tissue staining of the fibrotic scar in the lesion core is much denser and more prominent in control-lesioned animals (**Figure 2D**) than in mMS implanted animals (**Figure 2C**). Interestingly, there were no observed differences at long survival times in macrophage accumulation in the lesion site as visualized by immunohistological staining against ED-1 in mMS implanted versus control lesioned animals (not shown).

Immediately after implantation, lack of tissue in the mMS lumen was observed which was subsequently filled with cells and tissue after several days. At 5 weeks post-surgery massive axonal ingrowth into the mMS lumen could be detected (**Figure 3A**). Moreover, the mMS lumen was vascularized (**Figure 3B**) and axonal structures were found in close proximity of blood vessels (not shown). Assessment of the open field Basso-Beattie-Bresnahan locomotor score (BBB), revealed a significant functional improvement of mMS-implanted animals (black line in **Figure 3C**) *versus* control-lesioned animals (grey line in **Figure 3C**) at 2 and 4 weeks post-surgery.

Cell Invasion, Revascularization, Axonal Regeneration and Functional Improvement in Chronic Spinal Cord Injured Rats after Scar Resection and PEG Implantation

In chronic injury models of both partial and complete thoracic spinal cord transection the surgical removal of the lesion scar at week five after initial injury did not cause any detectable additional harm or discomfort to the animals. Scar resection and subsequent insertion of PEG 600 led to regeneration of tissue which was detectable within the matrix (**Figure 4**). In addition, deposition of extracellular collagen sheaths - which is typical for the basement membrane meshwork of scar tissues - was not as prominent after PEG-treatment (**Figure 4C**) compared to lesion-only controls (**Figure 4B**). In contrast to the chronic scar of lesion-only controls (not shown), the matrix-filled resection site was invaded by axon growth-promoting cells after the resection. Already as early as one week post resection and treatment several beneficial cell types in the matrix area could be identified such as endothelial cells (which were found to be present in regenerating blood vessels [**Figure 4D**]), astrocytes (**Figure 4F**) and peripheral Schwann cells (**Figure 4G**). Five weeks after resection the PEG-treated area is filled with numerous axonal profiles (**Figure 4E**).

The PEG matrix promoted the substantial regenerative growth of numerous axons (**Figure 4D,E,G**). With tracing studies and immunohistochemical staining, various ascending and descending axonal populations could be identified which regenerated not only *into* but also *beyond* the PEG-filled area[8]. Transmission EM analyses of the treated area of animals with a long survival period (8 months) not only confirmed the presence of Schwann cells but further revealed compact myelination of the regenerated axons inside the PEG-matrix[8]. Regenerated axon profiles were frequently associated with areas of angiogenesis (**Figure 4D**). Immunohistochemical stainings demonstrated that the axon profiles which were found in the treated resection area were closely associated with Schwann cells and appeared to be myelinated by these cells already at early time points after the resection and implantation[8].

A long-term (eight months) behavioral study revealed substantial long-lasting locomotor functional improvements after chronic scar resection and PEG-treatment (**Figure 5**).

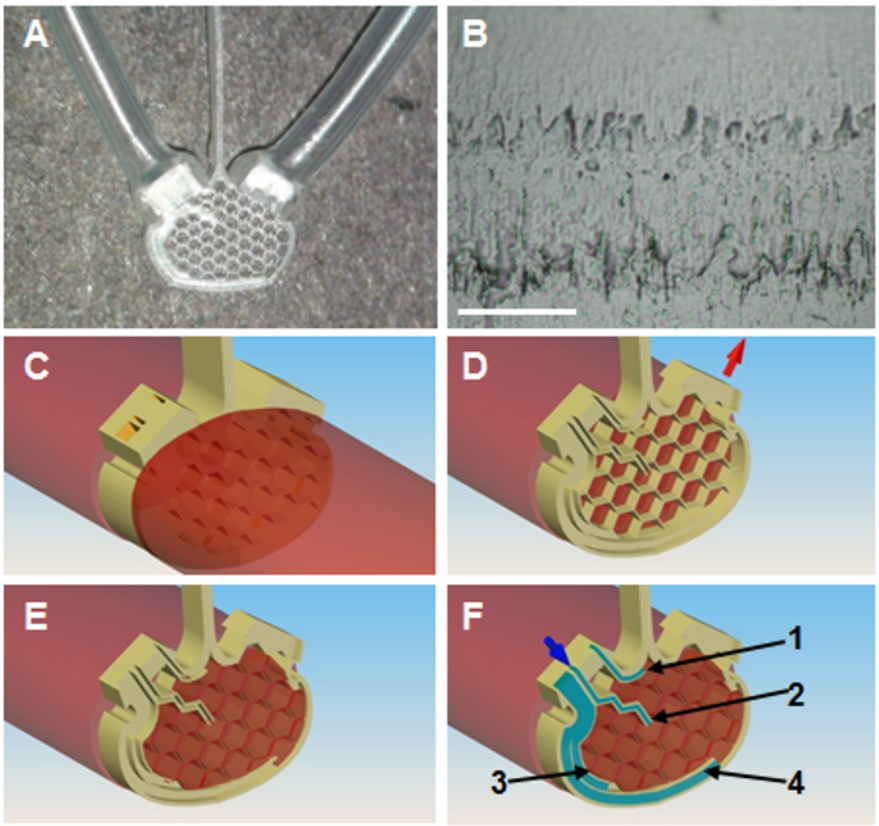

Figure 1. mMS Design and Principle of Operation. (**A**) photographic picture of the mMS, (**B**) adhesive surface microstructures of mMS sidewalls, scale bar: 50 µm, (**C-F**) schematic drawing: (**C**) implantation of the mMS into the spinal cord lesion, (**D**) application of vacuum to suck the tissue into the honeycomb structure (red arrow: negative pressure), (**E**) adhesive force keeps the spinal cord stumps in close proximity at a distance of only several micrometers, (**F**) distribution of pharmacological substances in the lumen via 4 internal micro-channels (black arrows point at micro-channels 1 - 4). Infusion is depicted by blue arrow at the inlet port. In **D - E** only one half of the mMS is shown. Reprinted from Brazda *et al*, 2013[7] with permission from Elsevier. Please click here to view a larger version of this figure.

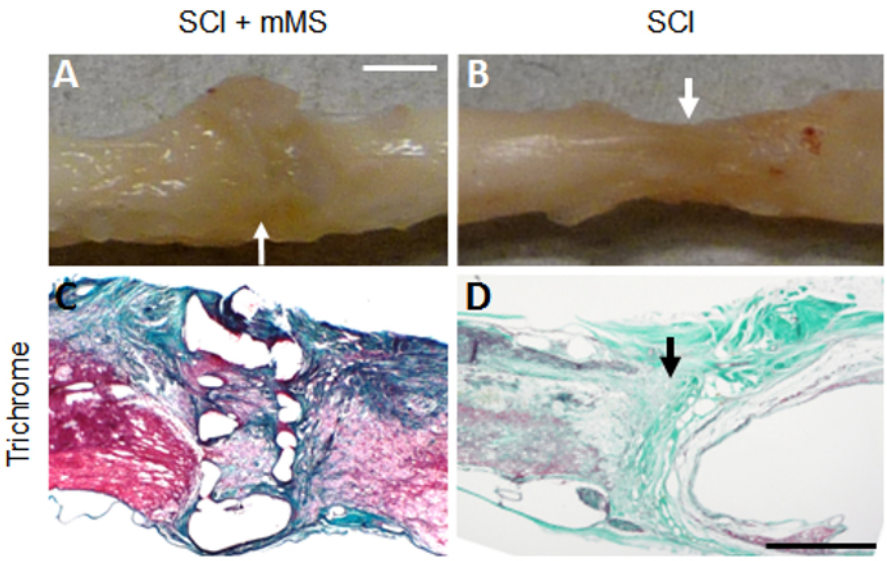

Figure 2. Spinal Tissue Preservation after mMS Implantation. Spinal cord tissue at 6, resp. 7 months after total transection with (**A**) and without mMS implant (**B**). The arrow in (**B**) indicates the spinal cord region corresponding to the implantation site of the mMS (arrow) in (**A**). Note the shrinkage of the spinal cord tissue in the untreated animal (**B**) in contrast to the well preserved structure in (**A**). Trichrome staining of sagittal spinal cord sections after mMS implantation (**C**) versus control animals without implant (**D**). Green: connective tissue, red: cytoplasma, black: nucleus.The lesion center is filled with scar tissue (green) in control-lesioned animals (**D**, black arrow depicts lesion epicenter), whereas only marginal scarring is evident around the mMS in treated animals after 14 days (**C**). Note that the mMS consists of honeycomb structures which are washed out during tissue processing if cut in 20 µm slices. Scale bar for (**A, B**) in (**A**), for (**C, D**) in (**D**): 1 mm. Modified from Brazda *et al*, 2013[7] with permission from Elsevier. Please click here to view a larger version of this figure.

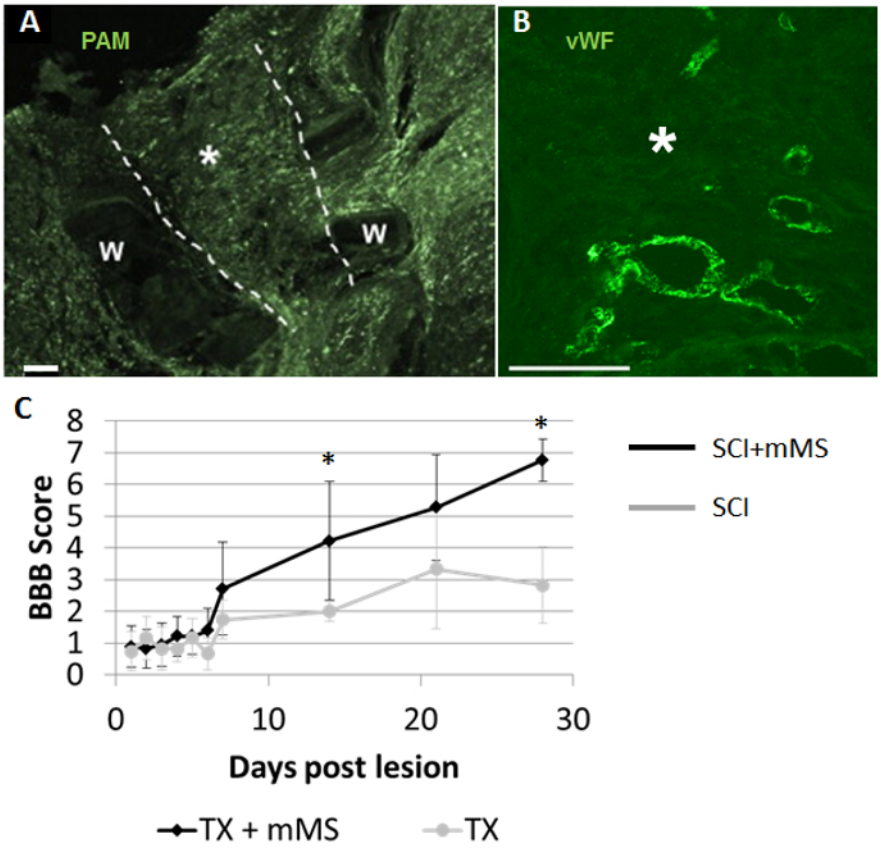

Figure 3. Axonal Regeneration, Revascularization and Open Field Locomotor Score after mMS Implantation. (**A**) Immunohistological staining of phosphorylated neurofilament with pan-axonal marker (PAM) in a sagittal spinal cord section at 5 weeks after complete spinal cord transection and mMS implantation. The mMS lumen is indicated by an asterisk. The walls (W) of the mMS are marked by dashed lines. Note the numerous stained axons in the previously tissue-devoid mMS lumen. Scale bar: 100 µm. (**B**) Immunohistological staining of blood vessels in the mMS lumen (identified with von Willebrand factor [vWF] staining, green). (**C**) Assessment of the BBB locomotor score for mMS implanted (black line, N = 9) *versus* control injured animals (grey line, N = 6). The mean BBB of left and right hindlimb (average per group) is depicted with standard deviation. Statistically significant differences are marked by asterisks (Mann-Whitney Rank Sum Test, $p < 0.05$). Modified from Brazda *et al,* 2013[7] with permission from Elsevier. Please click here to view a larger version of this figure.

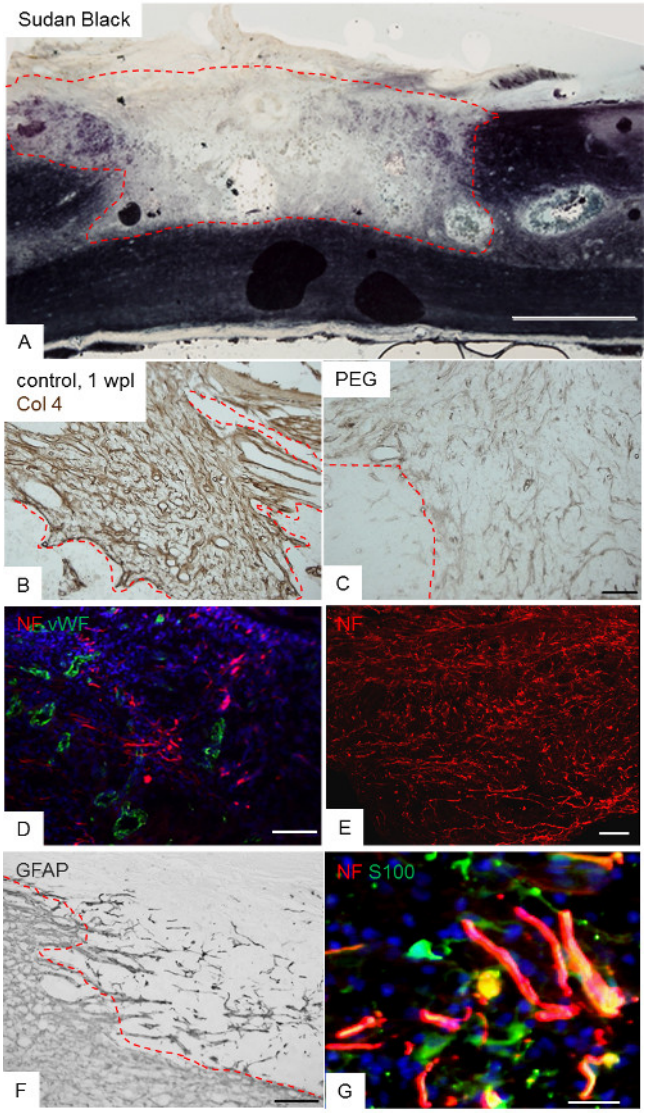

Figure 4. PEG Matrix Promotes Tissue Regeneration and Beneficial Cellular Invasion following Scar Resection and Treatment. (**A**) Sudan Black staining shows that large parts of the resected gap (devoid of Sudan Black staining) are filled with tissue at 1 week post resection. (**B, C**) Staining of the fibrous lesion scar with collagen type IV at 1 week post resection. A dense scar is present in control animals while PEG-treated animals reveal a much weaker and more distinct immunostaining. (**D**) Area of angiogenesis (wWF) contains profiles of regenerated axons (identified with neurofilament [NF]) at one week post resection. (**E**) Numerous axons have grown into the treated area at 5 weeks post resection. (**F, G**) Both glial fibrillary acidic protein positive ($GFAP^+$) astrocytes and $S100^+$ Schwann cells invade the PEG matrix and the latter are found in close association with the regenerating axons already after 1 week post treatment. Scale bars: (**A**) 1 mm; (**C-F**) 100 µm, (**G**) 50 µm. (**B-G**): adapted from Estrada *et al*., 2014[8] with permission from Elsevier. Please click here to view a larger version of this figure.

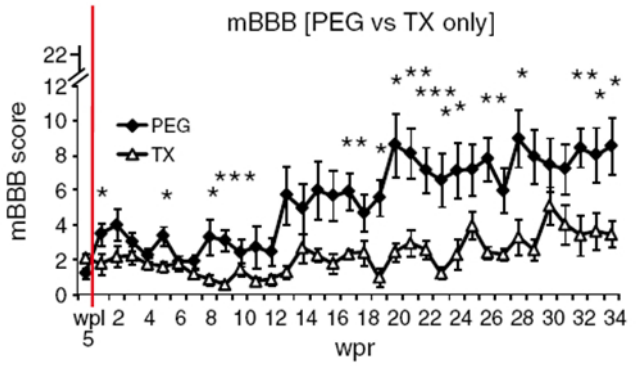

Figure 5. Improvement of Locomotor Function after Chronic Spinal Cord Injury, Scar Resection and PEG-treatment. Assessment of the modified BBB (mBBB) locomotor score for PEG-treated (PEG, black rhombs, N = 13-14 per timepoint) *versus* control animals, which received a total spinal cord transection without scar resection (TX, white triangles, N = 13-14 per timepoint) after chronic spinal cord injury. Averaged mBBB scores + standard error of the mean one-sided Mann-Whitney U test, *p ≤0.05, **p ≤0.01, ***p ≤0.001; Modified from Estrada *et al.*, 2014[8] with permission from Elsevier, wpl = week post initial lesion, wpr = weeks post resection. Error bar = SEM (Standard error of the mean). Please click here to view a larger version of this figure.

Discussion

Here two different surgical approaches are presented to bridge tissue gaps in the spinal cord after (1) acute complete transection and mMS implantation and (2) chronic spinal cord lesion and fibrous scar removal plus PEG matrix implantation. Both strategies lead to tissue preservation and axonal regeneration as well as to significant locomotor functional improvement of the treated animals. For mMS implantation an adequate fixation of the mMS within the spinal cord by the firm dura suture after surgery is a critical technical step.

The mMS holds further therapeutic potential due to its implemented internal microchannel system which allows the local infusion of therapeutically active liquids into the lesion core via, *e.g.*, an attached osmotic minipump[7]. For its intended future clinical use, the mMS material should be bioresorbable. Currently, the fabrication of connector systems composed of lactide-based materials is being tested. Moreover, coating of the mMS with an electronic conductor material will be established in order to apply therapeutic electric fields to the lesioned spinal cord.

As for chronic spinal cord lesions, another strategy was followed, since physical tension of the separated spinal cord stumps after surgical scar removal appeared too high if a gap of several millimeters must be crossed. Surprisingly, the low molecular weight PEG 600 proved to be a highly suitable biopolymer to fill the resulting gap. It allows the formation of a stable tissue bridge which promotes angiogenesis and cellular invasion of beneficial cell types. It is presumed that the physical properties of PEG600, like its viscosity, play important roles for the observed effectiveness since other PEG-types with higher or lower molecular weights and/or viscosities were not as beneficial.

Bridging the smaller gap after acute transection with the novel connector system lead to a clear functional improvement as early as 4 weeks after the injury. The respective animals reached a BBB score of approximately 7 by that time and clear differences between mMS-animals and control rats were apparent. Chronically injured animals which received a scar resection and PEG-implantation also recovered significantly better than untreated controls, but the improvements were notable mostly at later time points (after approximately 16 weeks). Such observations could be explained by the nature of the injury (*i.e.*, acute vs. chronic, and 1 mm vs. 4 mm tissue defect). In case of the larger lesions the regenerative growth of axons across the lesion site requires longer time periods. Also, it is very likely that the longer time periods of non-use of the hind limbs result in higher degrees of degenerative events and, therefore, in less prominent functional improvements.

Future optimization of the PEG treatment after chronic spinal cord injury, via combinatorial approaches, *e.g.*, additional seeding of the PEG with growth promoting (stem) cells like umbilical cord blood cells[10] *in vivo*, are currently being tested.

Disclosures

The authors have nothing to disclose.

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