Transplantation strategies to promote repair of the injured spinal cord

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Abstract—This review describes the results of the transplantation of Schwann cells and olfactory ensheathing glia in combination with other interventions. The complete transection injury model was used to test the combination of Schwann cell bridges with methylprednisolone, neurotrophins, or olfactory ensheathing glia. The contusion injury model was used to compare Schwann cell and olfactory ensheathing glia transplantation and to examine the results of combining Schwann cell transplants with elevated levels of cyclic adenosine monophosphate. The combination strategies were more effective than cell transplantation alone. The improved regeneration response usually involved a reduction in secondary tissue loss, axonal regeneration from brainstem neurons, an increase in myelinated fibers in the transplant, the exit of regenerated fibers from the transplant into the contiguous cord, and an improvement in locomotor function.

Key words: central nervous system (CNS) regeneration, contusion injury, cyclic adenosine monophosphate, methylprednisolone, neurotrophins, olfactory ensheathing glia, raphespinal tract, Schwann cells, spinal cord transection.

INTRODUCTION

Changes in injured spinal cord tissue start rapidly and are varied and many. It is likely, therefore, that effective therapeutic strategies will consist of a series of interventions. First, secondary tissue loss should be prevented early through neuroprotective, anti-inflammatory, or immunomodulatory interventions. After that, strategies to promote regrowth of axons and restore function will involve multiple approaches: (1) reducing scar formation, thereby diminishing the accumulation of proteoglycan molecules known to be inhibitory to axonal growth; (2) overcoming additional inhibitory molecules, myelin-related constituents, that also stymie axonal extension; (3) awakening damaged nerve cells to regenerate axons;

(4) providing sustenance to the nerve cells that are separated from their targets and, thus, are bereft of trophicfactors; (5) facilitating axonal growth across the site of injury; (6) guiding axonal growth to appropriate spinal cord regions; (7) enabling formation of new connections; and, finally, (8) retraining the nervous system to use the therapeutic interventions. Interestingly, in the early 1900s, Ramón y Cajal [1] speculated that to correct the central nervous system (CNS) deficiency in repair, we must "give to the sprouts, by means of adequate alimentation, a vigorous capacity for growth; and, place in front of the disoriented nerve cones and in the thickness of the tracks of the white matter and neuronic foci, specific orienting substances." This was undoubtedly the first suggestion of a combination strategy to effect repair in the adult CNS.

There are now exciting investigations under way in many laboratories to devise reparative strategies to address the aforementioned challenges for spinal cord repair. Surface receptors on growing nerve fibers that respond to inhibitory factors are being blocked by presenting antibodies to the inhibitory factors [2] or fragments thereof [3], or by introducing competitive antagonist peptides [4], thereby enabling the axons to grow through an inhibitory milieu. Another approach is to modulate intracellular signaling to (a) interfere with cascades that are initiated after a growing nerve fiber encounters an inhibitory molecule (such as targeting the Rho family of GTPases that regulate actin-mediated motility [5,6]), or (b) turn on pathways responsible for initiating axon growth and to enable fibers to grow through inhibitory environments (such as elevating levels of cyclic adenosine monophosphate (cAMP)). Another pursuit to overcome inhibitory factors is to deliver enzymes that prevent the formulation of or degrade chondroitin sulfate proteoglycans as they accumulate near the site of injury [7,8]. Other studies are investigating ways to promote axonal growth across the area of injury; transplantation plays a major role in these studies. Implantation of pieces of peripheral nerve [9], fetal tissue [10], olfactory ensheathing glia (OEG) [11,12], and Schwann cell (SC) bridges [13], for example, are being assessed. Transplants are often tested in conjunction with the neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3). These neurotrophins have been shown to awaken neurons to regrow axons [14], to increase numbers and different types of axons that grow into transplants [10,15,16], and to promote the growth of

regenerated axons from transplants into the spinal cord [17,18]. There are also important new approaches being tested in training and rehabilitation studies. The limitation of space in this article precludes additional discussion of new interventions being examined in other laboratories. Accordingly, the reference list is abbreviated. What follows is an overview of studies conducted in our laboratory at The Miami Project to Cure Paralysis.

COMPLETE TRANSECTION/SCHWANN CELL BRIDGE MODEL

We have, over the last decade, explored the efficacy of grafted SCs to repair the injured spinal cord in the adult rat. SCs have long been known to be key for the regeneration that occurs in the peripheral nervous system. SCs in peripheral nerves everywhere either ensheathe axons with their cytoplasm or myelinate axons. When an axon is damaged and it then degenerates, SCs nevertheless remain in their tunnels of extracellular matrix, and it is into these tunnels that the nerve fibers regenerate. Also, SCs secrete growth factors and extracellular matrix components that are known to promote nerve fiber growth [19]. SCs function in the CNS and have been shown to be more effective when genetically engineered to produce more growth factors than they normally do. Another advantage of SCs is their accessability. They could be obtained from a piece of peripheral nerve from a spinal cord injured person, expanded to very large numbers in tissue culture (which is now possible), and then be transplanted into the area of injury in the same person without the potential of immune rejection.

We have studied two models, the complete transection and the contusion models. Both have advantages and disadvantages. One of the strong advantages of the complete transection model is the availability of unambiguous results in detecting regenerated fibers below the area of injury. In a contusion injury, spared fibers exist around the spinal cord perimeter, which complicates the evaluation of regenerated fibers. The contusion injury, highly clinically relevant, leads to the development of a large cavity over weeks in both rats and humans.

An early complete transection/SC bridge paradigm used a cable of six million SCs encased within a polymer channel, into which both stumps of the severed spinal cord were inserted [20]. SCs and the channel were implanted into a gap created at the thoracic 8–11 levels in

an adult Fischer rat spinal cord. One month later, it was observed that axons grew onto the SC bridge from both stumps, there was mean of over 1000 spinal cord neurons that responded by regenerating axons onto the bridge, there was a mean of nearly 2000 myelinated axons on the bridge, and there were over eight times more unmyelinated axons on the bridge. However, there was a minimal response from brainstem neurons, and axons were not observed to leave the transplant. We next considered what intervention could be added to this paradigm to obtain a better regenerative result.

One of the first combinations we explored was the administration of methylprednisolone at the time of transplantation of the SC bridge [21]. Although its use is more controversial now, methylprednisolone has been administered in recent years to appropriate spinal cord injured persons within eight hours of injury. Some of the actions that this glucocorticoid is known to perform in experimental studies are to reduce lipid peroxidation, lessen edema and inflammation, lower excitatory amino acid release, and inhibit tumor necrosis factor-α (TNF-α) expression and nuclear factor kappa B (NF-κB) activation [22]. We found that the survival of spinal cord tissue inserted into the polymer channel was improved, there was less scar formation at the interface between the bridge and the host spinal cord, there were 50 percent more myelinated axons on the bridge, and there were twice as many spinal cord neurons that responded to the bridge when methylprednisolone was administered. Most importantly, there was an improved response from brainstem neurons that extended axons over 2 mm onto the bridge and there was, although modest, growth of axons from the bridge into the distal cord. These were both significant findings because the brainstem neurons are distant from the thoracic bridge; it is known that brainstem neurons respond to a peripheral nerve environment only when it is closer to these neuronal somata [23]. Also, the bridge/host interface had apparently been modified to permit the exit of the regenerated fibers.

We have explored the addition of the neurotrophins, BDNF and NT-3, in combination with SC bridges within polymer channels [24]. When the two neurotrophins were infused into the space between the SC bridge and the channel wall for 14 days (and the animals maintained for a month), there were twice as many myelinated axons on the bridge, there were three times as many responding spinal cord neurons, and brainstem neurons were responsive; when a tracer was placed inside the bridge, there

was labeling of a mean of 92 neurons in the brainstem. In other experiments [25], SCs were transduced with a human prepro BDNF cDNA that was introduced by means of a retrovirus. This paradigm differed somewhat from the one described above, in that the spinal cord was transected and the SCs (transduced or untreated) were deposited in the distal stump to create a 5 mm-long trail, as well as in the transection site. We found, one month later, that the trails had remained largely intact and that there was a very compact bundle of axons in close alignment with the trail for its full length. When the SCs had been transduced (and it was known that they were producing more BDNF than they normally do), there was a response of up to 135 labeled brainstem neuronal somata when the tracer was introduced at the far end of the trail. With the transplantation of untreated SCs, the response was much more modest, with up to 22 brainstem neuronal somata being labeled. With no SC transplantation, there was no labeling above the area of injury. Thus, the combination of completely transected spinal cord and SC transplantation with methylprednisolone or neurotrophins substantially improved the regenerative response. These experiments have been reviewed earlier [13,26].

Another combination strategy that we explored was the introduction of OEG into the spinal cord stumps at either end of the SC bridge [27]. One reason that OEG are attractive for CNS repair experiments is that they are situated in an area where nerve fiber growth continues throughout adulthood [11,12,28]. They are found in the olfactory mucosa and in the olfactory nerves spanning the mucosa and the central olfactory bulb. The sensory neurons in the mucosa turn over every few weeks, requiring newborn neurons to extend axons into the CNS throughout life. Similar to SCs in many ways, they are different in that they intermingle with astrocytes and are more migratory in CNS tissue. In the first OEG spinal cord transplantation study [29], it was found that the OEG accompanied centrally growing axons that had crossed the dorsal root entry zone when the OEG had been implanted in that area; this enabled the sensory fibers to enter the cord, in contrast to nontransplanted animals, in which the sensory fibers cannot. The sensory roots had been cut and re-anastomosed in all animals. A number of additional studies of OEG transplantation have been published [11,12,28,30,31].

In the completely transected T8–11 spinal cord in the SC bridge paradigm, transplanting OEG into both stumps of the severed cord, adjacent to both ends of the SC

bridge, resulted in a number of differences after six weeks [27]. In those animals receiving both SC bridges and OEG, the severed serotonergic fibers were found to regrow around the outside of the SC bridge/polymer channel in the connective tissue formed after transplantation and into which OEG had migrated. The serotonergic fibers favored the fibroblast/OEG to the SC bridge/OEG milieu. The serotonergic fibers then reentered the caudal spinal cord stump and grew at least 1.5 cm. When a tracer, wheat germ agglutinin-horseradish peroxydase (WGA-HRP), was introduced at the cervical 7 level, we observed that numerous labeled fibers emerged from the caudal SC bridge/cord interface. If OEG were not transplanted, there were no such labeled fibers exiting the SC bridge. Another finding was the detection of tracer-labeled neuronal somata in the lumbar region. Our interpretation of these data is that neuronal somata below the bridge and OEG injection, severed at the time of complete transection, regrew axons across the caudal interface, across the SC bridge, across the rostral interface, and up to the cervical level, where they picked up the tracer for retrograde transport back to their cell bodies. Growth to this cervical region reached 2.5 cm. Thus, the introduction of OEG substantially improved the regenerative response, including from brainstem neurons, particularly the raphe neurons, and enabled exit of fibers from the bridge and long-distance axonal regeneration.

CONTUSION/SC AND OEG TRANSPLANTATION MODEL

SCs and OEG have been transplanted into a moderate contusion injury induced at the T8 level by the New York University contusion device; a 10 g weight was dropped from a height of 12.5 mm. At first we asked whether OEG should be transplanted at the time of contusion or at 7 days, and whether they should be injected in a fluid medium or in a fibrin matrix [32]. The control animals received a contusion injury only or injection of medium only at 7 days after contusion. The grafts, following injection of two million OEG, largely filled the lesion site, reducing cavitation, and appeared continuous with the spinal cord tissue at 8 weeks. In control animals, 54 percent of the spinal tissue within a 2.5 mm segment of cord centered at the injury site was spared; significantly more tissue (66–73%) was spared when cells had been grafted either at 30 min or at 7 days. Serotonergic

axons were more evident in cell-grafted animals, in the graft, in the surrounding white matter, and at least 20 mm caudal to the graft.

Neuronal somata in the cord and the brainstem that possessed axons caudal to the graft were visualized by introducing a retrograde tracer beyond the lesion/graft. Animals receiving an OEG graft in medium at 7 days exhibited more than twice as many supraspinal axons caudal to the transplant than animals injected with only medium at 7 days. We do not know what proportion of these fibers were either spared or had regenerated. Seven-day transplantation also led to higher numbers of axons in the graft and modestly improved hindlimb function compared with implantation at 30 min. Outcomes of transplantation at 7 days in culture medium or in a fibrin matrix were largely similar. Thus, the results of this first study of OEG transplantation into a moderate contusion injury demonstrated that the transplantation of OEG promoted sparing/regeneration of supraspinal axons and that 7-day transplantation was more effective than acute transplantation. We decided to transplant SCs, as well as OEG, at 7 days after contusion and to use the more convenient injection in medium instead of fibrin.

In the next study [33], we injected two million SCs or two million OEG, or a combination of one million SCs and one million OEG, into a moderate contusion injury as above. Control animals received an injection of only medium. In the control animals, the typical large area of cavitation was seen at 12 weeks after injury. In all the other grafted animals, there was far less cavitation, and there was an improvement in tissue sparing. Quantitation at the injury level of the volume of spared spinal tissue as a percentage of the volume of the same length and level of cord from uninjured animals revealed that there was a statistically significant improvement with all types of grafts. In examining plastic sections to obtain myelinated axon counts, we found 2125 ± 697, 5212 ± 1783, 3884 ± 711, and 2965 ± 1110 myelinated axons in medium, SC, SC/OEG, and OEG transplants, respectively. The myelin sheaths in the control animals that received only an injection of medium were formed by host SCs that had migrated into the lesion area. This typically occurs in these contusion lesions. The number of myelinated axons in SC transplants was more than twice that number. When one million SCs were injected in the combination graft, the number of myelin sheaths was intermediate between the control and the SC-transplanted animals. The number in myelin sheaths in the OEG grafts was quite similar to

that of the medium-injected control animals. The survival of and myelination by OEG in a contusion injury needs to be carefully assessed, using appropriately prelabeled OEG. Most of the myelin seen in OEG transplants could have been formed by the migratory host SCs. Electron micrographs revealed typical peripheral myelin sheaths in all grafts.

A retrograde tracer was injected 6 to 7 mm below the caudal edge of the injury site or transplant, and the numbers of labeled neuronal somata at the C2, C6, T2, and T7–8 levels of the spinal cord, in the brainstem, and in the cerebral cortex were counted. In all areas of the spinal cord and the brainstem examined, the SC-containing (SC or SC/OEG) grafts led to a significant improvement in the numbers of neurons that had axons below the level of the injury/transplant site. Whereas labeled cells were not found in the cerebral cortex in any animals, with all types of grafts corticospinal axons terminated closer to the center of the lesion/graft than in control animals. Whether this represented regeneration or sparing (reduced dieback) of corticospinal axons is not known. There was a significant, though modest, improvement in hindlimb movement as assessed by the Basso, Beattie, Bresnahan (BBB) rating scale [34]. Thus, all types of grafts, SC, SC/OEG, or OEG, significantly reduced tissue loss and supported axonal growth into the graft. SC-containing grafts significantly improved axonal sparing and/or regeneration of spinal and supraspinal axons, and SC grafts led to an improvement in gross locomotor behavior [33].

CONTUSION/SC TRANSPLANTATION/cAMP ELEVATION EXPERIMENTS

There are recent exciting results from studies that have tested the effect of elevating cAMP, both *in vitro* and in spinal cord lesion models. This molecule is an intracellar second messenger that couples extracellular signaling by Ca2+ to changes in the gene expression of enzymes, neurotransmitters and -peptides, and receptors. These alterations contribute to long-lasting changes in a neuron's phenotype and function, including neuronal sprouting, synaptic density, and/or changes in neurotransmitter-receptor systems, and/or synthetic pathways, so-called neuronal "plasticity." The cAMP pathway first involves the initial coupling of adenylyl cyclase activation to Ca2+ ion channel efflux by calmodulin or through nonchannel, receptor-coupled G-proteins. Upon receptor

activation, heterotrimeric G-proteins bind guanosine triphosphate (GTP) and dissociate into constituent parts that in turn target adenyl cyclase. Adenyl cyclase converts adenosine triphosphate (ATP), the prevalent store of energy of the cell, into the second messenger, cAMP, which in turn activates protein kinase A (PKA), the cAMP-dependent protein kinase, by cleavage of its catalytic subunit from the repressor subunit. The catalytic subunit of PKA transduces the cAMP signals to downstream targets by phosphorylating target proteins capable of affecting gene activity, such as transcription factors, including the cAMP-responsive binding protein (CREB).

cAMP has been demonstrated, through the use of soluble analogs given at either the neuronal soma or axon growth cone in vitro, to (a) modulate the behavior of growth cones; in particular, turning responses to guidance cues or acting as a chemotaxic molecule [35,36]; (b) promote axon growth over otherwise inhibitory substrata such as myelin and myelin-associated glycoprotein (MAG) [37]; and (c) act as an intracellular signaling molecule transmitting extracellular cues to the nucleus for rapid neurite outgrowth, elongation, and the prevention of growth cone collapse [38].

In the animal, elevation of cAMP has been implicated in the priming lesion. Only when the peripheral branch (in contrast to the central branch) of a dorsal root ganglion is damaged in vivo do injured centrally directed fibers from the ganglion regenerate; inducing the peripheral branch injury is known as a priming or conditioning lesion. It has been shown recently that the priming lesion leads to an increase in cAMP levels in the ganglion [39]. When priming lesioning is not done, but cAMP is injected into the ganglion instead, the regenerative cue is mimicked, and sensory axons regrow across a dorsal column spinal cord lesion [39,40].

In beginning to test combination strategies with cellular transplantation into contusion injuries, we chose to investigate the elevation of cAMP levels in the contusion/SC transplantation paradigm [41]. We decided to not only inject a more cell-permeable analog of cAMP, di-butyryl-cAMP (db-cAMP), into the spinal cord on either end of the graft, but also to prolong the increase in cAMP by administering rolipram subcutaneously for 2 weeks at the time of contusion ("immediate") or at the time of SC transplantation, 7 days after injury ("delayed"). The injection of db-cAMP was performed at the time of cell transplantation. Rolipram, a phosphodiesterase inhibitor, inhibits cAMP hydrolysis, with

the result that a higher level of cAMP is maintained. Two million purified SCs were injected into the contusion lesion in 6 μl of medium at 7 days postinjury. The animals were maintained for 11 weeks.

Numbers of centrally myelinated axons in the lateral white matter were counted; the rolipram treatment alone, with transplanted SCs, or immediately with transplanted SCs and db-cAMP injection, led to significant increases in spared myelinated fibers. In the graft, axons with peripheral myelin sheaths that had formed after transplantation were counted as well. Significant increases in myelinated axon number were seen with four treatments: SCs + cAMP, SCs + rolipram, and SCs + cAMP + rolipram administered at the time of injury and at the time of transplantation. The sustained elevated level of cAMP after lesioning (that is, with the administration of rolipram) also promoted significant regeneration of numerous serotonergic fibers from the raphe nucleus in the brainstem into, across and—very importantly—beyond the implanted SC graft. Quantitation of these fibers was performed on immunostained preparations. The SC transplant alone is not supportive for supraspinal fiber regeneration.

When overland motor behavior recovery was assessed with the BBB rating scale [34], the only group that was significantly improved compared with the control SC-grafted animals were the animals that received rolipram at the time of contusion and SCs and cAMP one week later. In rolipram-treated groups (animals receiving rolipram, rolipram + SC grafts, and the immediate administration of rolipram with SC grafts and cAMP), there was significantly improved foot rotation, base of support (a sign of trunk stability), and fine motor skills on a gridwalk compared to SC-grafted control animals.

In summary, the transient elevation of cAMP after contusion (without rolipram) increased, to some extent, the number of myelinated axons; led to only very limited growth of serotonergic fibers into the grafts; and did not significantly improve functional recovery in SC implanted animals. However, the prolonged elevation of cAMP (by the administration of rolipram for 2 weeks) led to more significant lateral white matter sparing, greater axonal regeneration and myelination in the graft, an improved supraspinal axon response, and some motor recovery.

CONCLUSIONS

Results of cell transplantation in two spinal cord injury models are summarized in this brief overview. SCs bridging a complete gap in the spinal cord promote axonal regeneration from spinal cord neurons both rostral and caudal to the bridge. When this cellular bridging technique is combined with additional strategies such as the administration of a neuroprotective agent (methylprednisolone), neurotrophins (BDNF or NT-3), or OEG transplantation at either end of the SC bridge, the regeneration response is substantially improved. The combination strategies promote axonal regeneration from neuronal somata in the brainstem onto the bridge and also enable some of the regenerated fibers to exit the bridge.

When SCs or OEG are transplanted into a contusion injury site, compared with the injection of only medium, secondary tissue loss is reduced and corticospinal fibers are present in the rostral region of the transplant. When the transplants contain SCs, more spinal and supraspinal axons are found several millimeters below the graft, and there is a significant, although modest, improvement in overland locomotion. When SC grafting into a contusion injury is combined with strategies to sustain elevated cAMP levels in the cord, lateral white matter loss is significantly reduced, the number of myelinated axons is further increased in the SC graft, serotonergic fibers from the brainstem are found in and beyond the graft, and locomotor function is substantially improved. The efficacy of combined strategies has been demonstrated.

We do not yet know the combination of interventions that will lead to successful recovery from spinal cord injury. There are many different deleterious sequelae in the tissue that are set in motion after injury, and these require reversal. Secondary tissue loss must be halted and the inflammatory response modified as soon as possible. Following that, a regenerative strategy could be initiated that uses transplanted cells, perhaps transduced to provide additional growth factors; agents to overcome the milieu that is inhibitory to axonal growth; and strategies to modify scar formation. Also, pharmacological intervention to enhance certain transmitter systems could be helpful. Our work described here, along with cAMP results from other laboratories, suggests a new way to repair the cord; this intracellular molecule, responsible for directing many physiological functions within the neuron, may provide new insights into the management of the injured spinal cord. The engineering of new

biocompatible materials to bridge the cord injury needs to be explored further [42]. Finally, rehabilitation training will undoubtedly be required to make the interventions as functionally useful as possible.

ACKNOWLEDGMENTS

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