Video Article

Combining Peripheral Nerve Grafting and Matrix Modulation to Repair the Injured Rat Spinal Cord

John D. Houle, Arthi Amin, Marie-Pascale Cote, Michel Lemay, Kassi Miller, Harra Sandrow, Lauren Santi, Jed Shumsky, Veronica Tom
Department of Neurobiology and Anatomy, Drexel University College of Medicine

Correspondence to: John D. Houle at jhoule@drexelmed.edu

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Abstract

Traumatic injury to the spinal cord (SCI) causes death of neurons, disruption of motor and sensory nerve fiber (axon) pathways and disruption of communication with the brain. One of the goals of our research is to promote axon regeneration to restore connectivity across the lesion site. To accomplish this we developed a peripheral nerve (PN) grafting technique where segments of sciatic nerve are either placed directly between the damaged ends of the spinal cord or are used to form a bridge across the lesion. There are several advantages to this approach compared to transplantation of other neural tissues; regenerating axons can be directed towards a specific target area, the number and source of regenerating axons is easily determined by tracing techniques, the graft can be used for electrophysiological experiments to measure functional recovery associated with axons in the graft, and it is possible to use an autologous nerve to reduce the possibility of graft rejection. In our lab we have performed both autologous (donor and recipient are the same animal) and heterologous (donor and recipient are different animals) grafts with comparable results. This approach has been used successfully in both acute and chronic injury situations. Regenerated axons that reach the distal end of the PN graft often fail to extend back into the spinal cord, so we use microinjections of chondroitinase to degrade inhibitory molecules associated with the scar tissue surrounding the area of SCI. At the same time we have found that providing exogenous growth and trophic molecules encourages longer distance axonal regrowth into the spinal cord. Several months after transplantation we perform a variety of anatomical, behavioral and electrophysiological tests to evaluate the recovery of function in our spinal cord injured animals. This experimental approach has been used successfully in several spinal cord injury models, at different levels of injury and in different species (mouse, rat and cat). Importantly, the peripheral nerve grafting approach is effective in promoting regeneration by acute and chronically injured neurons.

Protocol

1) Preparation for microscopic surgery

1. The surgical station needs to be clean and sanitized with a dilute bleach solution prior to setting out instruments and accessory materials. Instruments will be autoclaved 1 day prior to surgery and stored in a sterile container. Turn on the Hot Bead Sterilizer (Fine Science Tools) used to remove pathogens and microbial contaminants from instruments between procedures on different animals.
2. Plug in the thermal barrier used to maintain body temperature during the surgical procedure. Set out cotton swabs and gauze pads used to control bleeding. Prepare small (< 2mm³) pieces of Gel Foam to be used for micro-hemostasis and immerse in a sterile saline solution.
3. Place rat in an induction chamber and anesthetize with Isoflurane (5% vaporized in oxygen, flow rate 2.0 L/min). Remove animal from chamber to preparation table and shave a wide area of fur from the intended surgical field. Wipe with 70% isopropyl alcohol beginning at the center of the field and radiating outward, followed by scrubbing with Polyhydroxidine Solution (Xenodine) in the same manner. Repeat this sequence of washes 3 times.
4. Place animal on the thermal barrier, cover nose and mouth with inhalation cone, reduce Isoflurane to 3% and flow rate to 0.2 L/min. Deliver appropriate dose of antibiotic (Ampicillin) by sub-cutaneous injection and analgesic (Buprenorphine 0.05 mg/kg) by intramuscular injection.
5. The surgeon will scrub their hands with Xenodine solution and rinse with water. A sterile surgical gown will be fitted onto the surgeon; hands will be dried with a sterile towel and covered with sterile gloves. This procedure is followed for each surgical procedure described in this text.
6. The lid covering the sterile instruments will be removed by an assistant and instruments will be laid out on a sterile covering by the surgeon.

2) Transection of PN to promote degeneration

1. With the animal lying on its side make a straight line incision from the head of the femur towards the knee, keeping parallel and approximately 3mm from the femur. Cut through the superficial biceps femoris muscles along the same line as the skin incision, to expose the underlying vastus lateralis muscle.
2. Using scissors make a small cut in the vastus lateralis near the knee and extend this up towards the head of the femur. Again with scissors gently separate the vastus lateralis until the conjoined tibial and peroneal nerve branches of the sciatic nerve are visualized (running parallel to the femur).
3. Trace these branches towards the head of the femur until the main trunk of the sciatic nerve is identified. Just below the bifurcation of the sciatic nerve use fine forceps to carefully separate connective tissue (epineurium) surrounding the tibial nerve branch (this is the larger branch).
4. Once isolated slip a 6-0 silk ligature around the tibial nerve and tighten. Use micro scissors to transect the nerve rostral to the ligature. Pass the suture line through the vastus lateralis muscle and tie off in a knot. This will provide a line for easy location of the cut end of the tibial nerve when the nerve is to be harvested 7-10 days later.
5. Degeneration of the tibial nerve prior to grafting provides an environment more suitable for axonal growth than does a fresh nerve preparation.

6. Close the vastus lateralis muscle with 6-0 silk sutures. Close the biceps femoris muscle with 6-0 silk sutures. Close the skin with Michel wound clips. Turn off the flow of Isoflurane, remove the inhalation nose cone and place animal in holding cage on thermal barrier and return to home cage once it is alert and active.

3) Removal of PN segment

1. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier.
2. Remove wound clips and separate previous skin incision. Cut through suture of superficial muscle to expose vastus lateralis and cut through suture in this muscle.
3. Trace the suture line to cut end of tibial nerve and remove suture from nerve. Use fine forceps to dissect epineurium from a 15 mm length of the tibial nerve and cut through this distal end. Remove the nerve segment and immerse in sterile Hanks Balanced Salt Solution.
4. If this is a heterologous graft experiment euthanize the animal by intraperitoneal injection of Euthasol. If this is an autologous graft experiment, the leg muscle and skin is closed as described in 2.6) and the animal prepared for cervical spinal cord injury, as described below.

4) Cervical hemisection injury and transplantation

Day 1

1. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier. Administer antibiotics and analgesics as above (1.4).
2. Locate the occipital notch and the prominent T2 dorsal vertebral process and make an incision the full length between these external landmarks. Cut through the midline of the superficial trapezius muscle and separate the underlying cervical rhomboideus muscles. Keep muscles apart by inserting a spreader.
3. The deep supraspinal muscles should be cut from the dorsal vertebral processes C4, C5 and C6. Using rongeurs perform a partial laminectomy of C5 to expose the entire right side and the medial portion of the left side of the spinal cord. Use the prominent dorsal vein as a landmark for the spinal cord midline.
4. Use a micro-scalpel blade or a 30 gauge needle to pierce the dura mater and extend the incision over the C5 spinal cord. Make a small incision in the underlying subarachnoid and pia mater.
5. With a pulled glass micro-cannula begin to aspirate the dorsal, right side of the spinal cord, using gentle pressure and micro-forceps to separate the tissue and facilitate its removal. Continue through intermediate and ventral portions of the spinal cord to create a 2 mm long cavity that is complete from dorsal to ventral and from midline to the lateral meninges.
6. Place saline-soaked gel foam into the cavity to achieve hemostasis.
7. Remove the segment of peripheral nerve from the Hanks solution. Gently peel the epineurium from the proximal 3mm of the nerve segment and appose this end to the rostral wall of the hemisection lesion cavity. Secure the segment with 10-0 suture through the epineurium to the dura mater, then suture the dura closed over the implanted end of the graft.
8. Sandwich the graft between 2 pieces of silastic membrane (Biobrane) for protection and easy isolation at a later time point. Place alongside of the C6, C7 and T1 vertebral processes. Do not appose the distal nerve end to spinal cord or muscle tissue.
9. Close muscles in layers with 4-0 suture and close skin incision with wound clips.
10. Place animal in holding cage on thermal barrier and return to home cage once it is alert and active.

Day 21

11. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier and administer antibiotics and analgesics as above (1.4). Cut through superficial sutures to expose silastic membrane surrounding the nerve graft. Carefully free the length of nerve from the silastic membrane using fine forceps and spring scissors and reflect from the surgical field.
12. Perform a partial laminectomy on the right side of the C7 vertebral process. Pierce the meninges as above (4.4) and by aspiration prepare a 1 mm³ dorsal quadrant lesion cavity in the C7 spinal cord. Use saline-soaked gel foam to achieve hemostasis and then replace with gel foam soaked with chondroitinase ABC. Treat the lesion site for 15 minutes, providing fresh chondroitinase-soaked gel foam every 5 minutes.
13. Cover the external length of the nerve graft with Silastic membrane. Close superficial muscles with 4-0 sutures and the skin incision with wound clips. Place animal in holding cage on thermal barrier and return to home cage once it is alert and active.

Day 23

14. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier and administer antibiotics and analgesics as above (1.4). Cut through superficial sutures to expose silastic membrane surrounding the nerve graft. Do not disturb the graft but rather expose the C7 graft site and use a Hamilton syringe with pulled glass cannula to microinject 1µl of chondroitinase ABC into the right side of the spinal cord approximately 1 mm distal to the apposition of graft to spinal cord.
15. Gently peel the epineurium from the distal 2mm of the nerve segment and appose this end to the C7 lesion cavity. Secure the segment with 10-0 suture through the epineurium to the dura mater.
16. Close superficial muscles with 4-0 sutures and the skin incision with wound clips. Place animal in holding cage on thermal barrier and return to home cage once it is alert and active.

5) Electrophysiological recording of action potential in graft and distal spinal cord

1. To determine if electrical activity is transmitted through axons that have regenerated into the PNG we must first expose and isolate the graft. Special care must be taken during this procedure to avoid physically damaging the graft and this is where the use of Silastic membrane after placement of the graft is important. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier.
2. After opening the skin and separating superficial muscles, Silastic membrane strips are identified and carefully dissected free of the graft.
3. Using rongeurs remove the dorsal vertebral process immediately rostral and caudal to the PNG-spinal cord apposition sites. Take care not to disturb the graft too much during this approach. Make a slit in the dura mater at both levels.
4. Approximately 1cm rostral to the C5 graft-spinal cord apposition site insert a single wire (platinum-iridium mix) stimulating electrode into the spinal cord ipsilateral to the lesion, to a depth of approximately 1mm. Carefully lift the center portion of the graft onto a bipolar hooked electrode. Provide single stimulating pulses of 10µA with 100µsec duration and determine if there are action potentials traveling through axons that have regenerated into the graft.

5. Switch the connections and position of wires so that the hooked electrode becomes the stimulating electrode and the single wire is inserted into the spinal cord distal to the apposition of PNG to the spinal cord and attempt to record action potentials in the spinal cord.
6. At the end of the experiment provide a train of 1mA stimuli for 100μsec duration at 50Hz, for a total of 1hr. Euthanize animals 1hr later, perfuse with 4% paraformaldehyde and process spinal cord tissue for the immunocytochemical detection of cFos in neurons that were activated by regenerated axons in the PNG.

6) Retrograde labeling of neurons that regenerate axons into the PNG and anterograde labeling of axons that regenerate back into the spinal cord

1. For animals not tested by electrophysiological measures it is possible to identify the source of axons that grow into the graft and the course of their termination in the spinal cord. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier and expose the PNG as described in 6.1 above.
2. Use micro scissors to cut through the graft at its mid-point and strip back the epineurium from the cut ends of the graft. Reflect the cut ends away from each other and lay each on a separate sheet of parafilm.
3. Place a pledget of gel foam soaked with True Blue on the proximal cut end to label neurons that contributed an axon to the PNG. Place a pledget of gel foam soaked in biotinylated dextran amine (BDA) on the distal cut end to label axons that extend from the graft back into the spinal cord.
4. Euthanize animals 3-4d later, perfuse with 4% paraformaldehyde and process brain and spinal cord tissue sections for fluorescent microscopy for True Blue labeled neurons or for BDA histochemistry for labeled axons.

7) Thoracic complete transection injury and transplantation

This offers an alternative approach to the cervical hemisection injury model above. Here a more severe, bilateral injury is produced and segments of peripheral nerve are placed into the lesion cavity to span and rostral and caudal stumps of the injured spinal cord.

Day 1

1. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier. Administer antibiotics and analgesics as above (1.4).
2. Identify T12 vertebral level at point of insertion of last rib. Make skin incision from this point rostral over vertebral processes T11, T10 and T9. Cut through superficial fascia through supraspinal muscles attached to these three processes.
3. Perform complete laminectomy of T10 vertebral process (overlying T12 spinal cord segment). Use a micro-scalpel blade or a 30 gauge needle to pierce the dura mater and extend the incision over the T12 spinal cord. Make a small incision in the underlying subarachnoid and pia mater.
4. With a pulled glass micro-cannula begin to aspirate the T12 spinal cord bilaterally, using gentle pressure and micro-forceps to separate the tissue and facilitate its removal. Continue through intermediate and ventral portions of the spinal cord to create a 3 mm long cavity that is complete dorsal to ventral and between the lateral meninges.
5. Use saline-soaked gel foam to achieve hemostasis and then replace with gel foam soaked with chondroitinase ABC. Treat the lesion site for 15 minutes, providing fresh chondroitinase-soaked gel foam every 5 minutes.
6. Remove the segment of peripheral nerve from the Hank's solution. Gently reflect the epineurium from the proximal 4mm of the nerve segment and cut a length to snugly fit between the rostral and caudal stumps of one side of the spinal cord. Place a second segment alongside the first grafted segment, apposed to the other side of the spinal cord. Suture the dura closed over the implanted grafts and cover dura with a small piece of silastic membrane.
7. Close muscles in layers with 4-0 suture and close skin incision with wound clips.
8. Place animal in holding cage on thermal barrier and return to home cage once it is alert and active.

Day 3

9. Anesthetize animal, shave and clean the intended surgical field as described above (1.3). Place the animal on thermal barrier and administer antibiotics and analgesics as above (1.4). Cut through superficial sutures to expose silastic membrane overlying the graft site. Do not disturb the graft but rather expose the spinal cord caudal to the graft and use a Hamilton syringe with pulled glass cannula to microinject 1μl of chondroitinase ABC into each side of the spinal cord approximately 1 mm distal to the apposition of graft to spinal cord.
10. Close superficial muscles with 4-0 sutures and the skin incision with wound clips. Place animal in holding cage on thermal barrier and return to home cage once it is alert and active. Perform electrophysiological stimulation and recording and tracer labeling as described in 5) and 6) above.

8) Representative Results

When this procedure is optimized the peripheral nerve segment will closely integrate with the host spinal cord. Ascending and descending spinal axons will enter the graft, grow in a relatively straight line parallel to the length of the graft and extend to the distal end of the graft at a rate of approximately 1 mm per day. Upon reaching the distal end, axons will penetrate the adjacent spinal cord if chondroitinase has been used to remove inhibitory proteoglycans from the surrounding scar tissue. Functional connectivity will be determined by electrophysiological and immunocytochemical detection of a response by spinal cord neurons to stimulation of the axons within the graft. Anatomical correlation for functional recovery will be assessed by tracing the number and length of axons that have extended beyond the nerve graft back into the spinal cord.

Discussion

1. For reproducibility of the experiment it is important that the level of spinal cord injury be consistent from animal to animal. Therefore it is critical that the appropriate vertebral process for laminectomy be identified. Because we are using a hemisection or transection lesion model for this study there is no ambiguity about the size of the lesion or whether specific spinal tracts were injured or spared.
2. It is necessary that the peripheral nerve be pre-degenerated before transplantation because this initiates digestion of peripheral axons to provide space for regenerating axons to grow and because it activates Schwann cells to produce several neurotrophic factors which support the ingrowth of axons.

3. Initial placement of the cut ends of the graft to spinal cord tissue should be made firmly (without damaging the graft) to optimize axon growth into and out of the graft. Direct apposition of the peripheral nerve graft to the spinal cord and securing the graft with sutures is critical.
4. Use of chondroitinase to modify the extracellular matrix around the graft site is another critical feature of this experimentation. Glial scar tissue formed in response to spinal cord injury is a structural and chemical barrier to axonal growth. Chondroitinase digestion of the inhibitory proteoglycan content of the scar facilitates the extension of regenerating axons into and beyond the graft.
5. There are several areas for possible modification of this procedure to improve the extent of axonal growth into and out of the peripheral nerve graft. We are experimenting with the delivery of neurotrophic factors to the site of apposition between graft and host spinal cord to promote the regenerative response of injured axons. An increase in neurotrophic factors can be achieved by injection of viral vectors to increase local production or by injection of nanoparticles for a gradual release of factors. We also are examining the role of exercise in promoting production of endogenous neurotrophic factors to facilitate axonal growth. In addition to the aspiration lesion models described above we have developed a cervical contusion injury model and have successfully applied our peripheral nerve graft approach to support axonal regeneration.
6. For all of our studies we employ several assays to measure changes in motor and sensory behavior after spinal cord injury and after intraspinal grafting of peripheral nerves.

Significance:

With the peripheral nerve grafting approach described above we have demonstrated that adult motor and sensory neurons will regenerate their injured axonal process for long distances when provided with an appropriate substratum for growth. We have shown that this approach can be carried out as an acute or delayed treatment strategy and that it can be applied successfully to small (mouse, rat) and large (cat) experimental animals. It is important that the functional activity of regenerating axons be tested and the peripheral nerve graft model provides relatively easy access to all of the axons bridging a spinal cord lesion. This is an obvious advantage over other transplantation approaches.

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